# BT3551- BIOPROCESS PRINCIPLES

## UNIT – I

## PART – A

**1. What is fermentation?**

Fermentation is a process in which microbes are grown in specifically designed vessels loaded with particular types of a nutritive media. These vessels are referred to as fermentors.

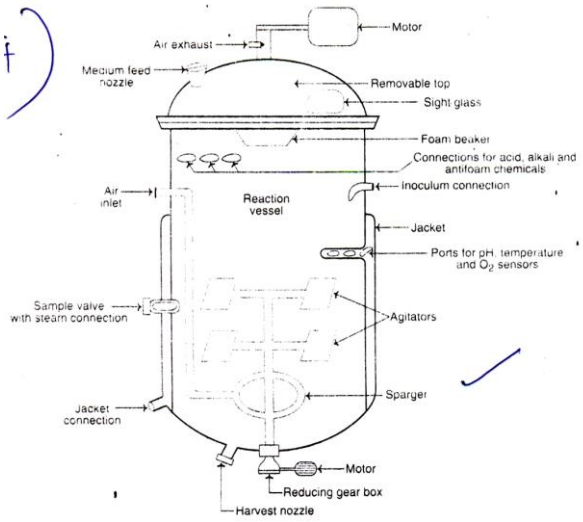
**2. Define Bioprocess Engineering.**

Bioprocess engineering includes the work of mechanical, electrical and industrial engineers to apply the principles of their disciplines to process based on using living cells or sub component of such cells.

**3. Difference between Bioprocess engineering and Biochemical engineering.**

| Bioprocess Engineering | Biochemical Engineering |
|---|---|
| It include the work of mechanical, electrical and industrial engineers to apply the principles of their disciplines to processes based on using living cells or sub components of such cells. The problems of detailed equipment design, sensor development, control algorithms and manufacturing strategies can utilize principles from these disciplines. | It is more limited in sense that it draws primarily from chemical engineering principles and broader in the sense that it is not restricted to well-defined artificially constructed processes, but can be applied to natural systems. |

**4. Draw the schematic Diagram of fermentor.**




**5. Write the parameters to be monitored and controlled in fermentation process.**

- ★ pH electrode
- ★ Oxygen probe
- ★ Temperature
- ★ Death rate
- ★ Cell growth

**6. Write the factors involved in fermentor design.**

- ★ The selection of the best type of reactor for the particular reaction.
- ★ The determination of the best operating conditions.

**7. Mention the general requirements of fermentation process.**

Most of the products formed by organisms are produced as a result of their response to environmental conditions such as nutrients, growth hormones and ions. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation. Nutrients required by cells can be classified in 2 categories:

1) Macro nutrients are needed in concentrations larger than $10^{-4}$ M. carbon, nitrogen, oxygen hydrogen, sulfur, phosphorus, $Mg^{2+}$ and $K^{+}$ are major macronutrients.
2) Micronutrients are needed in concentrations of les than $10^{-4}$ M. Trace elements such as $Mo^{2+}$, $Zn^{2+}$,$Cu^{2+}$,$Mn^{2+}$,$Ca^{2+}$,$Na^{+}$, vitamins, growth hormones and metabolic precursors are micronutrients.

**8. Point out the problems faced in fermentation process.**

1) The problem was typical of most new fermentation\n processes: a valuable pro\duct made at very low levels.
2) The lo\w rate of production\n per unit volume would necessitate very large and inefficient reactors, and the low concentration\n made product recovery and purification very difficult.

**9. Write down the steps involved in fermentation technology.**

1) Aseptic operation
2) Bio mass operation
3) Brewing
4) Medium Formulation
5) Product Recovery
6) Temperature programming.

**10. Compare aerobic and aerobic fermentation process with examples.**

| Aerobic | Anaerobic |
|---|---|
| Fermentation process done in presence of air is called aerobic fermentation process.<br>Eg: Citric acid production penicillin production. | Fermentation process done in absence of air is called anaerobic fermentation process.<br>Eg: Ethanol production Lactic acid production. |

**11. Mention any 4 valves and its uses in fermentor system.**

- ★ pH valve – used to control pH
- ★ Oxygen valve – used to control oxygen
- ★ Sampling valve - with drawing a sample for different laboratory test
- ★ Output valve – product recovery

**12. Give any 2 types of Bioreactors.**

- ★ Continuous stirred tank bio reactor
- ★ Bubble column Bioreactor

**13. List the applications of a bioreactor.**

- ★ It is used to do cell culture both animal and plant cells.
- ★ Vitamins are produced
- ★ Production of citric acid and lactic acid.

**14. Name any 2 unit operations involved in bioprocess.**

- ★ Downstream Processing
- ★ Upstream Processing

**15. Write the 4 kinds of Ideal Bioreactors.**

- ★ Fed-Batch Reactor
- ★ Enzyme –catalyzed reaction in CSTR's
- ★ CSTR reactors with recycle and wall growth
- ★ The Ideal plug-flow Tubular Reactor.

## PART – B

**1. Define Bioreactor/Fermenter. Explain its types in detail.**

A bioreactor is basically a device in which the organisms (cells) are cultivated and motivated to form the desired product (s). It is a containment system desired product (s). It is a containment system designed to give right environment for optimal growth and metabolic activity of the organisms.

A fermenter usually refers to the containment system for the cultivation of prokaryotic cells (bacteria, fungi), while a bioreactor grows the eukaryotic cells(mammalian, insect).

**TYPES OF BIOREACTORS**

Based on the designs of the bioreactors, they can be grouped into the following types

1. Continuous stirred tank bioreactors
2. Bubble column bioreactors
3. Airlift bioreactors
4. Fluidized bed bioreactors
5. Packed bed bioreactors
6. Photobioreactors.

In all types of bioreactors the ultimate aim is to ensure that all parts of the systems are subjected to the same conditions.

**Continuous stirred tank bioreactors**

A continuous stirred tank bioreactor consists of a cylindrical vessel with motor driven central shaft that supports one or more **agitators (impellers).** The shaft is fitted at the bottom of the bioreactor and depends on the size of the bioreactor i.e., height to diameter ratio, referred to as aspect ratio. The aspect ratio of a stirred tank bioreactor is usually between 3-5. However, for animal cell culture applications, the aspect ratio is less than 2. The diameter of the impeller is usually $\frac{1}{3}$rd of the vessel diameter. The distance between two impellers is approximately 1.2 impeller diameter. Different types of impellers (Rustom disc, concave bladed, marine propeller etc) are in use.

In stirred tank bioreactors or in short **stirred tank reactors (STRs),** the air is added to the culture sparger. The sparger may be ring with many hole or a tube with a single orifice. The sparger along with impellers (agitators) enables better gas distribution system throughout the vessel. The bubbles generated by sparger are broken down to smaller ones by impellers and dispersed throughout the medium. This enables the creation of a uniform and homogenous environment throughout the bioreactor.

**Advantages of STRs:** There are many advantages of STRs over other types. These include the efficient gas transfer to growing cells, good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors.

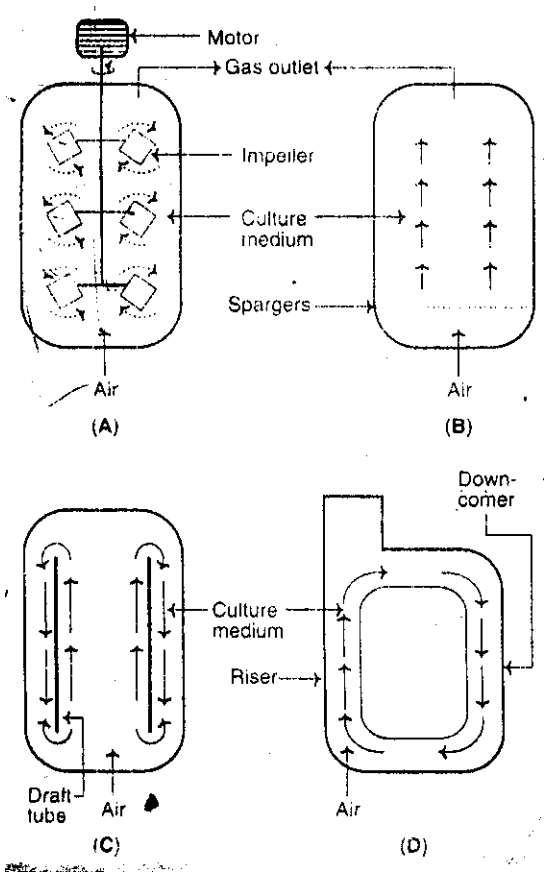



**Fig: Types of bioreactors (A) Continuous stirred tank bioreactor (B) Bubble column bioreactor (c) internal-loop airlift bioreactor (d) External-loop airlift bioreactor.**

**Bubble Column bioreactors**

In the bubble column bioreactor, the air or gas is introduced at the base of the column through periorated pipes or plates, or metal microporus spargers. The flow rate of the air/gas influences the performance factors –$O_2$ transfer, mixing. The bubble column bioreactors may be fitted with perforated plates to improve performance. The vessels used for bubble column bioreactors is usually cylindrical with an aspect ratio of 4-6 (i.e. height to diameter ratio).

**Airlift bioreactors**

In the airlift bioreactos the medium of the vessel is divided into two interconnected zones by means of a baffle or draft tube. In one of the two zones referred to a riser, the air/gas is pumped. The other zone that receives no gas is the downcomer. The dispersion flows up the riser zone while the down flow occurs in the downcomer. There are two types of airlift bioreactor.

**Internal-loop airlift bioreactor (fig)** has a single container with a central draft tube that creates interior liquid circulation channels. These bioreactors are simple in design, with volume and

circulation at a fixed rate for fermentation.

**External loop airlift bioreactor:** Possesses an external loop so that the liquid circulates through separate independent channels. These reactors can be suitably modified to suit the requirements of different fermentations. In general, the airlift biorectors are more efficient than bubble columns, particularly for more denser suspensions of microorganisms. This is mainly because in these bioreactors, the mixing of the contents is better compared to bubble columns.

**Airlift bioreactors** are commonly employed for aerobic bioprocessing technology. They ensure a controlled liquid flow in a recycle system by pumping. Due to high efficiency, airlift bioreactors are sometimes preferred e.g., methanol production, waste water treatment, single-cell protein production. In general, the performance of the airlift bioreactors is dependent on the pumping injection of air and the liquid circulation.

**Two-Stage airlift bioreactors**

Two-stage airlift bioreactors are used for the temperature dependent formation of products. Growing cells from one bioreactor (maintained at temperature 30°C) are pumped into another bioreactor (at temperature 42°C). There is a necessity for the two-stage airlift bioreactor, since it is very difficult to raise the temperature quickly from 30°C to 42°C in the same vessel. Each one of the bioreactors is fitted with valves and they are connected by a transfer tube and pump. The cells are grown in the first bioreactor and the bioprocess proper takes place in the second rector.

**Tower bioreactors**

**Pressure-cycle fermenter** with large dimensions constitutes a tower bioreactor . A high hydrostatic pressure generated at the bottom of the reactor increases the solubility of $O_2$ in the medium. At the top of the riser, with expanded top reduces pressure and facilitates expulsion of $CO_2$. The medium flows back in the downcomer and complete: the cycle. The advantage with lower bioreactor is that it has high aeration capacities without having moving parts.

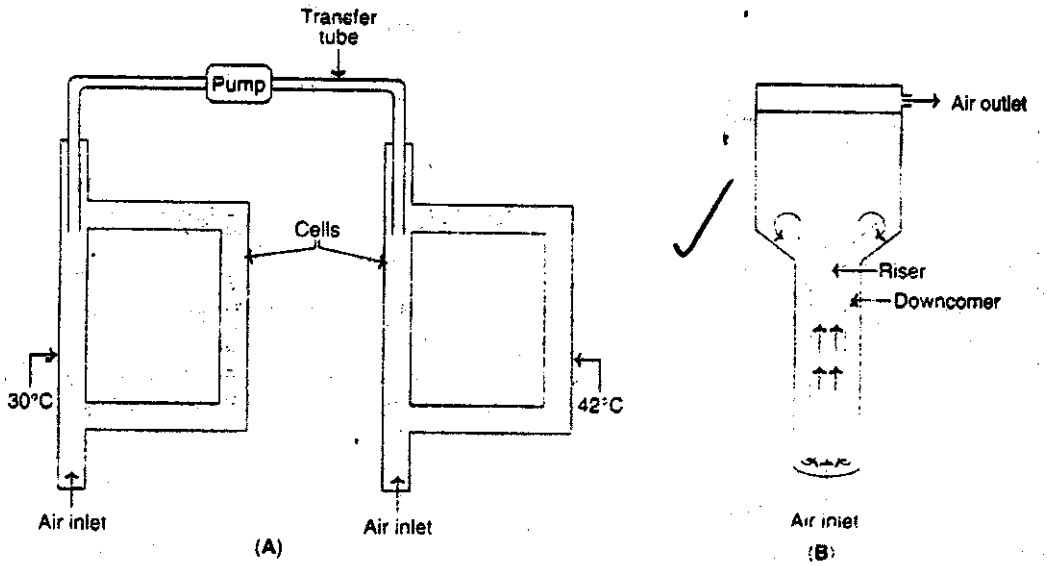



**Fig. Types of bioreactors (a) Two-stage airlift bioreactor (b) Tower bioreactor**

**Fluidized bed bioreactors**

Fluidized bed bioreactor is comparable to bubble column bioreactor except the top position is expanded to reduce the velocity of the fluid. The design of the fluidized bioreactors (expanded top and narrow reaction column) is such that the solids are retained in the reactor while the liquid flows out . These bioreactors are suitable for use to carry out reactions involving fluid suspended biocatalysis such as immobilized enzymes, immobilized cells, microbial flocs.

For an efficient operation of fluidized beds, gas is sparged to create a suitable gas-liquid-solid fluid bed. It is also necessary to ensure that the suspended solid particles are not too light or too dense (too light ones may float whereas to dense ones may settle at the bottom), and they are in a good suspended state. Recycling of the liquid is important to maintain continuous contact between the reaction contents and biocatalysts. The enable good efficiency of bioprocessing.

**Packed bed bioreactors:**

A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactor. **Fig.** the solids used may be porous or non-porous gets, and they may be compressible or rigid in nature. A nutrient broth flows continuously over the immobilized biocatalyst. The products obtained in the packed bed bioreactor are released into the fluid and removed. While the flow of the fluid can be upward or downward under gravity is preferred.

The concentration of the nutrients (and therefore the products formed can be increased by increasing the flow rate of the nutrient broth. Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali. However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions. The packed bed bioreactors do not allow accumulation of the products to any significant extent.

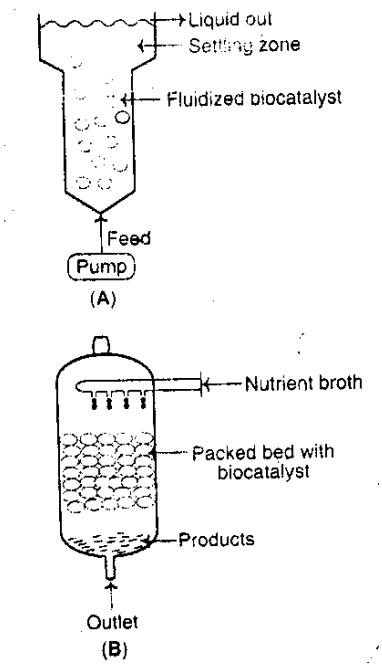



**Fig: Types of bioreactors (a) Fluidized bed bioreactor (b) Packed bed bioreactor**

**Photobioreactors**

These are the bioreactors specialized for fermentation that can carried out either by exposing to sunlight or artificial illumination. Since artificial illumination is expensive, only the out door photobioreactos are preferred. Certain important compounds are produced by employing photobioreactors e.g., β-carotene, asthaxanthin.

The different types of photobioreactos are depicted in figure. They are made up of glass or more commonly transparent plastic. The array of tubes or flat panels constitute light receiving systems (solar receivers). The culture can be circulated through the solar receivers by methods such as using centrifugal pumps or airlift pumps. It is essential that the cells are in continuous circulation without forming sediments. Further adequate penetration of sunlight should be maintained. The tubes should also be cooled to prevent rise in temperature.

Photobioreactors are usually operated in a continuous mode at a temperature in the range of 25-40°C. Microlagae and cyanbacteria are normally used. The organisms grow during day light while the products are produced during night.

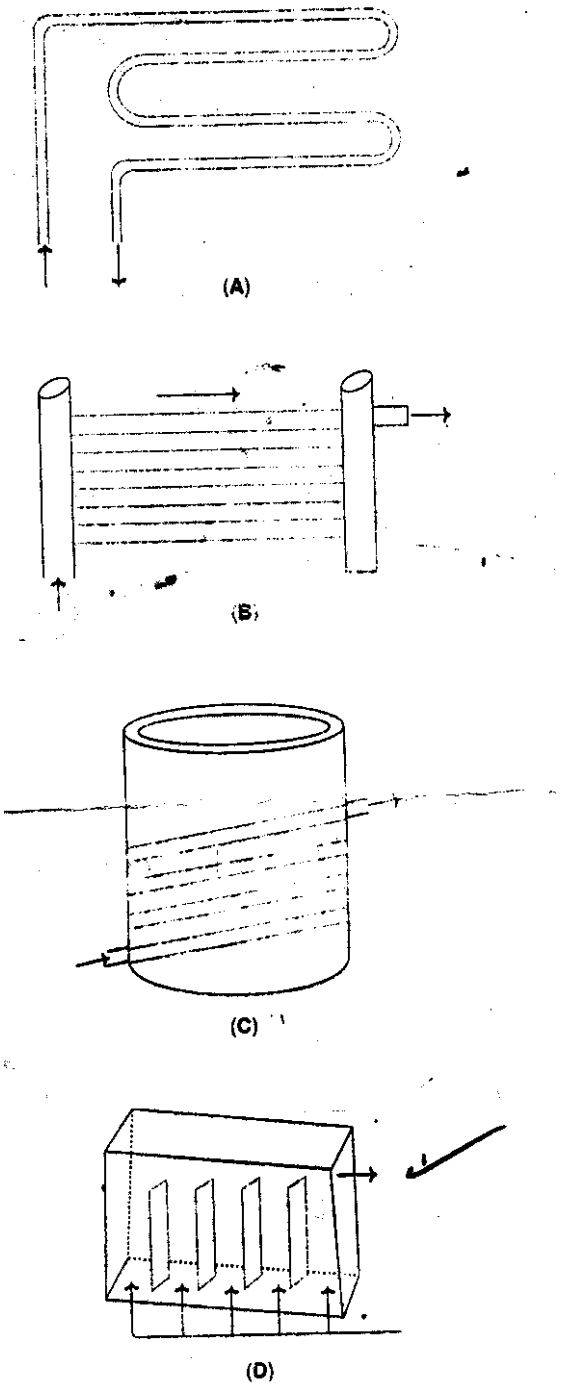



**Figure: Types of photobioreactors (A) Continuous run tubular loop (B) Multiple parallel tube (C) Helical wound tabular loop (D) Flat panel configuration.**

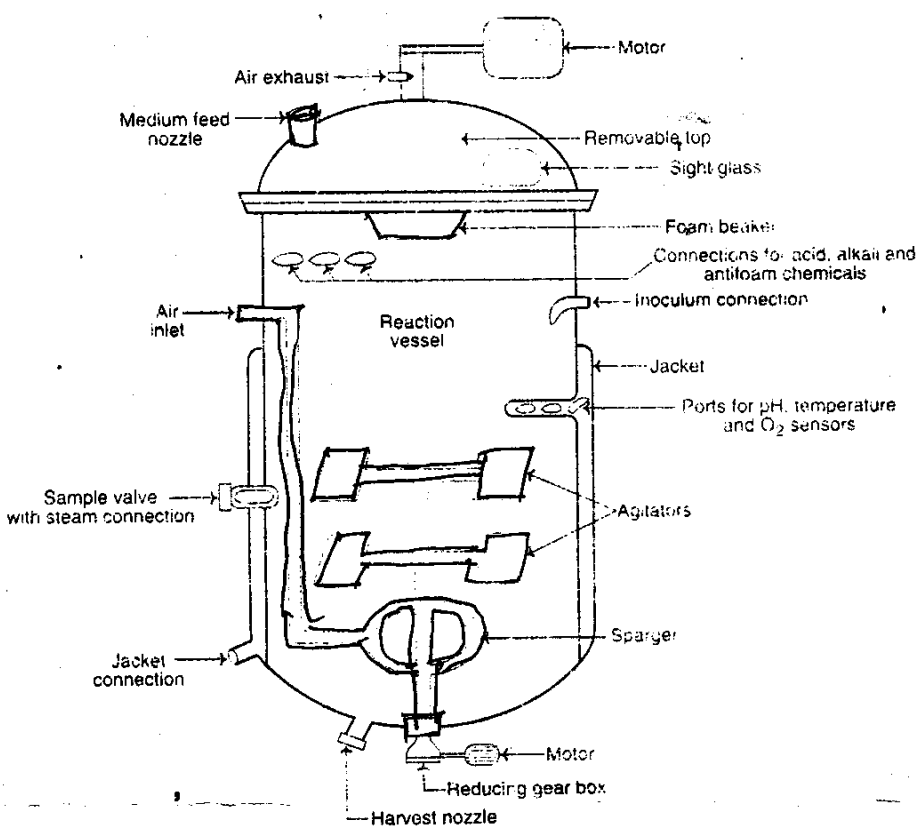



**Figure: Diagrammatic representation of a typical bioreactor**

**2. Explain in detail about operations involved in a Bioreactor.**

**OPERATION OF A CONVENTIONAL BIOREACTOR**

The operation of a bioreactor basically involves the following steps.

1. Sterilization
2. Inoculation and sampling
3. Aeration
4. Control systems
5. Cleaning.

**STERILIZATION**

Aseptic conditions are the basic requirements for successful fermentation. That is the bioreactor and its accessories, the growth medium and the air supplied during fermentation must be sterile.

**In situ sterilization**

The bioreactor filled with the required medium is injected with pressurized stream into the jacket or coil surrounding the reaction vessel. The whole system is heated to about 120° C and held at this temperature for about 20 minutes. In situ sterilization has certain limitation. It is not energy efficient (i.e. energy is wasted) since the bioreactor has to be heated for a long period to rise the temperature of the whole system to 120°C. Prolonged heating may destroy vitamins, besides precipitating the medium components.

**Continuous heat sterilization:**

In this technique, empty bioreactor is first sterilized by injecting pressurized steam. The medium is rapidly heated to 140° C for a short period, by injecting the pressurized steam. Alternatively, the medium can be sterilized by passing through a heat exchanger heated by pressurized steam. Subjecting the medium to high temperature for a short period does not precipitate medium components. Further, there is no energy wastage in continuous heat sterilization method.

**INOCULATION AND SAMPLING**

The bioreactor with the growth medium under aseptic conditions is ready for inoculation with the production organisms. The size of the inoculum is a generally 1-10% of the total volume of the medium. A high yielding production strain of the organism taken from stock culture (lyophilized and stored in a deep freezer or in liquid nitrogen) is used.

During the course of fermentation, samples are regularly drawn from the bioreactor. This is required drawn from the bioreactor. This is required to check the contamination (if any) and measurement of the product formed.

**AERATION**

Aeration of the termination medium is required to supply $O_2$ to the production organisms and remove $CO_2$ from the bioreactor. The aeration system is designed for good exchange of gases. Oxygen (stored in tanks in a compressed form) is introduced at the bottom of the bioreactor through a sparger. The small bubbles of the air pass through the medium and rise to the surface. The bioreactor usually has about 20% of its volume as vacant space on the upper part which is referred to as head space. The bioreactor has about 80% working volume. The gases released during fermentation accumulate in the headspace which pas out through an air outle.

**Air-lift System of aeration**

In this type of aeration, sparging of air is done at the bottom of the fermenter. This allows an upward flow of air bubbles. The more is the aeration capacity of the fermenter, the more is the dissolved $O_2$ in the medium. Further, the aeration capacity of the air-lift system is directly proportional to the air-flow rate and the internal pressure. Oxygen demand refers to the rate at which the growing culture requires $O_2$. For all the aerobic organisms, the operation capacity should be more than the oxygen demand or else the growth of the organisms will be inhibited due to oxygen depletion (starvation).

**Stirred system of aeration**

The aeration capacity of the medium can be enhanced by stirring. This can be done by using impellers driven by a motor. The aeration capacity of the stirred fermenter is proportional to the stirring speed, rate of air flow and the internal pressure. Stirred fermenters are better suited than air-lift fermenters to produce better aeration capacities.

**CONTROL SYSTEMS**

It is essential to maintain optimal growth. Environment in the reaction vessel for maximum product formation, Maximal efficiency of the fermentation can be achieved by continuously monitoring the variables such as the pH, temperature, dissolved oxygen, adequate mixing , nutrient concentrations and foam formation. Improved sensors are now available for continuous and automated monitoring of these variables (i.e. on line measurement of pH).

Most of the microorganisms employed in fermentation grow optimally between pH 5.5 and 8.5. In the bioreactor as the microorganisms grow, they release metabolities into the medium which change pH. Therefore, the pH of the medium should be continuously monitored and maintained at the optimal level. This can be done by the addition of acid or alkali base as needed, and a thorough mixing of the fermentation contents. Sometimes, an acid or alkaline medium component can be used to correct pH, besides providing nutrients to the growing microorganisms.

**Temperature**

Temperature control absolutely essential for a good fermentation process. Lower temperature causes reduced product formation while higher temperature adversely affects the growth of microorganism. The bioreactors are normally equipped with heating and cooling systems, that can be used per the requirement, to maintain the inaction vessels at optimal temperature.

**Dissolved oxygen**

Oxygen is sparingly soluble in water (0.0084 g/1 at 25°C); continuous supply of oxygen in the form of sterilized air is done to the culture medium. This is carried out by introducing air into the bioreactor in the form of bubbles. Continuous monitoring of dissolved oxygen concentration is done in the bioreactor for optimal product formation.

**Adequate mixing**

Continuous and adequate mixing of the microbial culture ensures optimal supply of nutrients and $O_2$, besides preventing the accumulated of toxic metabolic byproducts if any. Good mixing by agitation also creates favourable environments for optimal and homogenous growth environment and good product formation. However, excessive agitation may damage microbial cells and increase the temperature of the medium, besides increased foam formation.

**Nutrient concentration**

The nutrient concentration in a bioreactor is limited so that it wastage is prevented. In addition, limiting concentrations of nutrients may be advantages to optimal product formation, since high nutrient concentrational often associated with inhibitory effect on microbiogal growth. It is now possible to do on-line monitoring of the nutrient concentration and suitably modify as per the requirements.

**Foam formation**

The media used in industrial fermentation is generally rich in proteins. When agitated during aeration, it invariably results in froth or foam formation hat builds in head space of the bioreactor. Antifoam chemicals are used to lower surface tension of the medium, besides causing foam bubble was based commonly used as antifoam agents.

Mechanical foam control devices referred to as mechanical foam breakers, can also be used. Such devices fitted at the top of the bioreactor break the forma bubbles and the throw back into the fermentation medium.

**CLEANING:**

As the fermentation is complete, the bioreactor is harvested i.e., the contents are removed for processing. The bioreactor is then prepared for the next round of fermentation after cleaning (technically called turn round). The time taken should be as short as possible (since it is non-productive). Due to large size of the bioreactors, it is not possible to clean manually. The cleaning of the bioreactors is carried out by using high-pressure water jets from the nozzles fitted into the reaction vessel.

**3. Explain in detail about overview of fermentation industry and the general requirements of fermentation processes.**

**FERMENTATION**

The term 'Fermentation' is derived from the Latin work 'fervern', to boil, thus describing the appearance of the action of yeast on extracts of fruit or malted grain. The boiling appearance is due to presence of or the production of carbondioxide bubbles caused by the catabolism of the sugar presence in the extract. The biochemical meaning of fermentation reacts to the generation of energy by the catabolism of organic compounds.

The catabolism of sugar is an oxidation process which results in the production of reduced pyridine Nucleotidies which results in reoxidised for the process to continue. Under acronic conditions, reoxidation of reduced pyrine nucleotide occurs by electrons transfer, via the cytochrome system, with oxygen acting as the terminal electron accepts under anacrobic conditions, reduced pyridine Nucleordi oxidation is coupled with the reduction of an organic compound.

Thus the term fermentation has been used in strict biochemical sense to mean an energy – generation process in which organic compounds acts as both electron donars and terminal electron acceptors.

The production of allonol by the action of yeast on mal's or fruit extracts has been carried out on a large scale for very many years and was the first industrial process for the production of microbial metabolite. Thus industrial microbiologists have extended the term fermentation to desolube any process for the production of a product by the mass culture of a microorganism.

**THE RANGE OF FERMENTATION PROCESS**

There are five major groups of commercially important fermentations.

- Those that produce microbial cells (or biomass) as the product.
- Those that produce microbial enzymes
- Those that produce microbial metabolites
- Those that produce recombinant products
- Those that modify a compound which is added to fermentation – The transformation process.

**MICROBIAL BIOMASS**

The commercial production of microbial biomass may be divided into two major processes.

(i) The production of yeast to be used in the baking industry.
(ii) The production of microbial cells to be used as human n animal food [Single-cell protein]

Bakis yeast has been produced on a large scale since the early 1900s and yeast was produced as human food in Germany during the first world war. In the 1960s. the production of microbial biomass was used as the source of food protein. As a result of this, a few large –scale continuous processes for animal feed production was established in the 1970's. These process were based on hydrocarbon feed stock which could not compete against other high-protein animal feeds. The demui of animal food biomass was balanced by establishing a process for the production of fungal biomass for human food.

**MICROBIAL ENZYMES**

Enzymes have been produced commercially from plant, animal and microbial sources. However, the microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Also, it is infinitely easier to improve the productivity of a microbial system compared with a plant or animal one. The advent of recombinant DNA technology has enabled enzymes of animal origin to be synthesized by microorganism. The majority of the applications of enzymes are in food and related industries which are given below.

- Amylase is used in the baking and the mixing industry to reduce the dough viscosity, to accelerate fermentation, increase the load volume, improve the crumb softness and to maintain the freshness.
- Protease is used in the Leather industry, for denaming and baiting. Protease is also used in the Dairy industries for the manufacture of protein hydrolysates, stabilination of evaporated milk and curding of milk.
- Amylase is sued in the textile industry for Dexizing of fabrics and in the vegetable industry for the preparation of purees and soups.
- Streptokinasi is used in the pharmaceutical industry to avoid clotting of blood or it is used as Anti-blood clotting agent.
- Pectinace is used in the coffee industry for the preparation of coffee concentrates and in confectionaries for the preparation and manufacturers of soft centre candies.

Enzyme production is closely controlled in the microorganisms and in order to improve productivity these controls may have be, be exploited or modified. Such control medium, whereas repression control may be removed by mutation and recombination techniques. The number of gene copies coding for the enzyme may be increased by recombinant DNH techniques.

**MICROBIAL METABOLITES**

The growth of a microbial culture can be divided into a number of stages.

After the inoculation of a culture into a nutrient medium there is period is a period during which growth does not appear to occurs; this is referred to as the lag phase and considered as the time of ADAPTATION. Following the period during which the growth rate of the cells gradually increases the cell grow at a constant, maximum rate and this period is known as the log phase in the exponential phase. Eventually, growth phases in which the growth ceases and the cell enter the so called stationary phase. After a further period of time the viable cell number declines as the culture enters the death phase.

The behaviour of a culture may also be described according to the products which it produces during the various stages of growth curve. During the log phase of growth the products produced are essential to the growth of the cells and includes aminoacids, Nucleotidis, proteins, Nucleic acids, lipids, carbohydrates etc., These products are referred to as primary products of metabolism and the phase in which they are produced as the trophophase.

Many products of primary metabolism are of considerable economic importance and are being produced by fermentation. The synthesis of primary metabolities by wild-type microorganisms in such that their production is sufficient to meet the requirements of the organism. The wild-type microorganism has to be modified to provide cultural conditions to improve the productivity of these compounds. During the deceleration phase and stationary phase some murobial cultures synthesis compounds which are not produced during the trophophase and which do not appear to have any obvious function in cell metabolism. These compounds are referred to as the secondary compounds of metabolism and the phase in which they are produced as the idiophase.

The secondary metabolism may occur in continuous culture at low growth rates and is a property of slow- growing, as well as non-growing cells. The microorganisms grow at a relatively low rate in their natural environments, thus the idiophase state prevails in nature rather than the trophophase, thus the idiophase state prevails in nature rather than the trophophase, which is a property of the microorganisms in culture. The inter relationship between primary and secondary metabolism shows clearly that secondary metabolites tend to be elaborated from the intermediate and products of primary metabolism. All the micro-organism does not undergo secondary

metabolism- it is common among the filamentous bacteria and fungi and the sporing bacteria but it is found in Enterobactericial . The taxonomic distribution of secondary metabolism is quite different from that of primary metabolism. It is sometimes difficult to categories a product as primary or secondary and the kinetics of synthesis of certain compounds may change depending on cultural conditions.

The physiological role of secondary metabolism in the producer cells has been the subject of considerable debate, but the importance of these metabolites to the fermentation industry is the effects . They have an organism other man those that produce them. Many secondary metabolites have antimicrobial activity, others are specific enzyme inhibitors, some are growth promoters and many have pharmacological properties. Thus the products of secondary metabolism have formed the basis of a number of fermentation processes. As in the case of primary metabolites, wild-type microorganism tend to produce only low concentrations of secondary metabolites, their synthesis being controlled by induction, catabollte and feed back systems.

**RECOMBINANT PRODUCTS**

The advent of recombinant DNA technology has extended the range of potential fermentation produces. Genes from higher organism may be introduced into microbial cell such that the recipients are capable of synthesizing 'foreign' (n neterologous )proteins.

A wide range of microbial cells have been used as hosts for such systems including Escherichia coli, saccharomyces cervical and filamentous fungi. Products produced by such genetically engineered organism include interferon, insulin, human serum albumin, factors VIII and IX, epidermal growth factors, calf chymosin and bourni somatostatin. Important factors in the design of these processes include the secreation of the product, mini mixation of the degradation of the product and control on the set of synthesis during the fermentation.

**TRANSFORMATION PROCESS:**

Microbial cells may be used to convert a compound in to a structurally related, financially more valuable compound . because micro- organisms can behave as chiral catalysts with high positional specificity and stereo specificity ,microbial process are more specific than purely chemical ones and enables the addition, removal or modification of functional groups at specific sites on a complex molecule without the use of chemical protection. The reaction which may be catalysed include dehydrogenation, oxidation, hydroxylation dehydration and condensation, decarboxylation, animation deamination and isomerization.

Microbial process have the additional advantage over chemical reagents of operating at relatively low temperatures and pressures without the requirements for potentially polluting

heavy-metal catalysts. Although the production of uinegar is must well established microbial transformation processes. The majority of these processes involve the modulation of high value compounds including steroids, antchcoties and prostaglandine. The anamaly of the transformation fermentation process is that a large bismass has to be produced to catalyse a single reaction.

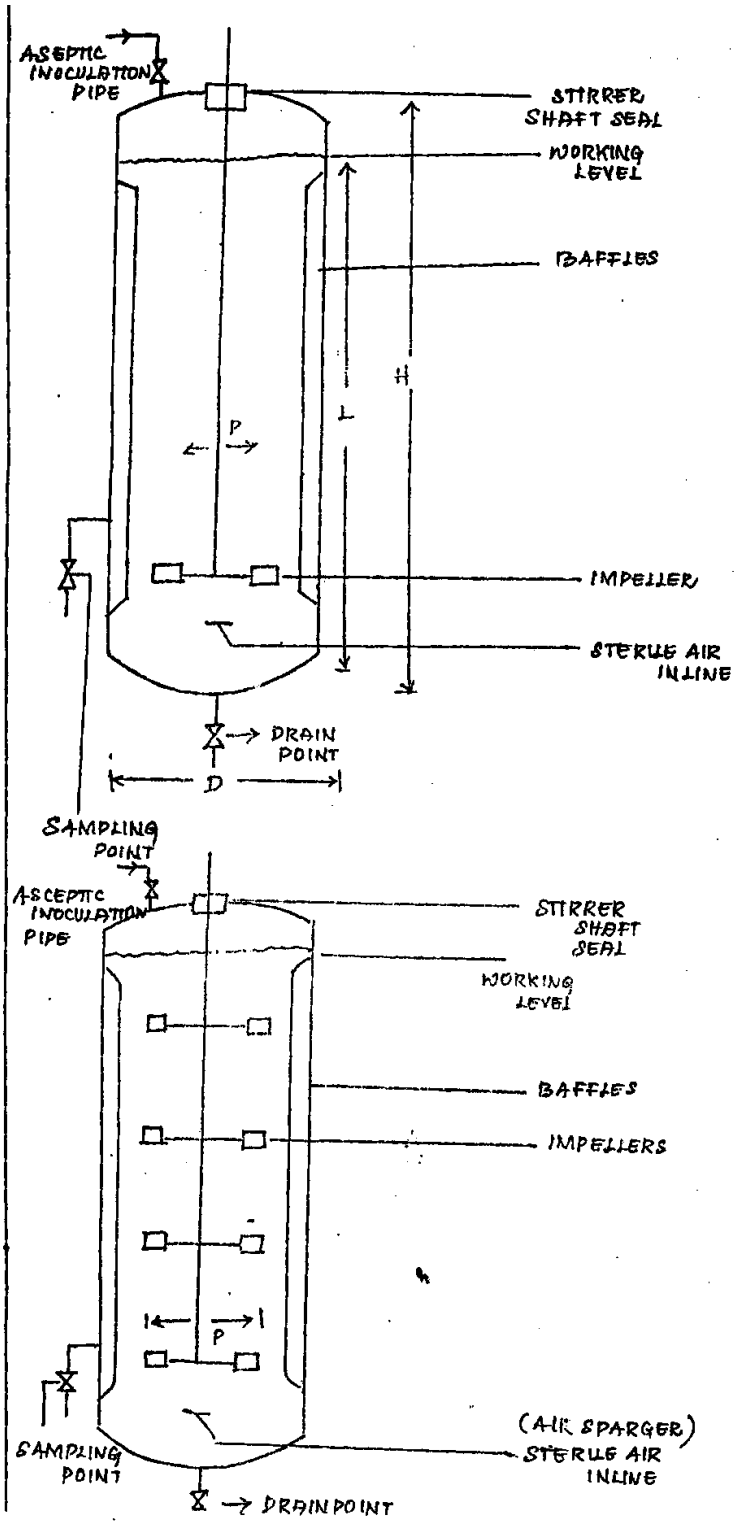
**GENERAL REQUIREMENTS OF FERMENTATION PROCESSES**

The main function of a fermenter is to provide a controlled environment for the growth of microorganisms or animal cells, to obtain a desired produce in designing and constructing a fermenter, a number of points must be considered.

- The vessel should be capable of being operated especially for a number of days and should be reliable in long-term operation and meet the requirement of containment regulations.
- Adequate aeration and agitation should be provided to meet the metabolic requirements of the microorganisms. However, the mixing should not cause damage to the organisms.
- Power consumption should be as low as possible
- A system of temperature control should be provided.
- A system of pH control should be provided.
- Sampling facilities should be provided
- Evaporation losses from the fermenter should not be excessive.
- The vessel should be designed to require the minimal use of labour in operation, harvesting, cleaning and maintenance.
- Ideally the vessel should be designed to require a range of processes, but this may be restricted because of containment regulations.
- The vessels should be constructed to ensure smooth internal surface, using welds instead of flange joints whenever possible.
- The vessel should be similar geometry to both smaller and larger vessels in the pilot plant or plant to facilitate scale-up.
- The cheapest material which enable satisfactory results to be achieved should be used.
- There should be adequate service provisions for individual plants.

**4. Explain in detail about Basic configuration and fermenter and ancillaries.**

**BASIC CONFIGURATION OF FERMENTER AND ANCILLARIES:**



**Construction Materials:**

In Fermentations with strict asceptic requirements it is important to select materials that can withstand repeated steam sterilization cycles. On a small scale(1 to 30 $dm^3$) it is possible to use glass and /or stainless steel. Glass is useful because it glois smooth surface, is non toxic, corrosion proof and it is usually easy to examine the interior of the vessel. Two basic types of fermentor are used,

[i] A glass vessel with a round or flat bottom and top flanged carrying plate.
[ii] A glass vessel with stainless-steel top and bottom plates.

[i] A glass vessel with a round or flat bottom and a top flanged carrying plate, the large glass containers originally used were borosilicate battery jars. All vessels of this type have to be sterilized by autoclaving. The largest diameter for glass cylinder is 60 cm.

[ii] A glass vessel with stainless steel top and bottom plates. These fermenter may be sterilized insiter, but 30 cm diameter is the upper limit to without working pressures. vessels with two stainless steel plates cost approximately 50% more than those with just a top plates.

Pilot scale and Industrial scale vessels are normally constructed of stainless steel or atleast have a stainless-steel cladding to limit corrosion. The America iron and steel institute (AlSl) states that steels containing less than 4% chromium are classified as steel alloys and those containing mox than 4% chromium are classified as stainless steels. Mild steels coated with glass or phenolic epoxy material has occasionally been used. Wood, plastic and concrete have been when contamination was not a problem in a process.

Although stainless steel is often quoted as the only satisfactory material, it was reported that mild steel vessels were very satisfactory after 12 years use for penicillin fermentations. And mild steel clad with stainless steel has been used atleast 25 years for acetone- butanol production. Pitting to a depth of 7 mm strip-tomyan production.

The corrosion resistance of stainless steal is thought to depend on the existence of a thin hydrous oxide film on the surface of the metal. The composition of this film varies with different steal alloys and different manufacturing process treatments such as rolling, pukung or heat treatment. The aim is stabilized by chromium and is considered to be continuous, non – porous, insoluble and self heating. If damaged, the film will re pain it self when exposed to air or an oxidizing agent.

The minimum amount of chromium needed to resist corrosion will depend on the corroding agent in a particular environment, such as acids, alkalis, gases, soil, salt or fresh water. Increasing

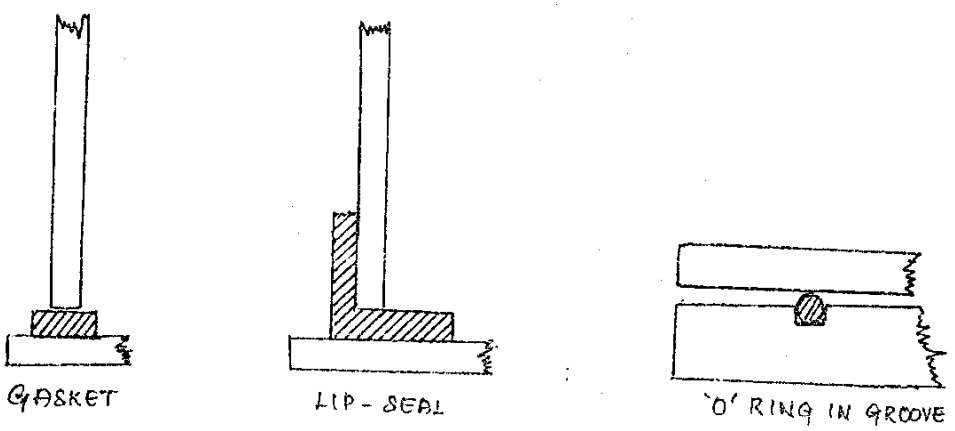
the chromium content enhances resistance to corrosion, but only grades of steal containing atleast 10 to 13 % chromium develop an effective film.

The inclusion of nickel in high pirunt chromium steel enhances their resistance and improves their engineering proprieties. The presence of mocybedenum improves the resistance of stainless steel to solution of halogen salts and pitting by chloride ions in brine or sea water corrosion resistance can be improved by tungsten, silicone and other elements. AISI grade 316 steels which contain 18% chromium, 10% nickel and 2-2.5% molybdenum are now commonly used in fermenter construction. In a citric acid fermentation where the pH may be $HO_2$ it will be necessary to use stainless steel with 3-4% molybdenum to prevent teaching of heavy metals from the steel which would interface with the fermentation.

The thickness of the construction material will increase with scale. At 300,000 to 400,000 $dm^3$ capacity, 7mm plate may be used for the side of a vessel and 10 mm plate, for the top and bottom, which should be hemispherical to withstand pressures.

**JOINT SEALS**

The next stage after body construction is to consider the ways in which a reliable asceptic seal is to made between glass and glass, glass and metal or metal and metal joints such as between the ferementer vessel and a detachable top or base plate. With glass and metal, a seal can be made with compressible gasket, a lip seal or an 'O' ring with metal to metal joints only an 'O' ring it suitable. This is placed on a a groove, machined in either the end plate, the ferementer body or on both. This seal ensures that a good liquid- and/or gas –tight joint is maintained inspite of the glass or metal expanding or contracting at different rates with changes in temperature during astruzation cycle or an incubation cycle.




Nitryl or butryl rubbers are normally used for these seals as they will withstand fermentation process conditions. The rubber seals have a finite life and should be checked regularly for damage or perishing.

The proposal to use two-seals without a steam trace was criticized for a number of reasons.

- Double seals are more difficult to assemble correctly.
- It is difficult to deft failure of one seal of a pair during operation or assembly.
- Neither of the two seals can be tested independently.
- Dead spaces between two seals must be considered to be contaminated.

In the design and construction of a fermenter there must be adequate provision for temperature control which will affect the design of the vessel body. Heat will be produced by microbial activity and mechanical agitation and if heat is particular manufacturing process, then heat may hair to be added to or removed from the system. On a laboratory seals, little heat is generated and extra heat has to be provided by placing the fermenter in a thermostatically controlled bath, or by the use of internal heating coils or a heating jacket through which water is circulated or by a silicone heating jacket. The silicone jacket consist of a double silicone rubber mat with heating wires between the two mats, its wrapped around the vessel and held in place by Velcro strips.

Once a certain size has been exceeded, the surface area covered by the jacket becomes too small to remove the heat produced by the fermentation. When this situation occurs internals coils must be used and wild water is circulated to achieve the correct temperature. Different types of fermentation will influence the maximum size of verses that can be used with jackets alone. It is impossible to specify accurately the necessary cooling surface of a fermenter since the temperatures of the cooling water, the sterilization process, the cultivation temperature, the type of micro-organism and the energy supplied by stirring can vary considerably in different processes.

A cooling area of 50 to 70$m^2$ may be taken as an average for a 55,000 $dm^3$ fermenter and a coolant temperature of 14°, the fermenter may be cooled from 120° to 30° in 2.5 to 4 hours without stirring. The consumption of the vessel during bacterial fermentation ranges from 500 to 2000 $dm^3h^{-1}$ of cooling water, while the cooling water consumption for fungi might need 2000 to 10,000 $dm^3$ $h^{-1}$, due to the lower optimum temperature for growth. To make the accurate estimate of heating/cooling requirements for a specific process, it is important to consider the contributing factors.

An overall energy balance for a fermenter during normal operation can be written as,

$$Q_{met} + Q_{ag} + Q_{gas} = Q_{ace} + Q_{exch} + Q_{evap} + Q_{sen}$$

Where,
$Q_{met}$ = heat generation rate due to microbial metabolism.

$Q_{ag}$ = heat generation rate due to mechanical agitation.
$Q_{gas}$ = heat generation rate due to aeration power input.
$Q_{acc}$ = heat accumulation rate by the system,
$Q_{exch}$ = heat transfer rate to the surrounding and/ or heat exchanger
$Q_{evap}$ = heat loss rate by evaporation
$Q_{sen}$ = rate of sensible enthalpy gain by the flow streams (exit –inlet).

This equation can be rearranged as,

$$Q_{exch} = Q_{met} + Q_{ag} + Q_{gas} - Q_{acc} - Q_{sens} - Q_{evap}$$

$Q_{exch}$ is the heat which will have to be removed by a cooling system, when designing a large fermenter, the operating temperature and the flow conditions will determines.

$Q_{evap}$ and $Q_{sen}$, the choice of agitator, its speed and the aeration rate will determine $Q_{ag}$ and the sparger design and aeration rate will determine $Q_{gas}$.
The cooling requirements to remove the excess heat from a fermenter may be determined by,

$Q_{exch} = u.A..\Delta T$

Where A= the heat transfer surface available, $m^2$
Q= Heat transferred , ω
U= Overall heat transfer co-efficient, $\omega/m^2k$.
ΔT= the temperature difference between the heating and the cooling agent, k.

The co-efficient U represents the conductivity of the system and it depends on the vessel geometry, fluid properties, flow properties, flow velocity, wall material and thickness. 1/U is the overall resistance to heat transfer. It is reciprocal of the overall heat-transfer co-efficient. It is the sum of the individual resistances to heat transfer as heat passes from one fluid to another and can be expressed as,

$$\frac{1}{U} = \frac{1}{h_0} + \frac{1}{h_i} + \frac{1}{h_{of}} + \frac{1}{h_{if}} + \frac{1}{h_\omega}$$

Where,

$h_o$ = Outside film co-efficient , $\omega/m^2k$,
$h_i$ = inside film co-efficient , $\omega /^2k$,
$h_{ef}$ = outside fouling film co-efficient $\omega/m^2$ k

$h_{if}$ = inside fouling film co-efficient $\omega/m^2k$
$h\omega$ = wall heat transfer co-efficient = $k/x_1$, $\omega/m^2=k$
k= thermal conductivity of the wall, $\omega/mk$
x= wall thicknes, m.

There are many methods to determine $\Delta T$ 1+ one side of the wall is at a constant temperature, as is often the case in a stirred fermentor, and the coolant temperature rises in the direction of coolant flow along a cooling coil, and arithmetic mean is appropriate.

$$\Delta T_{am} = \frac{(T_f - T_c) + (T_f - T_i)}{2}$$
$$= \frac{T_f - (T_c - T_i)}{2}$$

where, $T_f$ – The bulk liquid temperature in the vessel
$T_c$ – Temperature of coolant entering the system
$T_i$ = Temperature of coolant leaving the system.

The primary use of aeration is to provide microorganisms is submerged culture with sufficient oxygen for metabolic requirements, while agitation should ensure that uniform suspension of microbial cells is achieved in a homogenous nutrient medium. The type of aeration agitation system used in a particular fermenter depends on the characteristics of the fermentation process under consideration. Although fine bubble aerators without mechanical agitation have the advantage of lower equipment and power costs, agitation may be dispensed with only when aeration provides sufficient agitation i.e., in the process where broths of low viscosity and low total solids are used. Thus, mechanical agitation is usually required in fungi an actinomycete fermentations. Non-agitated fermentations are normally carried out in vessels of height/diameter ratio of 5:1. In such vessels aeration is sufficient to produce high turbulence.

The structural components of the fermenter involved in aeration and agitation are

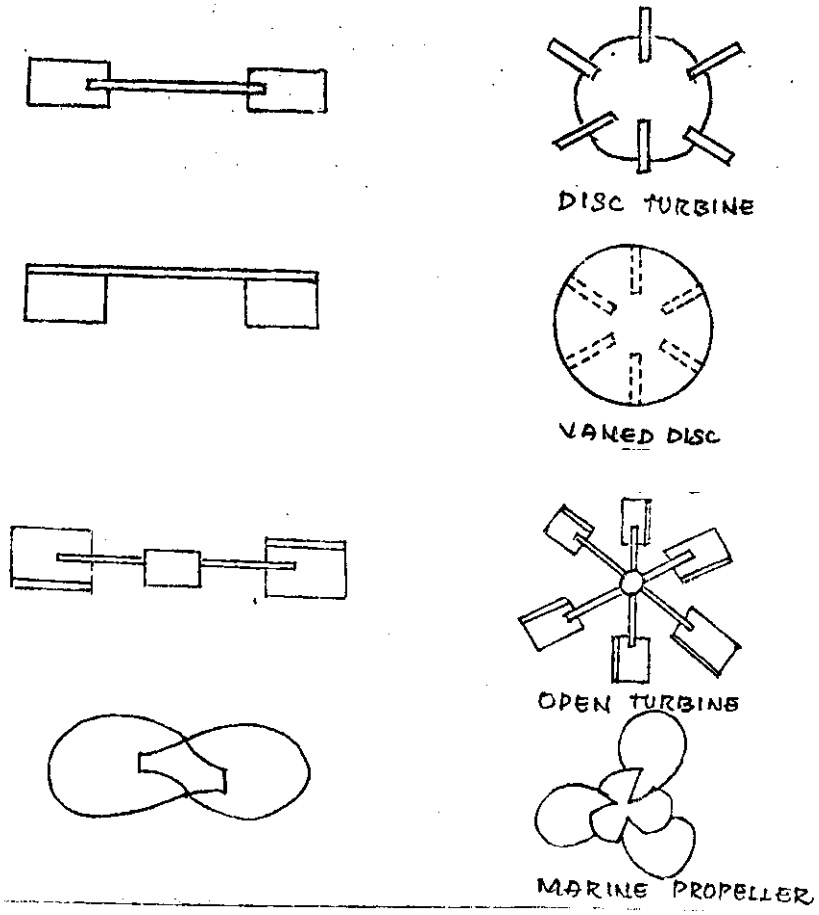
(a) The agitator (impeller)
(b) Stirrer Glands and Bearings
(c) Baffles
(d) The Aeration system (Sparger)

**AGMATOR (IMPELLER)**

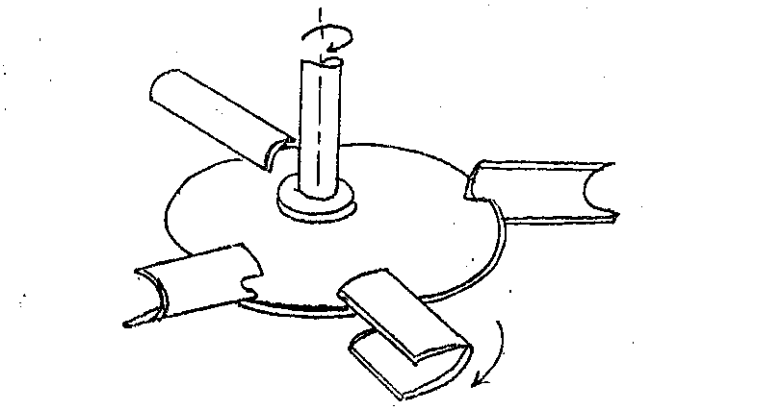
The agitator is required to achieve a number of mainly objective e.g. Bulk fluid and gas-phase mixing, air dispersion, oxygen transfer, heat transfer, suspension of solid particles and

maintaining an uniform environment through out the vessels contents. It should be possible to design a fermenter to achieve these condition, which need the most appropriate agitator, air sparges, baffles, the best positions for nutrient feeds, acid or alkali for pH control and anti-foam addition.

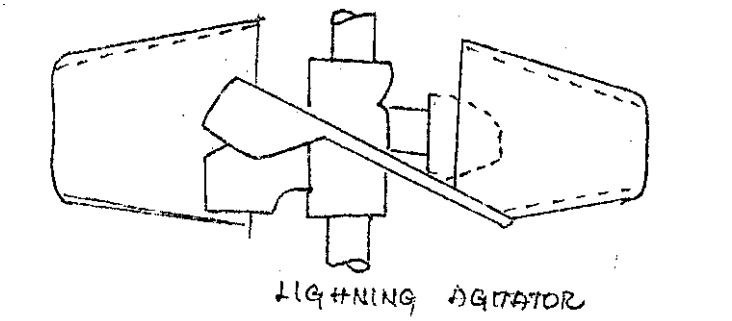
Agitators may be classified as due turbines vaned disis, open turbines of variables pitch and properties.



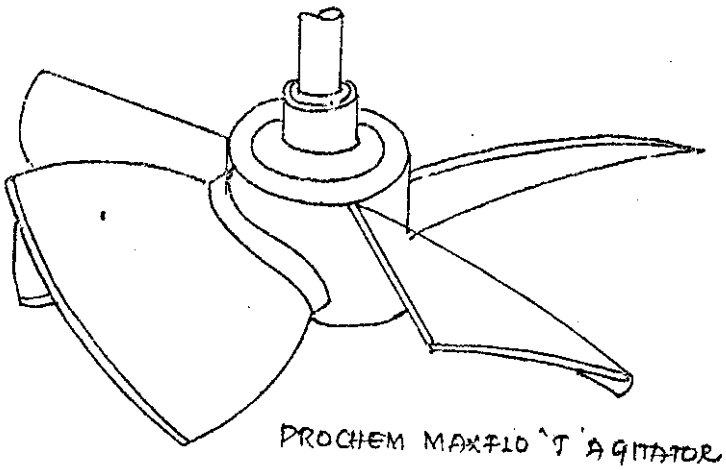

The disc turbine consists of a disc with a series of rectangular vanes set in a vertical plane around the circumference and the vanes disc has s series of rectangular vanes attached vertically to the underside. Air from the sprager hits the underside of the disc and is displaced towards the vanes where the bubbles are broken up into smaller bubbles. The vanes of the open turbine and the blades of the marine propeller are attached directly to a boss on the agitator shaft. Four other modern agitator developments which are derived from open turbines are also discussed below.



SCABA AGITATOR



LIGHNING AGITATOR



PROCHEM MAXFLO 'T' AGITATOR

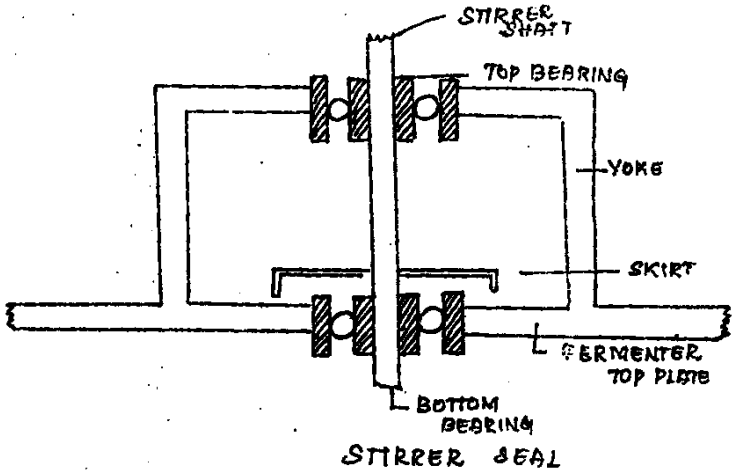
**STIRRER GLANDS AND BEARINGS**

The satisfactory sealing of the stirrer shaft assembly has been one of the most difficult problems to overcome in the construction of fermentation equipment which can be operated asceptically for long periods. A number of different designs have been developed to obtain asceptil seals. The stirrer shaft can enter the vessel from the top side or the bottom side of the vessel. To entry is most commonly used, but bottom entry may be advantageous if more space is

needed on the top plate for entry ports, and the shorter shaft permits higher stirrer speeds to be used by eliminating the problem of the shaft whipping at high speeds. The bottom entry shafts of stirrers were considered undesirable as the bearings would be submerged.

Three basic types of seal assembly have been used

(i) The stuffing Box (packed-gland seal)
(ii) The mechanical seal
(iii) Magnetic drive




**THE STUFFING BOX (PACKED-GLAND SEAL)**

The stuffing box was described by chainetal (1954). The shaft is sealed by several layers or packing rings of asbestos or cotton yavin, pressed against the shaft by a gland follower. At high stirrer speeds the packing wear quickly and excessive pressure may be needed the ensures tightness of fit stuffing box-bearing were commonly used in large-scale vessels, operational problems, particularly contamination, have led to their replacement by mechanical seal bearings for many processes.

**THE MECHANICAL SEAL**

The mechanical seal assembly is used in both small and large fermenter. The seal is composed of two parts, one part is stationary in the bearing housing, the other rotate on the shaft and the two components are pressed together by springs or precision machined, the moving surface normally consists of carbon-faced unit while the stationary unit is of stainless steel. Steam condensate is used to lubricate and cool the seals during operation and serve as a containment barrier.

Monitoring of the steam condensate flowing out of the seal is an effector ways for checking for seal failure. Disinfectants are alternatives for flushing the seals.

**MAGNETIC DRIVES**

The problems for providing a satisfactory seal when the impeller shaft passes through the top or bottom place of the fermenter may be solid by the use of a magnetic drive in which the impeller shaft does not piece the vessel. A magnetic drive consists of two magnets. One driving and one driven. The driving magnet is held in bearings in a housing on the outside of the head plate and connected to a drive shaft. The internal driven magnet is placed on one end of the impeller shaft and held in bearings in a suitable housing on the inner surface of the headplate. The magnetic drives are suitable for microbial fermentations upto 1500 $dm^3$ which could be used when higher containment levels are specified.

**BAFFLES**

Four baffles are normally incorporated into agitated vessels of all sizes to prevent a cortex and to improve aeration efficiency. In vessels over 3 $dm^3$ diameter six or eight baffles may be used . Baffles are metal strips roughly one-tenth of the vessel diameter and attached radially to the wall. The agitation effect is only slightly increased with wider baffles, but drops sharply with narrower baffles. Baffles should be installed so that a gap existed between them and the vessel wall, so that there was a scouring action around and behind the baffles thus minimizing the microbial growth the baffles.

**THE AERATION SYSTEM (SPARGER):**

A Sparges may be defined as a device for introducing air into the liquid in a fermenter. Three basic types of spargers have been used and may be described as the porous sparger, the orifice sparger (a perforated pipe) and the nozzle sparger (an open or partially closed pipe).

**POROUS SPARGER**

The porous sparger of sintered glass, caramics or metal, has been used primarily on a laboratory scale in non-agitated vessels. The bubble size produce from such spargers is always 10 to 100 times larger then the pore size of the aerator block.

**ORIFICE SPARGER**

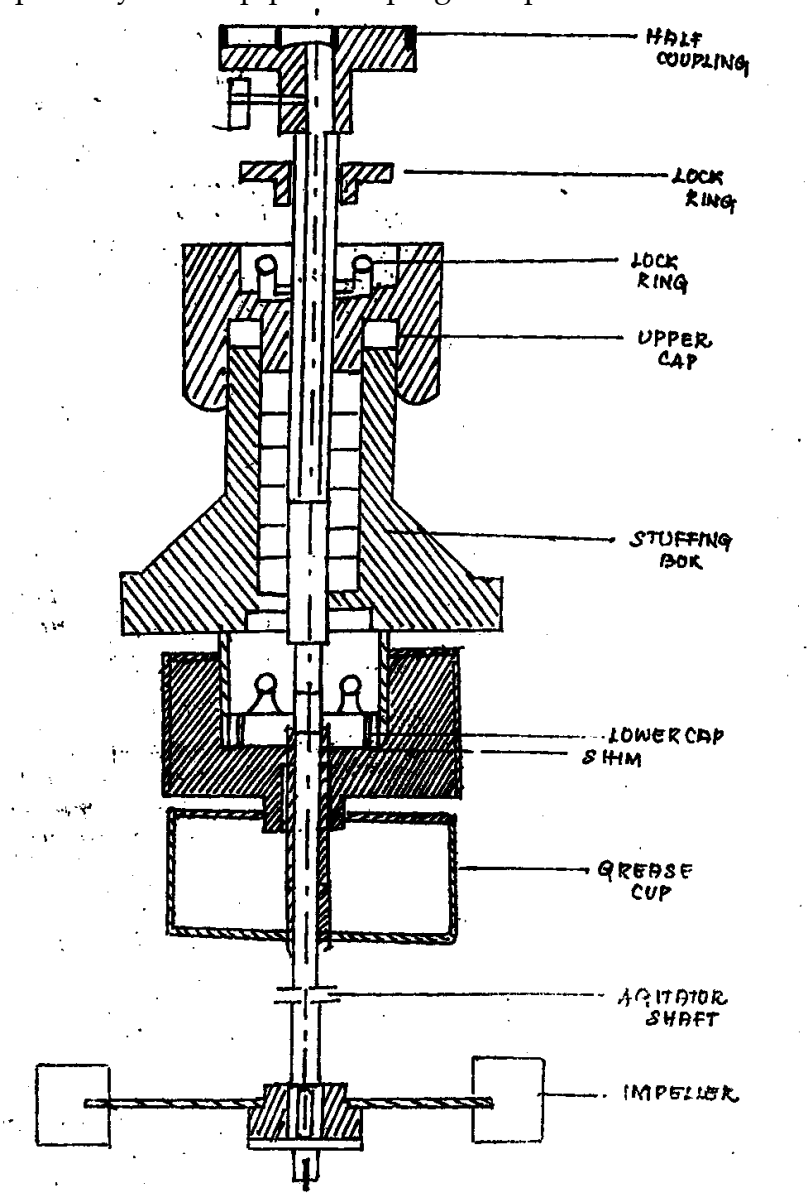
Various arrangements of perforated pipes have been tired in different types of fermentation vessel with or without impellers. In small stirred fermenters the perforated pipes were arranged

below the impeller in the form of crosses or rings, approximately three-quarters of the impeller diameter.

Orifice spargers without agitation have been used to a limited extent in yeast manufacture, efficient production treatment and the production of single-cell proteins.

**NOZZLE SPARGER**

Most modern mechanically stirred fermenter designs from laboratory to industrial scale have a single open or partially closed pipe as a sparger to provide steam or air bubbles.




**PACKED –GLAND STIRREER SEAL**

**FEED PORTS:**

Additions of nutrients and acid / alkali to small fermenters are normally made via silicons tubes which are autoclaved separately and pumped by a peristaltic pumps after asceptic connection. In larger units, the nutrients rescrons and associated piping are usually an integrated part which can be sterilized with the vessel.

**VALVES AND STEAM TRADS**

Valves are attached to fermenters and ancillary equipment are used for controlling the flow of liquids and gases in a variety of ways.

The valves may be

- Simple ON/OFF valves which are either fully open or fully closed.
- Valves which provide coarse control of flow rates
- Valves which may be adjusted very precisely so that the flow rates may be accurately controlled.
- Safety valves which are constructed in such a way that the liquids or gases will flow in only one direction.

A wide range of valves is available, but not all of them are used in fermenter construction.

**GATE VALVES:** In this valve, a sliding disc is moved in or out of the flow path by turning the stem of the valve. It is suitable for general purpose on a steam or a water line for use when fully open or fully closed and therefore should not be used for regulating flow.

**GLOBE VALVES:** In this value, a horizontal disc or plug is raised or lowered in its seating to control the rate of flow. This type of valve is very commonly used for regulating the flow of water or steam . Since it may be adjusted rapidly. It is not suitable for asceptic operation because of potential leakage round the valve stem which is similar to that of the gate valve.

**PISTON VALVES:** The piston valve is similar to that of the globe valve except that flow is controlled by a piston passing between the palking rings. This valve is efficient under asceptic conditions.

**NEEDLE VALVES:** The needle valve is similar to the globe valve except that the disc is replaced by a tapered plug or needle fitting into a tapered valve sear. The valve can ex used to give fine control of steam or liquid flow.

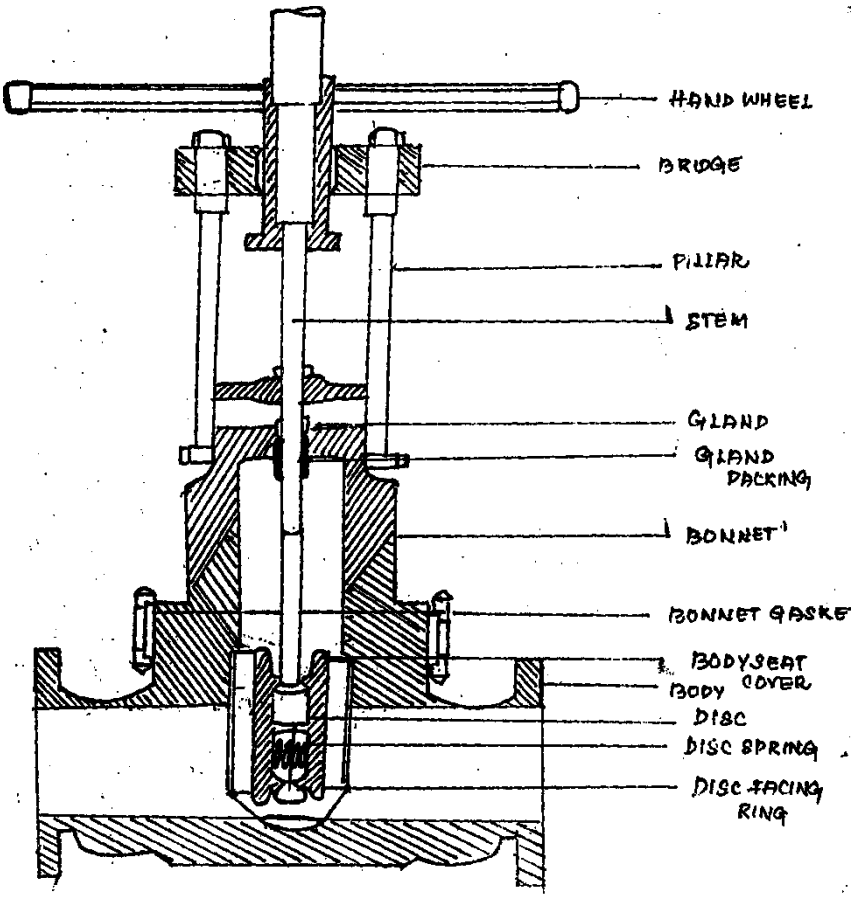
**PLUG VALVES:** In this valve, there is parallel to tapered plug sitting in housing through which an orifice has been maintained this type of valve has a tendency to seize or leak p, but the use of lubricants may overcome the problems.

**BALL VALVES:** This valve has been developed from the plug valves. The valve elements is stainless steel ball through which an orifice is machined. This valve is suitable for asceptic operation.

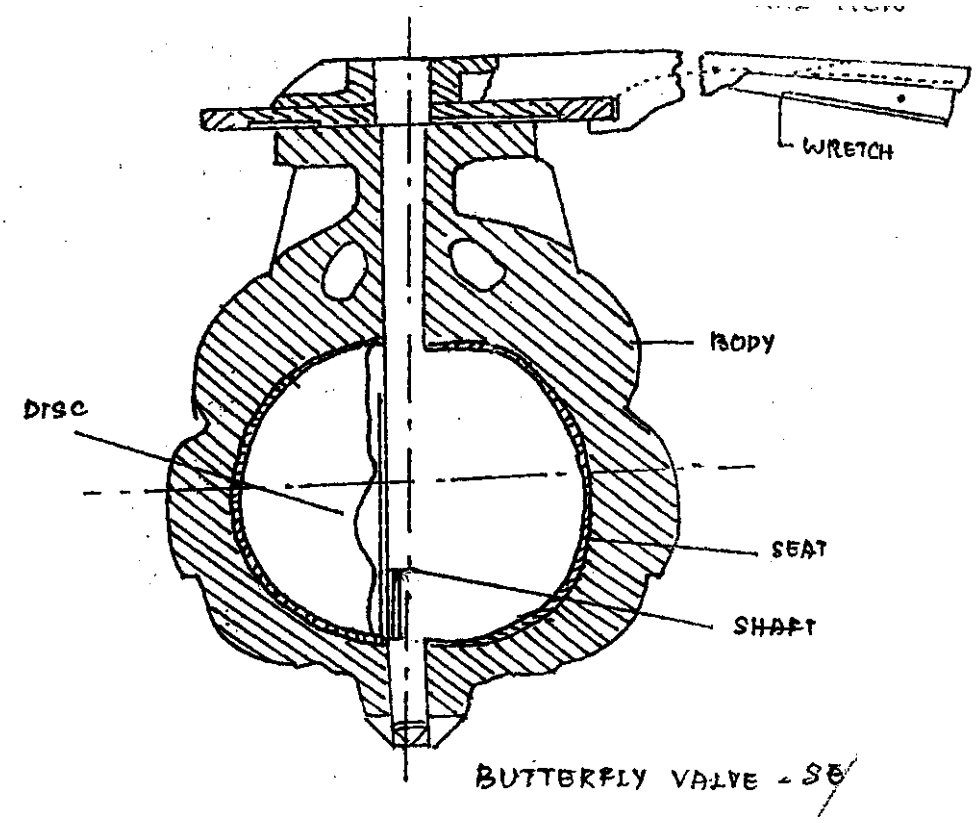
**BUTTERFLY VALVES:** This valve consist of a disc which rotates about a shaft in the housing., This is used a large diameter pipes operating under low pressure.

**PINCH VALVES:** The valve is suitable for asceptic operations with fermentation broths as there are nodded space to the valve structures.

**DIAPHRAMG VALVE:** This valve is like the pinch valve and is the mot suitable for asceptic operation provide the diaphragm of a material with withstand repeated sterilization our valves for crude flow, control , needle valves in securrate flow control. Diaphragma , pinch and ball valves for all sterilie users.




GATE VALVE – SECTIONAL VIEW




**5. Explain in detail about main parameters to be monitored and controlled in fermentations process**

**MAIN PARAMETERS TO BE MONITORED AND CONTROLLED IN FERMENTATION PROCESS:**

The success of a fermentation depends upon the existence of defined environment conditions for biomass and product formation. To achieve this goal it is important to understand what is happening to a fermentation process and how to control it to obtain optimal operating conditions. Thus the temperatures, pH, degree of agitation, oxygen concentration in the medium and other factors may have to be kept constant during the process.

A considerable number of process variables may need to be monitored during a fermentation. Methods for measuring these variables, the sensors or other equipment available and possible control procedure are outlined below.

There are three main classes of sensor:

- Sensors which penetrate into the interior of the fermenter
  Eg. pH electrodes , dissolved oxygen electrodes.

- Sensors which operate on samples which are continuously withdrawn from the fermenters
  Eg: Exhaust gas analysers

- Sensors which do not come into contact with the fermentation broth or gases.
  Eg: Tachometers, Load cells.

It is also possible to characterize a sensor in relation to its application for process control.

**IN-LINE SENSOR:** The sensor is an integrated part of the fermentation equipment and the measured valve obtained from it be used directly for process control.

**OFF-LINE SENSOR:** The sensor's is not part of the fermentation equipment. The measured valve cannot be used directly for process control. An operator is needed for actual measurement and for entering the measured valves into the control system for process control.

**METHODS OF MEASURING PROCESS VARIABLES TEMPERATURE**

The temperature in a vessel or pipe is one of the most important parameters to monitor and control in any process. It my be measured by mercury-in-glass thermometer, bimetallic thermometers, pressure bulb thermometers, thermocouplers, metal resistance thermometer, and thermistors. Metal resistance thermometer and thermistors are used in more fermentation application.

Accurate mercury in glass thermometer are used to cheek and calibrate the other forms of temperature sensors. A mercury-in-glass thermometer may be used in small bench fermenters, but its fragility restricts its use. In large fermenters it would be necessary to insert it into the thermometer pocket in the vessel, which introduces a time lag in registering the vessel temperatures. This type of thermometer can be used solely for indication, not for automatic control or recording.

**ELECTRICAL –RESISTANCE THERMOMETERS**

Electrical resistance of metals changes with temperature variation. This property has been used in the design of the resistance thermometers. The bulb of the instrument contains the resistance elements a mica framework (for very accurate measurement ) or a ceramic framework around which the sensing element is wound. A platinum wire of 100Ω resistance is normally used. Leads emerging from the bulb are connected to the measuring element. The reading is normally obtained by the use of a wheatstones bridges circuits and is the average temperature of the sensing element. This type of thermometer does have a greater accuracy (± 0.25%) than the other measuring devices.

**THERMISTORS**

Thermistors are semiconductors made from specific mixtures of pure oxides of iron, nickel and other metals. Their main characteristic is a large change in resistance with a small temperature change. The change in resistance is a function of absolute temperature. The temperature reading is obtained with a wheatstone bridge or a simple or more complex circuit depending on the application.

**TEMPERATURE CONTROL**

The use of water jackets or pipe coils within a fermenter as a means of temperature control. In many small systems there is a heating element, 300 to 400 W capacity being adequate for a 10-$dm^3$ fermenter, and a cooling water supply, these are ON or OFF depending on the need for heating or cooling. The heating element should be as small as possible to reduce the size of the heat 'sink' and resulting overshoot when heating is no longer required. In some cases it may be better to run the cooling water continuously at a study rate and to have the heating element only connected to the control unit. This can be an expensive made of operation if the water flows directly to waste. For a small scale use, the thermocirculation is used, which will pump recirculating thermostatically heated water through fermenters of upto 10 $dm^3$ capacity and give temperature control of ± 0.1.

In large fermenters, where heating during the fermentation is not normally required, a regularity valve at the cooling –water inlet may be sufficient to control the temperature. There may be provision for circulation of refrigerated brine if excessive cooling is required.

Low agitation speeds are often essential in animal cell culture vessels to minimize shear damage. In these vessels , heating fingers can create local hot spots' which may cause damage to cells very close to them. Heating jackets which have a lower heat output proportional to the surface area are used to overcome this problem.

**FLOW MEASUREMENTS AND CONTROL**

Flow measurement and control of both gases and liquids is important in process management.

**Gases:** one of the simplest methods for measuring gas flow to a fermenter is by means of a variable area meter. The most commonly used example is a rotameter, which consists of a vertically mounted glass tune with an increasing bore and enclosing a free – moving float which may be a ball or a hollow thimble. The position of the float in the graduated glass tune is indicative of flowrate. The accuracy depends on having the gas at a constant pressures, but errors of upto ±10% of full scale deflection ideally rotameters should not be sterilized and therefore

placed between a gas inlet and the stotile feltex. There is no – provision for on – une data logging with the simple rotameters. Metal tubes can be used in situations where glass is not satisfactory. Rotameters can also be used to measure liquid flow rates, provided that abrasive particles or fetrrous meters are not present.

The use of oxygen and carbon dioxide gas analyzers for efficient gas analysis requires the provision of very accurate gas – flow measurement if the analyzers are to be used effectively. Thermal – mass flow meters have been utilized for a range of 0 to 500 $dm^3$/min. these instruments have a ± 1% full scale accuracy and work on the principle of measuring a temperature difference across a heating device placed in the path of the gas flow.

Temperature probes such as thermistors are placed upstream and down stream of the heat source, which may be inside or outside the piping.

The mass flow rate of the gas, Q, can or calculated from the specific heat equation.

$$H=QC_P(T_2-T_1)$$

Where,

| | | |
|---|---|---|
| H | = | Heat transferred |
| Q | = | mass flow rate of gas |
| $C_P$ | = | Specific heat of the gas |
| $T_1$ | = | Temperature of gas before heat is transferred to it. |
| $T_2$ | = | Temperature of gas after heat is transferred to it. |

This equation can be rearranged as,

$$Q = \frac{H}{C_P\left(T_2 - T_1\right)}$$

A voltage signal can be obtained by the method of measurement which can be used in data togging control of gas flow is usually by needle values. Often this method of control is not sufficient, and it is necessary to incorporate a self – acting control value. At a small scale, such valves as the flowstat' are available

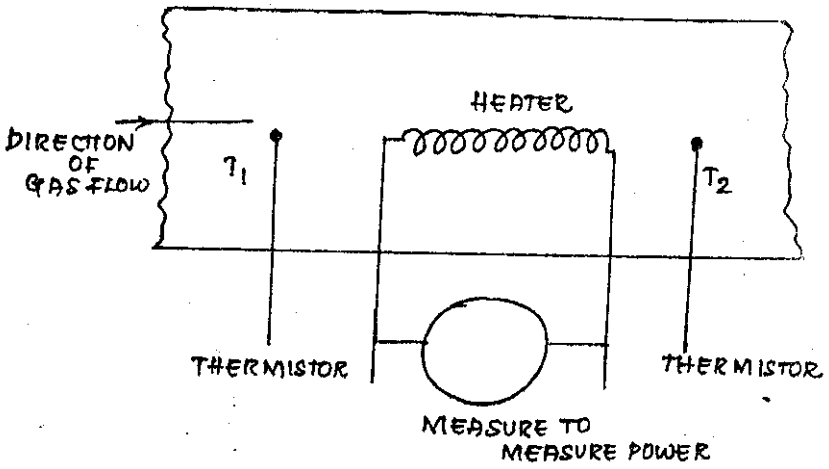



**Fig.**

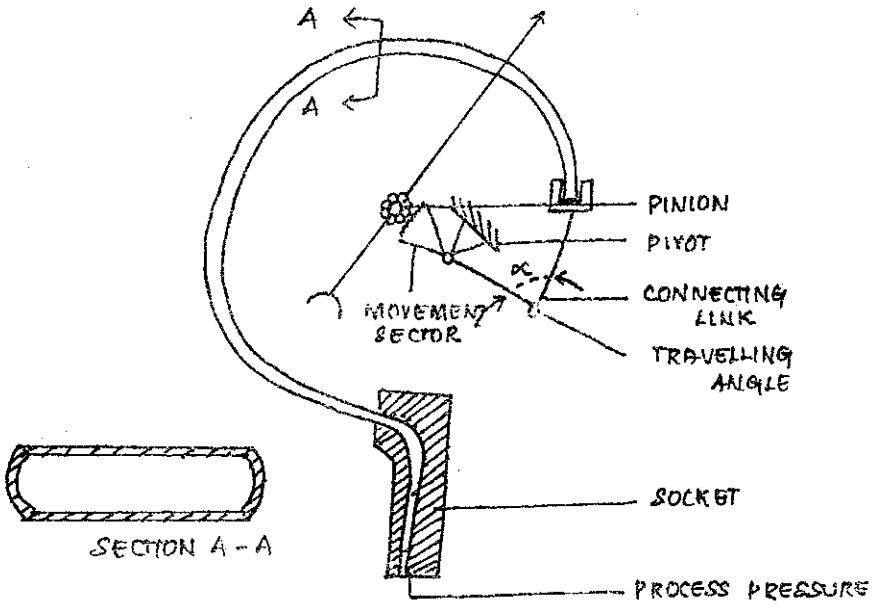
**Liquids:** The flow of non – sterile liquids can be monitored by a number of techniques but measurement of flow rates of sterile liquids presents a number of problems which have to be over come. On a laboratory state flow rates may be measured manually using a sterile burette connected to the feed pipe and timing the exit of a measured volume. A more expensive method is to use an electrical flow transducer which can cope with particulate matter in suspension and measure a range of flow rates from very low to high with an accuracy of ±1%. In this flow meter, there are two windings outside the tube, supplied with an alternating current to create a magnetic field. The voltage induced in the field is proportional to the relative velocity of fluid and the a magnetic field. The potential difference in the fluid can be measured by a pair of electrodes, and is directly proportional to the velocity of the fluid.

In batch and fed batch culture fermenters, a cheaper alternative is to measure flow rates indirectly by load cells. The fermenter and all ancillary reservoirs are attached to load cells which monitor the increases and decreases in weight of the various vessels at regular time intervals. Another indirect method of measuring flow rate ascetically is to use a metering pump which pumps liquid continuously at a predetermined and accurate rate. A variety of metering pumps are commercially available rate A variety of metering pumps are commercially available including motorized syringes, peristaltic pumps, piston pumps and diaphragms pumps.

Motorized syringes are used only when very small quantities of liquids have to be added slowly to the vessel. In a peristaltic pump, liquid is moved forward gradually by squeezing a tubing held in a semicircular housing. A piston pump contains an accurately machined ceramic or stainless steel piston moving in a cylinder normally fitted with a double ball inlet. Leakage can occur via the shaft housing of a piston pump. This problem can be prevented by the use of diaphragm pumps. This pump uses a flexible diaphragm to pump fluid through the housing with a ball to pump and to control the direction of flow. The diaphragm may be made of Teflon, neoprene, stainless steel and is actuated by a piston.

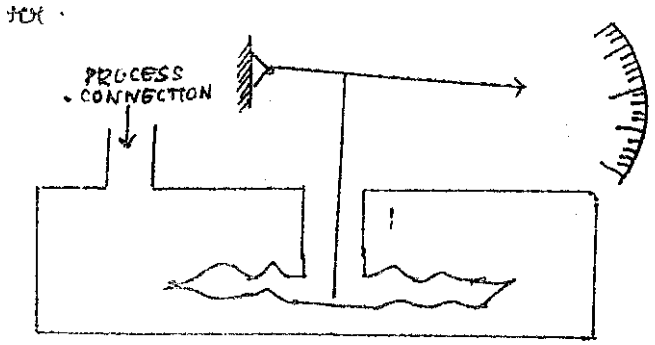
**PRESSURE MEASUREMENT**

Pressure is one of the crucial measurements that must be made when operating many processes. Pressure measurements may be needed for several reasons, the most important of which is safety. It is therefore important to fit the equipment with devices that will sense, indicate and record and control the pressure. The measurement of pressure is also important in media sterilization. In a fermenter, pressure will influence the source buffing of gases and contribute to the maintenance of sterility when a positive pressure is present.




'C' BOURDON TUBE PRESSURE GAUGE

One of the standard pressure measuring sensors is the Bourdon tube pressure gauge which is used as direct (A-A) which tends to become circulate with increasing pressures radii gradually straightens out. The process pressure is connected to the fixed socket end of the tube while the sealed tip of the other end of connected by a geared sector and pinion movement which actuates an indicates pointer to show linear rotational response when a vessel or pipe is to be operated under ascetic conditions a diaphragm gauge can be used. Changes in pressure cause movements of the diaphragm capsule which are monitored by mechanically levered pointer.




NESTED - DIAPHRAGM PRESSURE SENSOR

Alternatively, the pressure could be measured remotely using pressure bellows connected to the core of the variable transformer the movement of the core generates a corresponding output it is also possible to use pressure sensors incorporating strain gauges. If a wire is subjected to strain its electrical resistance changes, this is due in part to the changed dimensions of the wire and the change in resistivity which occurs due to the stress in wire. Another method is the use of piezoelectric transducer. Certain solid crystals such as quarts have an asymmetrical electrical charge distribution. Any change in shape of the crystal produces equal, external, unlike electric charges on the opposite faces of the crystal this is called the piezoelectric effect. Pressure can therefore be measured by means of electrodes attached to the opposite surface of the crystal.

**Pressure control**

Different working pressures are required in different parts of a fermentation plant. During normal operation a positive head pressure of 1.2 atmospheres absolute is maintained in fermenter to assist in the maintenance of aseptic conditions.

**Safety Valves**

Safety valves should be incorporated at various suitable places in all vessels and pipe layouts which are likely to be operated index pressure. The value should be set to release the pressure as soon as it increases above a specified working pressure.

**Agitator Shaft Power**

A variety of sensors can be used to measure the power consumption of a fermenter. On a large scale, a watt mete attached to the agitator mover will give a fairly good indication of power uptake. Torsion dynamometers can be used in small scale applications since the dynamometer has to be placed on the shaft outside the fermenter the measurement will once again include the friction in the bearings.

**Rate of Stirring**

In all fermenters it is important to monitor the rate of rotation (rpm) of the stirrer shaft. The tachometer used for this purpose may employ electromagnetic induction voltage generation, light sensing or magnetic force as detection mechanisms. The final choice of tachometer will be determined by the type of signal which is required for recording and / or process control for regulating the monitor speed and other ancillary equipment.

**IOAM sensing and control**

The formation of foam is a difficulty in many types of microbial fermentation which create serious problems if not controlled. It is a common practice to add an antifoam to a fermenter when the culture starts foaming about a certain predetermined levi.

A foam sensing and control unit is shown below. a problem is inserted through the top plate of the fermenter. The probe is a stainless steel rod, which is installed except at the tip, and set at a defined level above the broth surface. When the foam rises and touches the problem tip, a current is passed through the circuit of the probe, with the foam acting as an electrolyte and the vessel acting as on earth. The current actuates a pump or valve and the antifoam is released into the fermenter for a few seconds.

Process tuners are routinely included in the circuit to ensure that the antifoam has tune to mix into the medium and break down the foam before the probe is programmed affix a percent time internal to sense the foam level again and possibly actuate the pump n value.

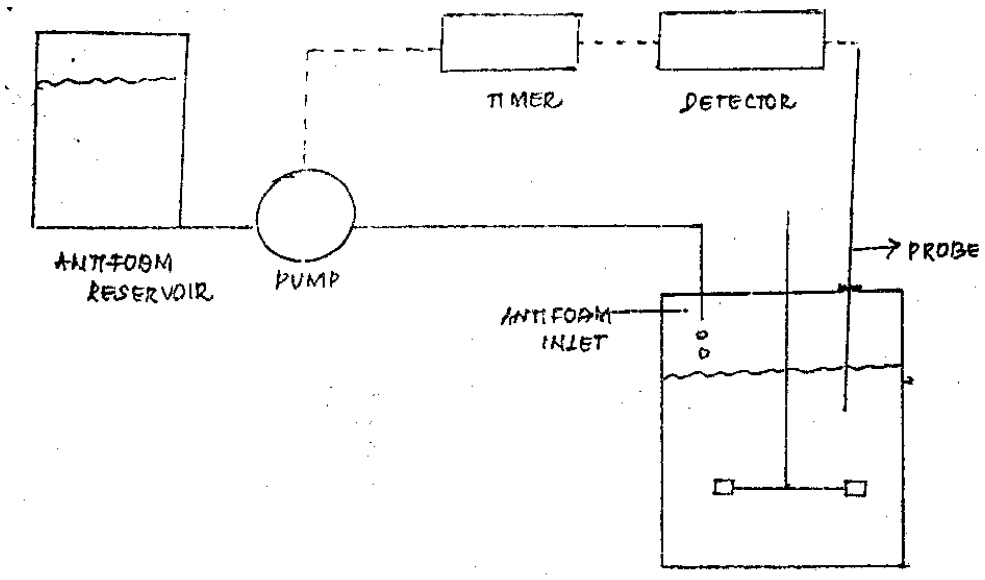



**Figure:**

A number of mechanical antifoam devices have been described including discs, propellers, brushes or hollow cones attached to the agitate shaft above the surface of the broth. The foam is broken down when it is thrown, against the walls of the fermenter.

**Weight:**

A load cell affers a convenient method of determining the weight of a fermenter a tild vessel. This is done by planning compression load cress in or at the foot of the vessel supports. When designing the support system for a fermenter or other vessel, the weight of which is to be measured by load cells, the principle of the three legged stool should be remembered. Three feet will always rest in stable equilibrium each though the supporting surface is uneven.

A load cell is essentially an elastic body, usually a solid or tabular steel cylinder, the compressive strain of which under axial load may be measured by a series of electrics resistance strain gauges which are cemented to the surface of the cylinder.

**BIOMASS:**

The estimation of microbial biomass in a fermenter is an obvious requirement, yet it has proved very difiallt to develop a satisfactory sensor. Most monitoring has been done indirectly by any weight samples, cell density (spectrophotometers) cell numbers (coulter counters) or by the use of gateway sensors.

**Measurement and control of dissolved oxygen:**

In most aerobic fermentations it is essential to ensure that the dissolved oxygen concentration does not exceed or fall below a specified level. The steam sterllizoble oxygen electrodes are available for the monitoring of dissolved oxygen.

The electrodes measure the partial pressures of the dissolved oxygen and not the dissolved oxygen concentration. Thus at equilibrium, the probe signal of an electrode will be determined by

$P(O_2) = C(O)_2 \times P_T$

Where, $P(O)_2$ is the partial pressure of dissolved oxygen sensed by the probe

$C(O_2)$ is the volume or mole fraction of oxygen in the gas phase.

$P_T$ is the total pressure.

The actual reading is normally expressed as percentage saturation with air at atmospheric pressure, so that 100% dissolved oxygen means a partial pressure of approximately 160 mm Hg. Pressure changes can have significant effects on readings. If the total pressures of the gas equilibrating with the fermentation broth varies, the electoral reading will change even through there is no change is the gas composition. Changes is atmospheric pressure can often cause 5% changes and back pressure due to the exit fitters can also cause increase in the readings.

In small fermenters ($1dm^3$), the commonest electrodes are the galvanic and have a lead anode, silver cathode and employ potassium hydroxide, chloride, bicarbonate or acetate as on electrolyte. The sensing tip of the electrodes is a Teflon, polyethylene and polystyrene membrane which all our the passage of gas phase so that an equilibrium is established with the gas phases inside and outside the electrode. Because of the scow movement of oxygen across the membrane, this type of electrodes has a slow response of the under of 60 seconds to achieve 90% of readings of true value.

Polorographic electrodes, which are (buffer) most bulkier than the galvanic electrodes, are most commonly used in the pilot and production fermenters, needing instrument parts of 12, 19 or 25 mm diameter. They have silver anodes which are negatively polarized with reference of the cathodes of platinum or gold, using aqueous potassium chlorial as the electrotype. Dissolved oxygen concentrations may also be determined by the tubing method. The probe consists to a coil of permeable Teflon or propylene tubing with in the fermenter through which is passed a stream of tietrum or nitrogen. The oxygen which diffuses from the fermentation medium through the tubing will in to the inert gas stream, is then determined using a paramagnetic gas analyzer.

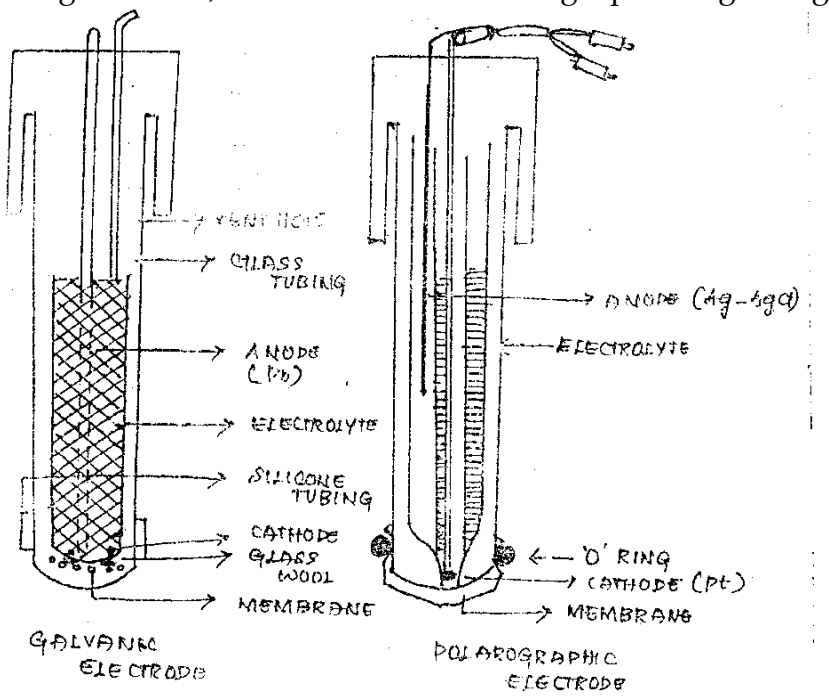



**Figure:**

The measurement and recording of the inlet and exit gas composition is important is many fermentation studies. Pay observing the concentrations of carbon dioxide and oxygen is the entry and exit gases in the fermenter and knowing the gas flow rate it is possible to determine the oxygen uptake of the system, the carbon dioxide evolution rate and the respiration rate of the microbial culture. The oxygen concentration can be determined by a paramagnetic gas analyzer. Oxygen has strong affinity for a magnetic field, a property which is shared with only nitrous and nitric oxides. The analyzers may be of deflection or thermal type.

In the deflection analyzer, the magnetic force acts on a dumb – bell test body that is free to rotate about an axis. The magnetic force which is created around the test body is proportional to the oxygen concentration. When the test body swings out of the magnetic field a corrective electrostatic force must be applied to return it to the original position. Electrostatic force readings can therefore be used as a measure of oxygen concentrations.

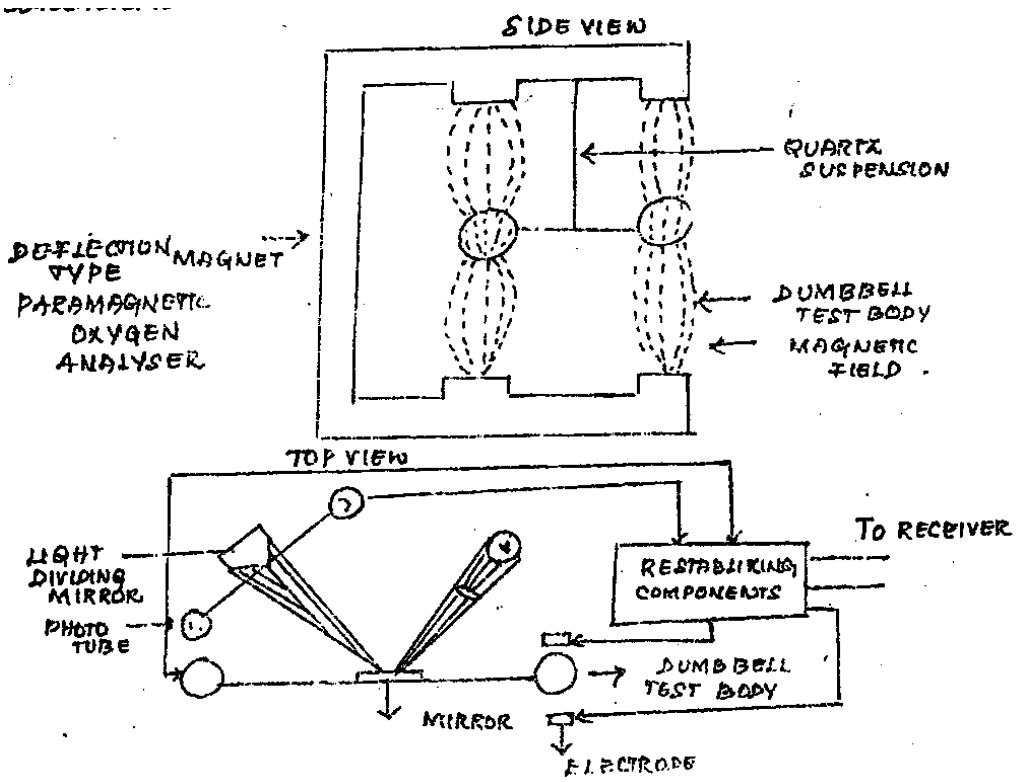



**Figure:**

In a thermal analyzer, a flow through ring element is a detection component. After entering the ring, the paramagnetic oxygen content of the sample is attracted by the magnetic field to the central glass tube where resistors heat the gases. The resistors are connected into a wheat stone bridge circuit to defect varatices in resistance due to flow rate change.

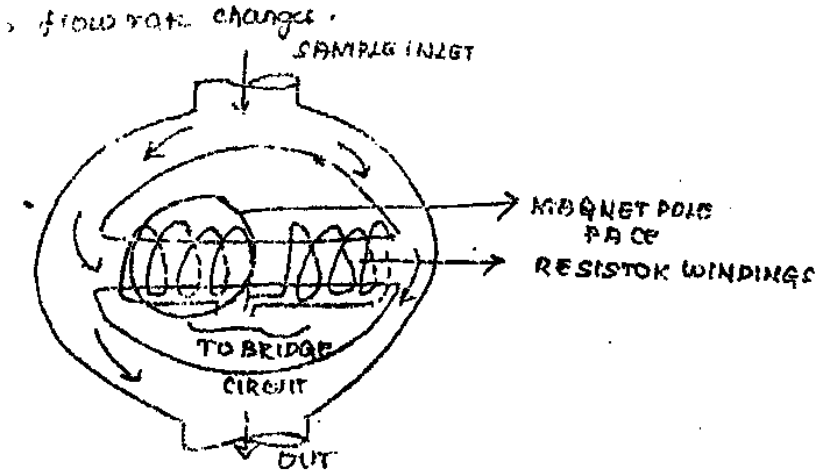



**Figure:**

**pH measurement and control:**

In batch culture, the pH of an actively growing culture will not remain constant for very long. In most processes there is a need for pH measurement and control during the fermentation if maximum yield of a product is to be obtained rapid changes in pH can often be reduced by a careful design of media. Particularly is the choice of carbon and nitrogen sources, and also is the in corporation of buffers or by batch feeding. The pH may be further controlled by the addition of appropriate quantities of ammonia or sodium hydroxide if too acidic or sculptures acid if the change is to an alkaline condition, normally the pH draft is only is one direction.

pH measurements is now routinely carried out using a combined glass reference electrode that will withstand repeated sterilization at temperatures of $121^0$ and pressures of 138 $kN/m^2$. The electrodes may be silver / silver chloride with potassium choroids or species formulations as an electrolyte.

## UNIT – II

## PART - A

**1. Write the possible criteria for good medium.**

- It will produce the maximum yield of product (or) biomass per gram of substrate used.
- It wills product the maximum concentration of product (or) biomass.
- It will permit the maximum rate of product formation
- There will be the minimum yield of undesired products

**2. Define defined media.**

Defined media contain specific amounts of pure chemical compounds with known chemical compositions. A medium containing glucose, $(NH_4)_2 SO_4$, $KH_2PO_4$ & $Mgcl_2$.

**3. Define complex media.**

Complex medium contain natural compounds whose chemical composition is not exactly known. A medium containing yeast extracts, peptones, molasses or corn steep liquor is a complex medium.

**4. Define cellular yield co – efficient.**

The cellular yield co – efficient is defined as the quantity of cell dry matter produced to the quantity of carbon substrate

$$\therefore\ Y = \frac{\text{Quantity of cell dry matter produced}}{\text{Quantity of carbon substrate utilized}}$$

**5. Write any two patterns of foaming in fermentations.**

- Foaming remains at a constant level through – out the fermentation. Initially it is due to the medium and later due to microbial activity.
- The fermentation has a low initial foaming capacity which rises.

**6. Write the two ways of approaching the problem due to excessive foaming.**

1. To try and avoid roam formation by using a defined medium and a modification of some of the physical parameters. (PH, temperature, aeration and agitation. This assumes that the foam is due to a component in the medium and not a metabolite.
2. The foam is unavoidable and antifoam should be used. This is the more standard approach.

**7. Define antifoams.**

Antifoams are surface active agents, reducing the surface tension in the foams and destabilizing protein films by

a. Hydrophobic bridges between two surfaces
b. Displacement of the absorbed protein and
c. Rapid spreading on the surface of the film.

**8. Write the properties of an ideal antifoam.**

- Should disperse readily and have fast action on existing foam.
- Should be active at low concentrations.
- Should be long acting in preventing new foam formation.
- Should not be metabolized by the micro – organism

**9. What is meant by chelators?**

The problem of insoluble metal phosphates may be eliminated by incorporating low control of chelating agents such as ethylene diamine tetraacetic acid (EDTA), citric acid etc., is to the medium. These chelating agents preferentially form complexes with the metal ions in a medium.

**10. Mention how oxygen is transferred from air to the cell during a fermentation.**

- The transfer of oxygen from air bubble into solution.
- The transfer of dissolved oxygen through the fermentation medium to the microbial cell.
- The uptake of the dissolved oxygen by the cell.

**11. Write the rate of oxygen transfer from air bubble to the liquid phase.**

The equation is given by

$$d c_L / dt = K_L a (C^* - C_L)$$

$C_L$ = concentration of dissolved oxygen in the fermentation broth (mmoles $dm^{-3}$) is time (hours)
$dC_L$ / dt – is the change in oxygen concentration over a time period ie., the oxygen transfer rate (mmoles $O_2$ $dm^{-3}$ $h^{-1}$)
$K_L$ – is the mass transfer coefficient ($cmh^{-1}$)
a - is the gas / liquid interface area per liquid volume ($cm^2$ $cm^{-3}$)
$C^*$ - is the saturation dissolved oxygen concentration (mmoles $dm^{-3}$)

**12. What is meant by DOT?**

DOT means dissolved oxygen tension or dissolved oxygen activity. The dissolved oxygen is usually monitored using a dissolved oxygen electrode which records dissolved oxygen activity or DOT.

**13. What is meant by OTR?**

$OTR = K_La.C^*$
OTR = Oxygen Transfer Rate
$K_L$ = mass transfer coefficient (cm $h^{-1}$)
a = is the gas / liquid interface area per liquid volume ($cm^2$ $cm^{-3}$)
C* - is the saturated dissolved oxygen concentration (mmoles $dm^{-3}$)

**14. What is meant by OUR?**

OUR – is the oxygen uptake rate.
$t_{cro} = C_L(ag) / OUR$

$t_{cro}$ – is the time for oxygen to be exhausted
$C_L(ag)$ – is the dissolved oxygen comic in the zone of the agitator

**15. Write the equation for power number.**

$NP = P/(PN^3D^5)$
NP = power number
P – External power from the agitator
P – Liquid density
N – Impeller rotational speed
D – Impeller diameter

**16. Write the equation of a volumetric oxygen transfer rate of a fermentor.**

The volumetric oxygen transfer rate of a culture is described by the term,
$Qo_2xxx$, where $Qo_2$ – specific oxygen uptake rate (mmo les $O_2$ $g^{-1}$ biomass$n^{-1}$)
X – biomass concentration (g $dm^{-3}$)
Thus units of $Qo_2x$- $dm^{-3}h^{-1}$.
It is given by the equation
$dc_L / dt = K_La (C^* - C_L)$

**17. Write some carbon sources used in media.**

- Starch waste (maize & potato)
- Molasses (cane & beet)
- Whey
- n – Alkanes
- Gas oil

**18. Write some hydrogen sources used in media.**

- Soya meal
- Yeast extract
- Distillers soluble
- Dried Blood
- Groundnut meal

**19. Write some mineral sources used in media.**

- ❖ Magnesium
- ❖ Phosphorus
- ❖ Potassium
- ❖ Sulphur
- ❖ Calcium
- ❖ Chlorine

**20. Write some vitamin sources used in media.**

- ➢ Ascorbic acid
- ➢ Thiamine
- ➢ Riboflavin
- ➢ Niacin
- ➢ Biotin
- ➢ Folic acid

## PART – B

**1. Write a detailed not on medium formulation.**

Medium formulation is an essential stage in the design of successful laboratory experiments, pilot – scale development and manufacturing processes. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production and there must be an adequate supply of energy for biosynthesis and cell maintenance. The first step to consider is an equation based on the stoichiometry for growth and product formation. Thus for an aerobic fermentation:

Carbon and energy source + nitrogen source + $O_2$ + other $\rightarrow$ requirements

Biomass + products + $CO_2$ + $H_2O$ + heat

This equation should be expressed in quantitative terms, which is important in the economical design of media if component wastage is to be minimal. Thus, it should be possible to calculate the minimal quantities of nutrients which will be needed to produce a specific amount of biomass. Knowing that a certain amount of biomass is necessary to produce a defined amount of product, it should be possible to calculate substrate concentrations necessary to produce required product yields. There may be medium' components which are needed for product formation which are not required for biomass production. Unfortunately, it is not always easy to quantity all the factors very precisely.

A knowledge of the elemental composition of a process micro – organism is required for the solution of the elemental balance equation. This information may not be available so that data which is given in table. Will serve as a guide to the absolute minimum quantities of N, S, P, Mg and K to include in an initial medium recipe. Trace elements (Fe, Zn, Cu, Mn, Co, Mo, B) may also be needed in smaller quantities. An analysis of relative concentrations of individual elements in bacterial cells and commonly used cultivation media quoted by Cooney (1981) showed that some nutrients are frequently added in substantial excess of that required, e.g. P,K; however, others are often near limiting values, e.g. Zn, Cu. The concentration of P is deliberately raised in many media to increase the buffering capacity. These points emphasize the need for considerable attention to be given to medium design.

Some micro – organisms cannot synthesize specific nutrients, e.g. amino acids, vitamins or nucleotides. Once a specific growth factor has been identified it can be incorporated into a medium in adequate amounts as a pure compound or as a component of a complex mixture.

The carbon substrate has a dual role in biosynthesis and energy generation. The carbon requirement for biomass production under aerobic conditions may be estimated from the cellular yield coefficient (Y) which is defined as:

$$\frac{\text{Quantity of cell dry matter produced}}{\text{Quantity of carbon substrate utilized}}$$

**Table: Cellular yield coefficient (Y) of bacteria on different carbon substrates (data from Abbott and Clamen, 1973)**

| Substrate | cellular yield coefficient (g biomass dry wt. $g^{-1}$ substrate) |
|---|---|
| Methane | 0.62 |
| n – Alkanes | 1.03 |
| Methanol | 0.40 |
| Ethanol | 0.68 |
| Acetate | 0.34 |
| Malate | 0.36 |
| Glucose (molasses) | 0.51 |

Some values are given in table. Thus for bacteria with a Y for glucose of 0.5 g cells $g^{-1}$ glucose, the concentration of glucose needed to obtain 30 g $dm^{-3}$ cells will be 30 / 0.5 = 60 g $dm^{-3}$ glucose. One litre of this medium would also need to contain approximately 3.0 g N, 1.0 g K, 0.3 g S and 0.1 g Mg. More details of Y values for different micro organisms and substrates are given by Atkinson and Mavituna (1991 b)

An adequate supply of the carbon source is also essential for a product – forming fermentation process. In a critical study, analyses are made to determine how the observed conversion of the carbon source to the product compares with the theoretical maximum yield. This may be difficult because of limited knowledge of the biosynthetic pathways. Cooney (1979) has calculated theoretical yields for penicillin G biosynthesis on the basis of material and energy balances using a biosynthetic pathway based on reaction stoichiometry. The stoichiometry equation for the overall synthesis is:

$$a_2\, C_6 H_{12} O_6 + b_2\, NH_3 + c_2 O_2 + d_2 H_2 SO_4 + e_2\, PAA \rightarrow n_2 \text{Pen G} + p_2\, CO_2 + q_2\, H_2O$$

Where $a_2$, $b_2$, $c_2$, $d_2$, $e_2$, $n_2$, $p_2$ and $q_2$ are the stoichiometric coefficients and PAA is phenylacetic acid. Solution of this equation yields:

$10/6\ C_6H_{12}O_6 + 2NH_3 + 1/2O_2 + H_2SO_4 + C_8H_8O_2 \rightarrow C_{16}H_{18}O_4N_2S + 2CO_2 + 9H_2O$

In this instance it was calculated that the theoretical yield was 1.1 g penicillin G $g^{-1}$ glucose (1837 units $mg^{-1}$).

Using a simple model for a batch – culture penicillin fermentation it was estimated that 28, 61 and 11% of the glucose consumed was used for cell mass, maintenance and penicillin respectively. When experimental results of a fed – batch penicillin fermentation were analyzed, 26% of the glucose has been used for growth, 70% for maintenance and 6% for penicillin. The maximum experimental conversion yield for penicillin was calculated to be 0.053$gg^{-1}$ glucose (88.5 units $mg^{-1}$). Thus, the theoretical conversion value is many times higher than the experimental value. Hersbach et al. (1984) concluded that there were six possible biosynthetic pathways for penicillin production and two possible mechanisms for ATP production from NADH and $FADH_2$. They calculated that conversion yields by different pathways varied from 638 to 1544 units of penicillin per mg glucose. At that time the best quoted yields were 200 units penicillin per mg glucose. This gives a production of 13 to 29% if the maximum theoretical yields.

The other major nutrient which will be required is oxygen which is provided by aerating the culture, and this aspect is considered in detail in chapter. The design of a medium will influence the oxygen demand of a culture in that the more reduced carbon sources will result in a higher oxygen demand. The amount of oxygen required may be determined stoichiometrically, and this aspect is also considered in chapter. Optimization is dealt with later in the chapter.

**2. Explain**
**a) Criteria for good medium**
**b) Medium requirements for fermentation process**

**Raw materials and media design for fermentation process:**

A detailed investigation is needed established the most suitable medium for an individual fermentation process, but certain basic requirements must be met by any such medium. All micro organisms require water, sources of energy, carbon, nitrogen, mineral elements and possibly vitamins plus oxygen if aerobic. On a small scale it is relatively simple to devise a medium containing pure compounds, but the resulting medium, although supporting satisfactory growth, may be unsuitable true in a large scale process. On a large scale one must normally use source of nutrients to create a medium which will meet as many as positive of the following criteria

**Criteria for Good Medium**

The following are the requirements of a good medium.

- It should produce the maximum yield of product or biomass per gram of substrate used.

- It should produce the maximum concentration of product in biomass.
- It should permit the maximum rate of product formation.
- These should be a minimum yield of undesired products
- It should be of consistent quality and be readily available throughout the year.
- It will cause minimal problems during media making and sterilization.
- It should case minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment

The use of cane molasses, beet molasses, areas grains, stanch, glucose, sucrose and lactose as carbon sources and ammonium salts, area nitrates, corn – steep waurs, Soya bean meal, slaughter – house waste and fermentation residues an nitrogen sources, have tended to meet most of the above criteria for production media because they are cheap subscribes.

**MEDIUM REQUIREMENTS FOR FERMENTATION PROCESS:**

**Water:** water is the major component of all fermentation media, and is needed in many of the ancillary services such as heating, cooling, cleaning and rising. Clean water of consistent composition is therefore required in large quantities from reliable permanent sources. When assessing the suitability of water supply it is important to consider PH, dissolved salts and efficient contamination.

The unaxial content of water is very important is brewing, and must actual in mashing process.

Hard waters containing high $CaSo_4$ concentration are better for English Burton Beces and prices type lagers, while water with high carbonate content are better for darker beers such as stouts

Nowadays the water may be treated by deionixation another techniques and state added, or pH adjusted, to favour different deer so that breweries are not dependent on local water sources. The reuse or efficient use of water is normally of high priority. In the continuous culture of single ceu protein (SCP), the production scale of 60,000 tonnes per year it was realized that very high costs would be inceveredif fresh purified water was used on a once – through basis this approach was therefore adopted in full scale process to reduce the capital cost and operating cost and it was estimated that water used in a once through basis without reeycling would have increased water costs by 50% and efficient treatment costs 10 fold

**Energy sources:**

Energy for growth comes from either the oxidation of medium components or from light. Most industrial micro – organisms are chemo – organotrophs, therefore the commonest sources of

energy will be the carbon source such as carbohydrates, lipids and proteins some micro – organisms can also use hydrocarbons or methanol as carbon and energy sources.

**Carbon sources:**

The rate at which the carbon source is metabouxed can of ten influence the formation of biomass or production of secondary and primary metaboutes. I art growth due to high concentrations of rapidly metabolixed sugars is often associated with low productivity of secondary metabolites. The problem was overcome by using the less readily metabolized sugars such as lactose, but many processes. Now use semi – continuous or continuous feed of glucose or sucrose. The main product of fermentation process will often determine the choice of carbon source, particularly if the product resulted from the direct dissimilation of it.

In fermentations such as ethanol or single – cell protein production where raw materials are 60 to 77% of the production cost, the selling price of the product will be determined largely by the cost of the carbon source. The purity of carbon source may also affect the choice of the substrate. For example, metallic ions citric acid processes.

The method of media preparation, particularly sterilization, may affect the sustability of carbohydrates for individual fermentation processes. If is often best to sterilize sugars separately because they may react with ammonium ions and amino acids to form black nitrogen containing compounds which coal partially inhibit the growth of many micro – organisms.

Starch suffers from the handicap that when heated in the sterilization process it gelatinizes, giving rise to a very viscous liquid (or) liquids. So that only concentrations of up to 2% can be used without modification.

**Carbohydrates**

It is a common practice to use carbohydrates as the carbon source in a microbial fermentation process. The most widely available carbohydrate is starch obtained from maize grain. It is also obtained from other cereals, potatoes and cassava Maize and other cereals may also be used directly in a partially ground state e.g. maize chips starch may also be readily hydrolyzed by ductile acids and enzymes to give a variety of glucose preparations.

Hydrolyzed cassava starch is used as a major carbon source for glutanie and production in Japan. Syrups produced by acid hydrolysis may also contain toxic products which may make them suitable for particular processes.

Barley grains may be partially germinated and heat treated to give a material known as malt, which contains a variety of sugars besides starch. Malt is the main substrate for brewing beer and layer in many countries. Malt extracts may also be prepared from malted grain.

Sucrose is obtained from sugarcane and sugar beet. It is commonly used in fermentation media in a very impure form as beet or cane molasses, which are residues left after the crystallization of sugar solutions in sugar refining. Molasses is used in the production of high – volume / low value products such as ethanol, scp, organic and amino acids and some maizebial gum. Molasses or sucrose can be used for the production of higher value / cow – bulk products such as antibiotics, specialty enzymes, vaccines and fine chemicals. The cost of molasses will be very high when compared with pure carbohydrates. However molasses contains many impurities and molasses based fermentations, will need a most expensive and complicated extraction / purification stage to remove impurities and efficient treatment will be more expensive because of unuttilized waste materials which oxestil present. The use of lactose and crude lactose (milk whey powder) in media formulations is now extremely limited such the introduction of continuous feeding process.

Corn steep liquor is a by – product of starch extraction from maize. Although primarily used as a nitrogen source, it does contain lactic acid, small amounts of reducing sugars and complex poly sacchoxides.

**Oils and Fats**

Oils were first used as carrier for antifoams in antibiotic processes vegetable oils may also be used as carbon substracts particularly for their content of fatty acids, oleic, unoleic and unolenic and because costs are competitive with those of carbon hydrates.

There are factors favoring then use of oil instead of carbohydrates. A typical oil contains approximately 2.4 times the energy of glucose on a per weight basis oils also have a volume advantage as it would take 1.24 $dm^3$ of soya bean oil to add 10k cal of energy to a fermenter, whereas it would take 5$dm^3$ of glucose sucrose assuming that they are being added as 50% w/w solutions. Ideally in any fermentation process, the maximum working capacity of a vessel should be used.

Oils also have an antifoam property which may make down stream processing simpler – on a purely technical basis glyaxol trifoliate was the most suitable substrate. When both technical and ewonmuat factors are considered, soyaben oil or rapeseed oil are the preferred substrates. Glyuxol violeate is known to be used in some fermentations where substrate purity is an important consideration. Methyl oleate has been used as the sole carbon substrate in cephalosporin production.

**Hydrocarbon and their derivatives**

A-alkanes hair been used for the production of organic acids, amino acids, vitamins and 10 factors, nucleic acids, antibiotics, enzymes and proteins. Methane, methanol and n-alkanes hair been used as been used as substrates for biomass production. There are certain advantages and disadvantages of hydrocarbons and their derivatives as fermentation substrates, particularly with returnee to cost / process aspects and purity.

A detailed study of hydrocarbons show that the cost of hydrocarbons does not make them economically attractive bulk feed stocks for the production of established products or potential new products where feedstock costs are an appreciable fraction of manufacturing costs of now value bulk products.

**Nitrogen Sources**

Most industrially used micro – organisms can iltelixe in organic and organic sources of nitrogen inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates. Ammonia has been used for pH control and as a major nitrogen source is a defined medium for the commercial production of human serum albumin by saccharomyces cerevisai. Ammonium salts such as ammonium sulphate will usually produce acid conditions as the ammonium ion is utilized and the free acid will be librated.

Nitrate will normally cause an alkane drift as they are metabolized. Ammonium nitrate will first cause an acid drift as the ammonium ion is utellxed and the free acid will be liberated and nitrate assimilates is repressed. When the ammonium ion has been exhausted there is an alkane drift as the nitrate is used as an alternative nitrogen source.

Organic nitrogen may be supplied as amino acid, protein or urea in many instances growth will be faster with the supply of organic nitrogen, and a few micro organism have an absolute requirement for amino acids.

Other proteinaceous nitrogen compounds serving as sources of amino acids include corn – step liquor, soya meal, peanut meal cotton seed meal. Chemically defined amino acid media devoid of protein are necessary in the production of certain vaccines when they are intended for human use. Control mechanism exist by which nitrate reductose an enzyme involved in the conversion of nitrate to ammonium ion, is repressed is the presence of ammonia. In this reaction, ammonia and ammonium ion is the preferred nitrogen source.

In Aspergillus's nidulans, ammonia also regulates the production of alkaline and neutral proteases. Therefore the mixtures of nitrogen sources, individual nitrogen components may

influence metabolic regulation. It has been shown that antibiotic production by many micro – organisms is influenced by the type and concentration of the nitrogen source in the culture medium. Antibiotic production may be inhibited by a rapidly utilized nitrogen source. In shake flask media experiments, salts of weak acids may be used to serve as a nitrogen source and eradicate the source of the strong acid PH change due to chloride or sulphate ions which would be present if ammonium chloride or sulphate were used as nitrogen sources.

The use of complex nitrogen sources for antibiotic productions has been a common practice. In the production of polyene antibiotics. Soyabean meat is considered a good nitrogen source because of the balance of nutrients.

In gibberellin production, the nitrogen sources has been shown to have an influence on the directing of production of different gibberellins and the relative.

**Minerals:**

All microorganisms require certain mineral elements for growth and metabolism. In many media, magnesium, Phosphorus, potassium, sulphur, calcium, and chlorine are essential components and because of the concentration required they must be added as distinct components. Others such as cobalt, copper, iron, manganese, molybedenum, and zinc are also essential but are usually present as impurities in other major ingredients.

In specific processes the concentration of certain minerals may be very critical. Some secondary metabolic processes have a lower tolerance range to inorganic phosphate than vegetative growth the inorganic phosphate concentration also influences production of bactelicins, citric acid (surface culture), agot, monomycin, novobiocin, onylitracyline, polyenes, ristomycin rifamyciny, streptomycin, vanomycin and viomycin.

In the recent review of antibiotic biosynthetis recognised target enzymes which were repressed by (a) repressed by phosphate (b) inhibited by phosphate (c) repression of an enzyme occurs but phosphate repression is not clearly proved.

The concentration of manganese, iron and zinc are the most critical in secondary metabolism.

**3. Explain detail about medium optimization methods.**

**Medium optimization:**

At this stage it is important to consider the optimization of a medium such that it meets as many as possible of the seven criteria given in the introduction to this chapter. The meaning of optimization in this context does need careful consideration (Winkler, 1991). When considering

the biomass growth phase in isolation it must be recognized that efficiently grown biomass produced by an 'optimized' high productivity growth phase is not necessarily best suited for its ultimate purpose, such as synthesizing the desired product. Different combinations and sequences of process conditions need to be investigated to determine the growth conditions which produce the biomass with the physiological state best constituted for product formation. There may be a sequence of phases each with a specific set of optimal conditions.

Medium optimization by the classical method of changing one independent variable (nutrient, antifoam, pH, temperature, etc.,) while fixing all the others at a certain level can be extremely time consuming and expensive for a large number of variables. To make a full factorial search which would examine each possible combination of independent variable at appropriate levels could require a large number of experiments, $x^n$, where x is the number of levels and n is the number of variables. This may be quite appropriate for three nutrients at two concentrations ($2^3$ trials) but not for six nutrients at two concentrations. In this instance $3^6$ (729) trials would be needed. Industrially the aim is to perform the minimum number of experiments to determine optimal conditions. Other alternative strategies must therefore be considered which allow more than one variable to be changed at a time. These methods have been discussed by Stowe and Mayer (1966), McDaniel et al. (1976), Hendrix (1980), Nelson (1982), Greasham and Inamine (1986), Bull et al (1990) and Hicks (1993).

When more than five independent variables are to be investigated, the Plackett – Burmar design may be used to find the most important variables in a system, which are then optimized in further studies (Plackett and Burman, 1946). These authors give a series of designs for up to one hundred experiments using an experimental rationale known as balanced incomplete blocks. This technique allows for the evaluation of X – 1 variables by X experiments. X must be a multiple of 4, e.g., 8, 12, 16, 20, 24, etc. Normally one determines how many experimental variables need to be included in an investigation and then selects the Plackett – Burman design which meets that requirement most closely in multiples of 4. Any factors not assigned to variable can be designated as hummy variables. As will be shown shortly in a worked example, the incorporation of dummy variables into an experiment makes it possible to estimate the variance of an effect (experimental error).

Table shows a Plackett – Burman design for seven variables (A – G) at high and low levels in which two factors, E and G, are designated as 'dummy' variables. These can then be used in the design to obtain an estimate of error. Normally three dummy variables will provide an adequate estimate of the error. However, more can be used if fewer real variables need to be studied in an investigation (Stowe and Mayer, 1966). Each horizontal row represents a trial and each vertical column represents the H(high) and L (low) values of one variable in all the trials.

**Table: Plackett – Burman design for seven variables (Nelson, 1982)**

| Trial | Variables | | | | | | | Yield |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | |
| 1 | H | H | H | L | H | L | H | 1.1 |
| 2 | L | H | H | H | L | H | L | 6.3 |
| 3 | L | L | H | H | H | L | H | 1.2 |
| 4 | H | L | L | H | H | H | L | 0.8 |
| 5 | L | H | L | L | H | H | H | 6.0 |
| 6 | H | L | H | L | L | H | H | 0.9 |
| 7 | H | H | L | H | L | L | H | 1.1 |
| 8 | L | L | L | L | L | L | L | 1.4 |

**H denotes a high level value; L denotes a low level value**

This design requires that the frequency of each level of a variable in a given column should be equal and that in each test (horizontal row) the number of high and low variables should be equal. Consider the variable A; for the trials in which a is high, B is high in two of the trials and low in the other two, similarly, C will be high in two trials and low in two, as will all the remaining variables. For those trials in which A is low, B will be high two times and low two times. This will also apply to all the other variables. Thus, the effects of changing the other variables cancel out when determining the effect of A. The same logic then applies to each variable. However, no changes are made to the high and low values for the E and G columns. Greasham and Inamine (1986) state that although the difference between the levels of each variable must be large enough to ensure that the optimum response will be included, caution must be taken when setting the level differential for sensitive variables, since a differential that is too large could mask the other variables. The trials are carried out in a randomized sequence.

**Table: Analysis of the yields shown in table 1 (Nelson 1982)**

| | Factor | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G |
| Σ (H) | 3.9 | 14.5 | 9.5 | 9.4 | 9.1 | 14.0 | 9.2 |
| Σ(L) | 14.9 | 4.3 | 9.3 | 9.4 | 9.7 | 4.8 | 9.6 |
| Difference | -11.0 | 10.2 | 0.2 | 0.0 | - 0.6 | 9.2 | -0.1 |
| Effect | -2.75 | 2.55 | 0.05 | 0.00 | - 0.15 | 2.30 | -0.10 |
| Mean square | 15.125 | 13.005 | 0.005 | 0.000 | 0.045 | 10.580 | 0.020 |
| Mean square for 'error' $= \frac{0.045 + 0.020}{2} = 0.0325$ | | | | | | | |

The effects of the dummy variables are calculated in the same way as the effects of the experimental variable. If there are no interactions and no errors in measuring the response, the effect shown by a dummy variable should be 0. If the effect is not equal to 0, it is assumed to be a measure of the lack of experimental precision plus any analytical error in measuring the response (Stowe and Mayer, 1966).

This procedure will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in a more detailed study to determine the optimum values to use.

The stages in analyzing the data using Nelson's (1982) example are follows:

1. Determine the difference between the average of the H (high) and L (low) responses for each independent and dummy variable.

There fore the difference = Σ A (H) - Σ A (L)

The effect of an independent variable on the response is the different between the average response for the four experiments at the high level and the average for four experiments at the low level.
Thus the effect of

$$A = \frac{\Sigma A(H)}{4} - \frac{\Sigma A(L)}{4} = \frac{2\left(\Sigma A(H) = \Sigma A(L)\right)}{8}$$

This value should be near zero for the dummy variables.

2. Estimate the mean square of each variable ( the variance of effect).

For A the mean square will be = $\frac{\left(\Sigma A(H) = \Sigma A(L)\right)^2}{8}$

3. The experimental error can be calculated by averaging the mean squares of the dummy effects of E and G.

Thus, the mean square for error = $\frac{0.045 + 0.020}{2} = 0.0325$

This experimental error is not significant;

4. The final stage is to identify the factors which are showing large effects. In the example this was done using an F – test for

$$\frac{\text{Factor mean square}}{\text{error mean square}}$$

This gives the following values:

$$A = \frac{15.125}{0.0325} = 465.4,$$

$$B = \frac{13.005}{0.0325} = 400.2$$

$$C = \frac{0.0500}{0.0325} = 3.255$$

$$D = \frac{0.0000}{0.0325} = 0.00$$

$$F = \frac{10.580}{0.0325} = 325.6$$

When Probability Tables are examined it is found that factors A, B and F show large effects which are very significant, whereas C shows a very low effect which is not significant and D shows no effect. A,B and F have been identified as the most important factors. The next stage would then be the optimization of the concentration of each factor, which will be discussed later.

Nelson (1982) has also referred to the possibility of two factor interactions which might occur when designing table. This technique has also been discussed by McDaniel et al. (1976), Greasham and Inamine (1986),Bull et al. (1990) and Hicks (1993).

The next stage in medium optimization would be to determine the optimum level of each key independent variable which has been identified by the Plackett – Burman design. This may be done using response surface optimization techniques which were introduced by Box and Wilson (1951). Hendrix (1980) has given a very readable account of this technique and the way in which it may be applied. Response surfaces are similar to contour plots or topographical maps. Whilst topographical maps show lines of constant elevation, contour plots show lines of constant value. Thus, the contours of a response. In this context, response means the results of an experiment carried out at particular values of the variables being investigated.

The axes of the contour plot are the experimental variables and the area within the axes is termed the response surface. To construct a contour plot, the results (responses) of a series of

experiments employing different combinations of the variables are inserted on the surface of the plot at the points delineated by the experimental conditions. Points giving the same results (equal responses) are then joined together to make a contour line. In it simplest form two variables are examined and the plot is two dimensional. It is important to appreciate that both variables are changed in the experimental series, rather than one being maintained constant, to ensure that the data are distributed over the response surface. In figure the profile generated by fixing $X_1$ and changing $X_2$ and then using the best $X_2$ value and changing $X_1$ constitutes a cross which may not encroach upon the area in which the optimum resides.

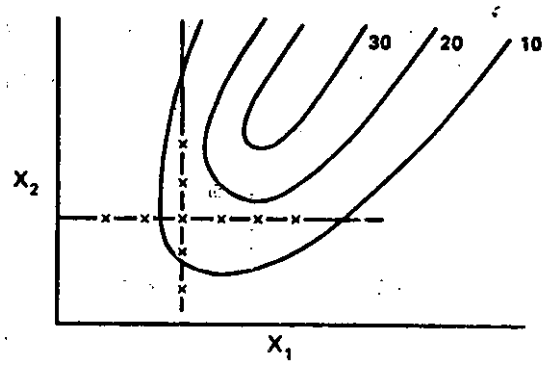



**Figure: Optimal point of a response surface by one factor at a time.**

The technique may be applied at different levels of sophistication. Hendrix applied the technique at its simplest level to predict the optimum combination of two variables. The values of the variables for the initial experiments are chosen randomly or with the guidance of previous experience of the process. There is little to be gained from using more than 15 – 20 experiments. The resulting contour map gives an indication of the area in which the optimum combination of variables resides. A new set of experiments may then be designed within the indicated zone. Hendrix proposed the following strategy to arrive at the optimum in an incremental fashion.

1. Define the space on the plot to be explored.
2. Run five random experiments in this space.
3. Define a new space centered upon the best of the five experiments and make the new space smaller than the previous one, perhaps by cutting each dimension by one half.
4. Run five more random experiments in this new space.
5. Continue doing this until no further improvement is observed, or until you cannot afford any more experiments.

The more sophisticated applications of the response surface technique use mathematical models to analyze the first round of experimental data and to predict the relationship between the response and the variables. These calculations then allow predictive contours to be drawn and facilitate a more rapid optimization with fewer experiments. If there or more variables are to be

examined then several contour maps will have to be constructed. Hicks (1993) gives an excellent account of the development of equations to model the different interactions which may take place between the variables. Several compute software packages are now available which allow the operator to determine the equations underlying the responses and, thus, to determine the likely area on the surface in which the optimum resides. Some examples of the types of response surface profiles that may be generated are illustrated in figure.

The following examples illustrate the application of the technique:

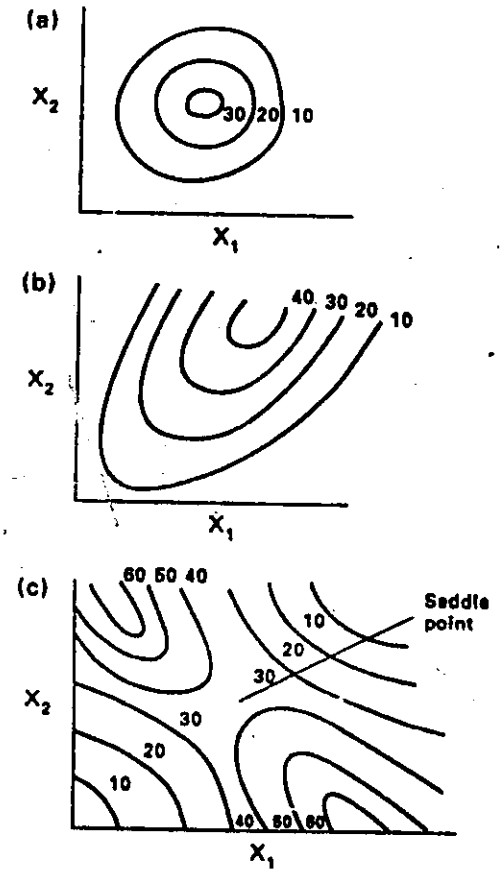



**Figure: Typical response surfaces in two dimensions (a) mound, (b) rising ridge, (c) saddle.**

1. McDaniel et al. (1976), figure the variables under investigation were cerelose and soybean level, with the analysis indicating the optimum to be 6.2% cerelose and 3.2% soybean.
2. Saval et at. (1993). The medium for streptomycin production was optimized for four components resulting in a 52% increase in streptomycin yield, a 10% increase in mycelial dry weight and a 48% increase in specific growth rate.

**4. Explain Growth Media.**

**Growth Media:**

Two major types of growth media are defined and complex media. Defined media contain specific amounts of pure chemical compounds with known chemical compositions. A medium containing glucose, $(NH_4)_2SO_4$, $KH_2PO_4$, and $MgCl_2$ is a defined medium. Complex media contain natural compounds whose chemical composition is not exactly known. A medium containing yeast extracts, peptone, molasses, or corn steep liquor is a complex medium. A complex medium usually can provide the necessary growth factors, vitamins, hormones, and trace elements, often resulting in higher cell yields, compared to the defined medium. Often, complex media are less expensive than defined media. The primary advantage of defined media is that the results are more reproducible and the operator has better control of the fermentation. Further, recovery and purification of a product is often easier and cheaper in defined media. Table summarizes typical defined and complex media.

**Compositions of Typical Defined and complex Media**

| | **Defined medium** | |
|---|---|---|
| **Constituent** | **Purpose** | **Conan (g/liter)** |
| Group A | | |
| Glucose | C, energy | 30 |
| $KH_2PO_4$ | K, P | 1.5 |
| $MgSO_4.7H_2O$ | Mg, S | 0.6 |
| $CaCl_2$ | Ca | 0.05 |
| $Fe_2(SO_4)_3$ | Fe | $15 \times 10^{-4}$ |
| $ZnSO_4.7H_2O$ | Zn | $6 \times 10^{-4}$ |
| $CuSO_4.\ 5H_2O$ | Cu | $6 \times 10^{-4}$ |
| $MnSO_4.\ H_2O$ | Mn | $6 \times 10^{-4}$ |
| Group B | | |
| $(NH_4)_2HPO_4$ | N | 6 |
| $(NH_4)H_2PO_4$ | N | 5 |
| Group C | | |
| $C_6H_5Na_3O_7.\ 2H_2O$ | Chelator | 4 |
| Group D | | |
| $Na_2HPO_4$ | Buffer | 20 |

| | | |
|---|---|---|
| $KH_2PO_4$ | Buffer | 10 |

Complex medium used in a penicillin fermentation

| | |
|---|---|
| Glucose or molasses (by continuous feed) | 10% of total |
| Corn steep liquor | 1 -5% of total |
| Phenylacetic acid (by continuous feed) | 0.5 – 0.8% of total |
| Lard oil (or vegetable oil) antifoam by continuous addition | 0.5% of total |
| pH to 6.5 to 7.5 by acid or alkali addition | |

**5. Write in detail about oxygen requirements for a fermenter.**

**Oxygen requirements:**

It is sometimes forgotten that oxygen, although not added to an initial medium as such, is nevertheless a very important component of the medium in many processes, and its availability can be extremely important in controlling growth rate and metabolite production. This will be discussed in detail in chapter.

The medium may influence the oxygen availability in a number of ways including the following.

1. Fast metabolism. The culture may becomes oxygen limited because sufficient oxygen cannot be made available in the fermenter if certain substrates, such as rapidly metabolized sugars which lead to a high oxygen demand, are available in high concentrations.
2. Rheology. The individual components of the medium can influence the viscosity of the final medium and its subsequent behaviour with respect to aeration and agitation.
3. Antifoams. Many of the antifoams in use will act as surface active agents and reduce the oxygen transfer rate. This topic will be considered in a later section of this chapter.

**Fast Metabolism:**

Nutritional factors can alter the oxygen demand of the culture. Penicillium chrysogenum will utilize glucose more rapidly than lactose or sucrose, and it therefore has a higher specific oxygen uptake rate when glucose is the main carbon source (Johnson, 1946). Therefore, when there is the possibility of oxygen limitation due to fast metabolism, it may be overcome by reducing the initial concentration of key substrates in the medium and adding additional

quantities of these substrates as continuous or semi – continuous feed during the fermentation. It can also be overcome by changing the composition of the medium, incorporating higher carbohydrates (lactose, starch, etc). and proteins which are not very rapidly metabolized and do not support such a large specific oxygen uptake rate.

**Rheology:**

Deindoerfer and West (1960) reported that there can be considerable variation in the viscosity of compounds that may be included in fermentation media.

**Table: Some processes using batch feed or continuous feed or in which they have been tried**

| Product | Additions | Reference |
|---|---|---|
| Yeast | Molasses, nitrogen sources, P and Mg | Harrison (1971)<br>Reed and Peppler (1973 |
| Glycerold | Sugar, $Na_2CO_3$ | Eoff et al. (1919) |
| Acetone – butly alcohol | Additions and withdrawals of wort | Soc. Richard et al (1921) |
| Riboflavin | Carbohydrate | Moss and Klein (1946) |
| Penicillin | Glucose and $NH_3$ | Hosler and Johnson (1953) |
| Novobiocin | Various carbon and nitrogen sources | Smith (1956) |
| Griseofulvin | Carbohydrate | Hockenhull (1956) |
| Rifamycin | Glucose, fatty acids | Pan et al. (1959) |
| Gibberellins | Glucose | Borrow et al. (1960) |
| Vitamin $B_{12}$ | Glucose | Becher et al. (1961) |
| Tetracyclines | Glucose | Avanzini (1963) |
| Citric acid | Carbohydrates, $NH_3$ | Shepherd (1963) |
| Single – cell protein | Methanol | Harrison et al. (1972) |
| Candicidin | Glucose | Martin and McDaniel (1975) |
| Streptomycin | Glucose, ammonium sulphate | Singh et al. (1976) |
| Cephalosporin | Fresh medium addition | Trilli et al. (1977) |

Polymers in solution, particularly starch and other polysaccharides, may contribute to the rheological behavior of the fermentation broth (Tuffile and Pinho, 1970). As the polysaccharide is degraded, the effects on rheological properties will change. Allowances may also have to the made for polysaccharides being produced by the micro – organism.

## UNIT – III

## PART – A

**1. Define Del factor (or) Nabla factor.**

Del factor is a measure of the fractional reduction inviable organism count produce by a certain heat and time regime.

$$\therefore \nabla = \ln\left(N_o / N_t\right)$$

**2. Define Decimal reduction time.**

It is the time taken at a particular temperature to reduce the initial population by a factor of 10. Thus

$$N_t / N_o = 1 / 10$$

$$\therefore \ln\left(1 / 10\right) = -kt$$

$\therefore$ Decimal reduction time 'D' is related to the specific death rate (K) by

$$D = \frac{2.303}{K}$$

**3. Define Thermal death kinetics of micro organism.**

Thermal death of micro organisms at a particular temperature can be described by first order reaction kinetics

$$\frac{dN}{dt} = -KN$$

where,

k- reaction rate constant of the reaction (or) specific death rate
N-number of viable organism present
t-time of sterilization treatment.

**4. How the reduction in batch sterilization represented graphically?**

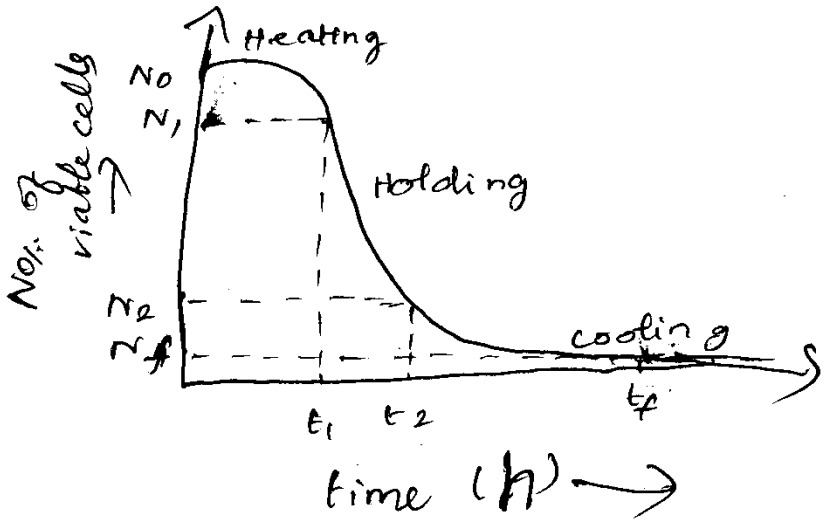



**Fig.**

**5. Define sterilization.**

It is the destruction of micro organisms by means of heat, chemical agents, radiation and mechanical means. Another approach is to remove the living micro organisms by means of filteration or high speed centrifugation.

**6. What are the types of sterilization?**

1. Physical methods
    a) Heat
    b) Filteration
    c) Irradiation
2. Chemical methods

**7. What is the use of Hot air oven in sterilization?**

It is used to sterilize glass ware, foreups, scalepels, scissors and materials such as fat, oil, powder etc. that are impermeable to moisture. The instruments to be sterilized are placed in an overn at 160$^{o}$-180$^{o}$C for 2-3 hrs. microbial death apparently results from the oxidation of cell constituents and denaturation of proteins.

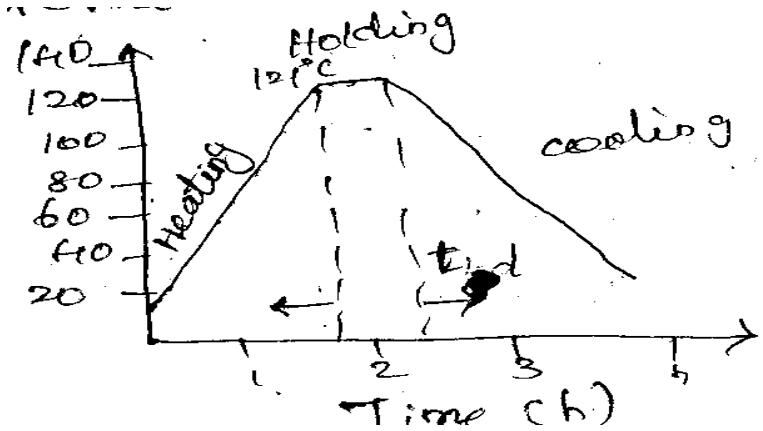
**8. What is meant by batch sterilization?**

Liquid medium is most commonly sterilized in batch in the vessel where is will be used. Medium is seated to sterilization temperature by introducing steam, and reach sterilization temperature (121°C), kept for certain period of time. Then cooled formentation period with the help of cooling water.

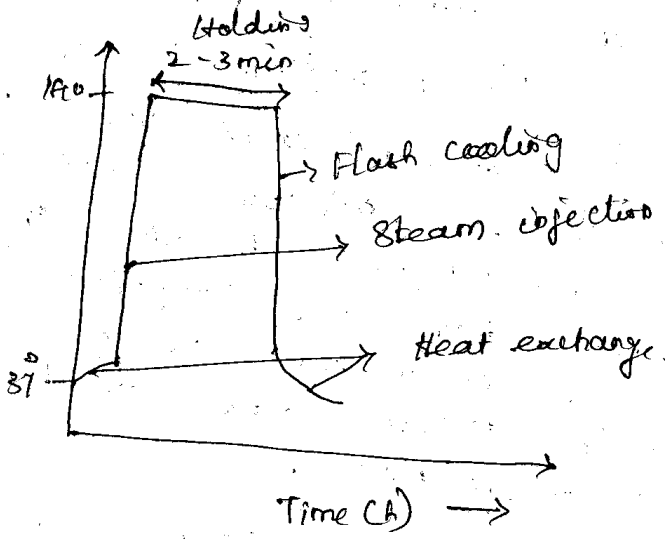
**9. What is meant by continuous sterilization?**

Continuous sterilization particularly a high temperature, short lopesure time, can achieve complete sterilization with much best damage to the medium. Both the neat up and cool down periods are very rapid. Continuous sterilization is easier to control and reduces down time in the fermentors.

**10. Draw temperature – time profile for batch sterilization process.**




**11. Draw temperature and time profile for continuous sterilization process.**



**12. Define Peelet number.**

$$Pe = \frac{uL}{\Delta_z}$$

where,

| | | |
|---|---|---|
| $P_e$ | - | Peelet number |
| U | - | average linear fluid velocity |
| L | - | Pipe length |
| $\Delta_z$ | - | axial dispersion coefficient |

**13. What is meant by damkohler number?**

The extent of cell destruction in the sterilizer can be relate and to the specific death constant $K_d$ by another dimensionless number called damkohler number Vs $N_2/N_1$, graph.

$$\Delta_a = \frac{KL}{u}$$

| | | | |
|---|---|---|---|
| where, | $\Delta_a$ | - | Damkohlex number |
| | K | - | Specific death constant |
| | L | - | Length of holding pipe |
| | u | - | average linear fluid velocity |

**14. Write the advantages of batch over continuous sterilizers.**

1. Lower capital cost
2. Lower risk of contamination – Continuous processes requires aseptic transfer of the sterile broth to the sterile vessel.
3. Easier manual control
4. Easier to use with media containing a high proportion of solid matter.

**15. Write the advantages of continuous sterilizers over batch sterilizers.**

1. Superior maintenance of medium quality
2. Easy of scale up to large volumes
3. Easier automatic control
4. The reduction of sterilization cycle time

**16. Write mechanism of filling by moist heat.**

Moist heat bills the organism by coagulating and denaturing their enzymes and structural protein. Sterilization by moist heat of the most resistant spores generally requires 121°C for 15-30 minutes. Moist heat is used for the sterilization of culture media, and all other materials through which steam can penetrate.

**17. Name the filters used for filter sterilization.**

1. Earthenware candles filters
2. Asbestos and asbestos – paper discs filter
3. Sintered glass filters
4. Cellulose membrane filters
5. Fibre glass filters

**18. Write merits and demerits of Heat sterilization.**

**Merits:**

1. Sterilization is very effective
2. Heat deliver system can be monitored effectively with varied controls like pressure gauge, temperature etc.
3. Established quality control methods available.

**De merits:**

i) Steam impermeable materials like fats, oil and powders cannot be sterilized by autoclaving.
ii) Heat sensitive materials can not be sterilized by heat.
iii) Dangers of explosion when high pressure is used.

**19. Differentiate deapth filters and surface filters.**

Deapth filters consisting of compacted beds or pads of fibrous material such as glass wool have been used widely. Cells are collected in depth filters by a combination of impaction, interception, electrostatic effects, and for particles smaller than about 1.0pm, diffusion to the fibers.

Where surface filters are polymeric membrane, which trapping contaminants as on a sieve. Membrane filter cartridges typically contain a pleated, hydro phobic filter with small and uniformly sized pores 0.4pm or less in diameter.

## PART - B

**1. Write short note on thermal death kinetics of micro organisms.**

Thermal death of micro organisms at a particular temperature can be described by first order reaction kinetic

$$\frac{dN}{dt} = -KN \qquad \rightarrow (1)$$

where,

K- reaction rate constant of the reaction (or) the specific death rate.
N-Number of viable organism present
t-time of sterilization treatment.

Rearranging on

$$\frac{dN}{N} = -Kdt \qquad \rightarrow (2)$$

integrating the above equation, yields

$$\frac{Nt}{N_o} = e^{-kt} \qquad \rightarrow (3)$$

where $N_o$ - Number of viable organisms present initially at time t=0
$N_t$ - Number of viable organisms present finally at time , t.

Taking natural logarithms, equ (3) is,

$\ln\left(\frac{N_t}{N_o}\right) = -kt \rightarrow (4)$ This kinetic description makes 2 predictions

(i) An infinite time is required to achieve sterile conditions ie/$N_t$=0
(ii) After a certain time there will be less than one viable cell present.

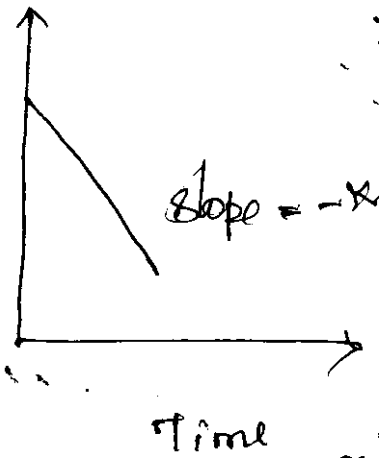



**Fig.**

The relation ship between temperature and the reaction rate constant was demonstrated by arrhenies equation,

$K=A \exp^{(-E/RT)}$ $d \ln K/dT=E/RT^2$ on integration.

Where, E= activation energies,
R=gas constant,
T=absolute temperature
A= Arrhenices constant

**Decimal reduction time:**

It is the time taken at a particular temperature is reduce the initial population by a faster of 10. Thus,

$$N_t/N_o = 1/10 ; \left(\frac{N_t}{N_o}\right) = -Kt$$

$$\therefore \ln\left(1/10 = -Kt\right)\left(\therefore N_t/N_o = 1/10\right)$$

Therefore, the decimal reduction time 'D' is related is the specific death rate (K), by

$$D = \frac{2.303}{K}$$

**Del factor (or) Wabla factor**

Del factor is a measure of the fractional reduction is viable organism count produced by a certain heat and time regime.

$$\therefore \nabla = \ln\left(No/N_t\right)$$

$$\text{But } \ln = kt\left(No/N_t\right)$$

$\ln(N_t/N_o)=-kt$ & $K=Ae^{-E/RT} \overset{\text{This is got by cobining}}{\Leftarrow}$ & $kt= A.t.e^{(-E/RT)}$

Thus $\Rightarrow$ $\nabla =A.t.e^{(-E/RT)}$

On integrating above equation,

$$Lnt= E/RT+/n(\nabla /A)$$

**2. For the inactivation of B subtiles spores $A=9.5\times10^{37}$ $min^{-1}$, & E= 68.7 kcal/md, assuming that a liquid containing these spores is instantaneously sterilized at temperature 115°C. Calculate the time required is give a destruction ratio of $10^6$.**

**Solution:**

**Given:**

$A= 9.8 \times 10^{37}$ $min^{-1}$

$E=68.7$ kcal / min

$T= 115^oC$

$K=Ae^{(-E/RT)}$

$=9.5\times10^{37}\ e^{(-68.7/1.9872\times388)}$

$K=0.1922$

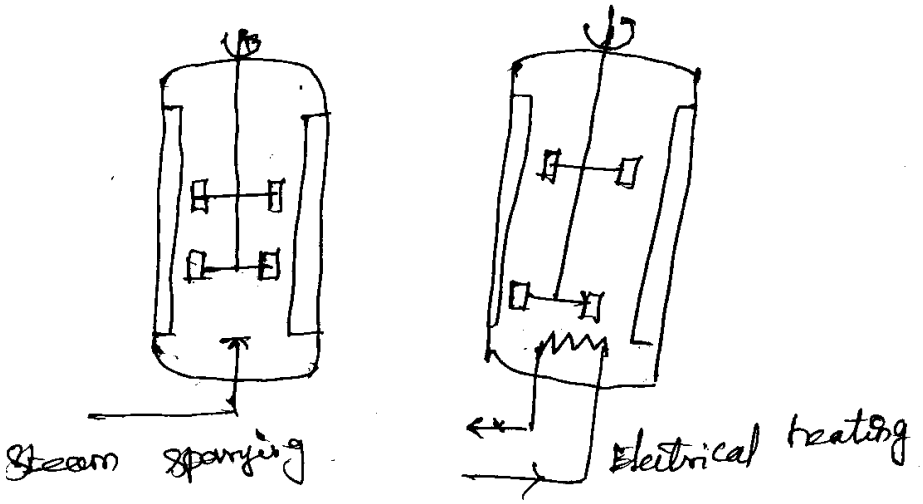
$Ln(N_o/N)=kt$

$Ln(10^6)=0.1922t$

$$\boxed{t = 71.9\,\text{min}}$$

**3. Explain is detailed about batch sterilization process.**

Liquid medium is most commonly sterilized is batch in the vessel where it will be used the medium is created to sterilization temperature by introducing steam into the coils or jacket of the vessel; alternatively steam spary directly into the medium, or the vessel is heated electrically.




The sterilization cycles are composed of heating, holding and cooling. A typical temperature –line profile for batch sterilization is shown below,

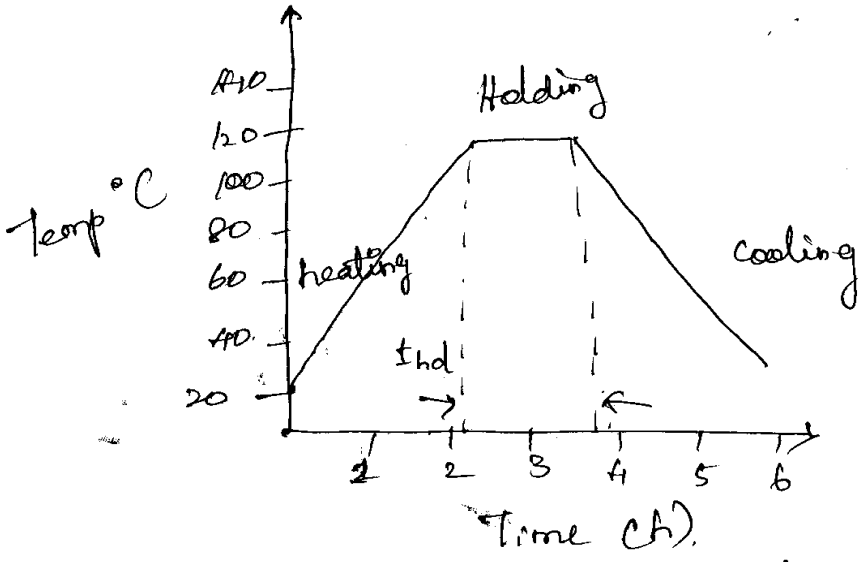



**Fig.**

Depending on the rate of heat transfer from the steam or electrical coil element, raising the temperature of medium takes a significant period of time.

Once the holding or sterilization temperature is reached, the temperature is held constant for a period of time end. Destruction of nutrients along with contaminant organisms occurs during batch sterilization to minimize these loss, the holding times at a highest sterilization should be kept as short as possible.

Cooling water in the coils or packet is then used as reduce the medium temperature is the required value.

Therefore, the total del factor required should be equal to the sum of the del factor for heating, holding and cooling as

$$\nabla_{total} = \nabla_{heat} \,\&\, \nabla_{hold} \,\&\, \nabla_{cool}$$

The values of $\nabla_{heat} \,\&\, \nabla_{cool}$ determined by the methods used for the heating and cooling. The values of $\nabla_{hold}$ is determined by the length of the controlled holding period.

Reduction in the number of viable cells during batch sterilization represented graphically by,

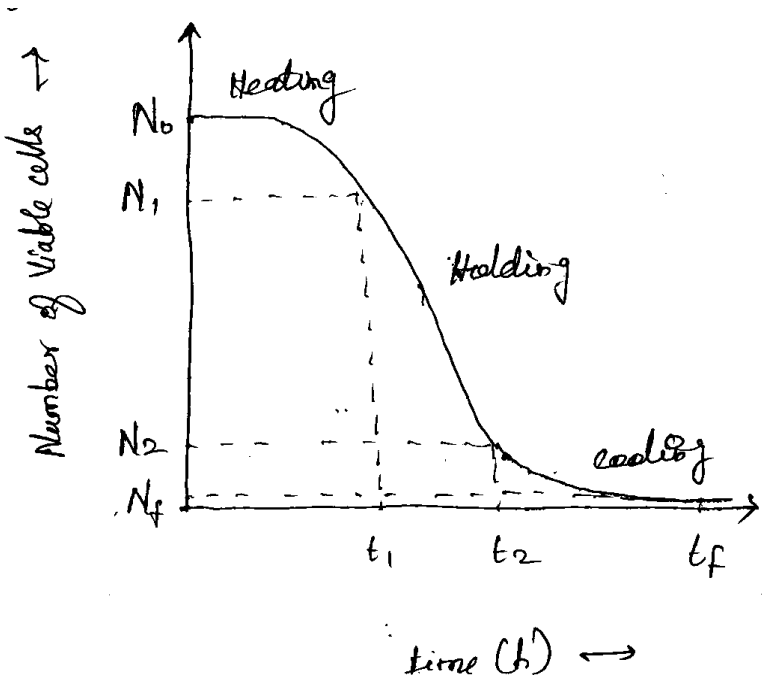



**Fig.**

Where,

$N_o$ = Number of contaminants present is the raw material

$N_1$ = Number of contaminants reduced during heating

$N_2$ = Number of cells at the end of holding period
$N_f$ = final number of cell after cooling (i.e), ideally zero

If $N_o$ to $N_f$ are known, the holding time required to reduce the number of cells from $N_1$ to $N_2$ can be determined by considering the kinetics of call death.

Thermal death of microorganism can be described by first order kinetics,

$$dN/dt = -kN \quad \rightarrow (1)$$

where,
N= Number of viable cells,
t= time
K= specific death constant

The above equation applies to each stage of the batch sterilization cycles heating, holding and cooling.

For holding period,

$$\ln N_1/N_2 = kt_{nd} \quad \rightarrow (2)$$

(or)

$$t_{hd} = \frac{\ln M_1/N_2}{k} \quad \rightarrow (3)$$

where, $t_{hd}$=holding time,
$N_1$=number of viable cells at the start of holding
$N_2$=number of viable cells at the end of holding

K is $Ae^{-E/RT}$ $\longrightarrow$ (4)

Combining equation (1) and (4) we can get

$$\frac{dN}{dt} = -Ae^{-E/RT}N \quad \rightarrow (5)$$

Integrating equ (5)

$$\ln N_o/N_1 = \int_o^{t_1} Ae^{-E/RT}dt \quad \rightarrow (6)$$

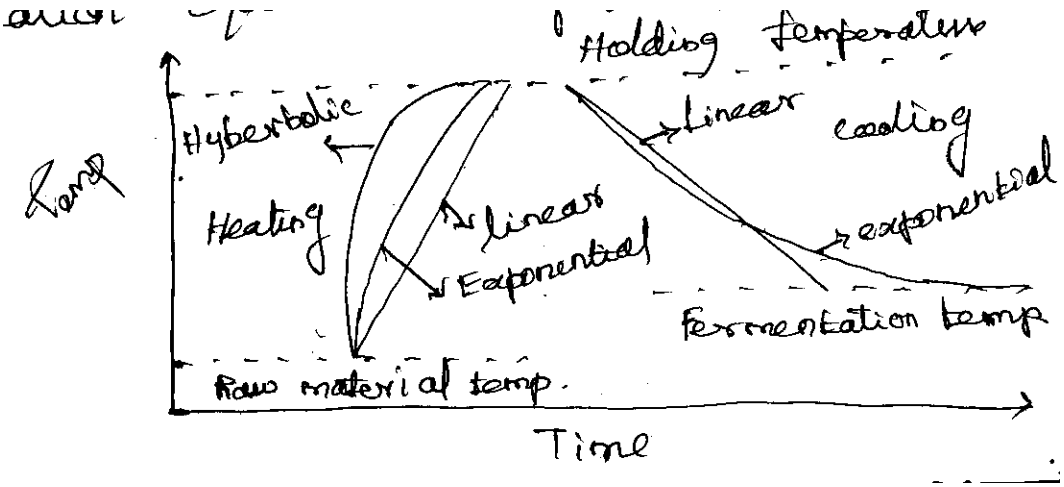
and for cooling period

$$\ln \frac{N_2}{N_f} = \int_{t_2}^{t_1} Ae^{-E/RT} dt \qquad \rightarrow (7)$$

where,

$t_1$= is the time at the end of heating
$t_2$= is the time at the end of holding
$t_f$= is the time at the end of cooling

generalized temperature time profile for heating and cooling stages of a batch sterilization cycle. Is as follows,




**4. During sterilization of a fermentation medium in a given temperature, the heat up from 100°C and 121°C book 25 min, while cool down period from 121°C to 100°C took 15 men. The characteristics values of rate constant (K) and the degree of sterilization (∇) at different temperature are ∇ 100-121°C iC/min=12.549& K121°C=2.538 min $^{-1}$. If the total value of ∇ required for the whole sterilization process is 45.0, what should be the holding period at 121°C?**

**Solution:**

$$\nabla_{100-121^oC} = 12.549$$

heating (ic/min)

$$\nabla_{heating} = \frac{12.549 \times 25}{21} = 1495$$

Since the cooling cycle is cool the medium from 121°C to 100°C tables 15min,

$$\nabla_{cooling} = \frac{12.549 \times 15}{21} = 8.970$$

$$\Rightarrow \nabla_{holding} = \nabla_{total} - \nabla_{heating} - \nabla_{cooling}$$

=45-14.950-8.970

=21.08

At holding temp 121°C the given value of θ is 2.538 $min^{-1}$.

$$\therefore \text{ holding period}=t_{hd} = \nabla \frac{holding}{K}$$

$$= \frac{21.08}{2.538}$$

$t_{nd}$=8.3 min

**5. A fermenter containing 40$m^3$ of medium (25°C) is going to be fertilized by direct infection of saturate ϕ steam. The typical bacterial count of the medium is about $5\times10^{12}$ $m^{-3}$, which needs to be reduced t such an extent that the chance and a contaminant surviving the sterilization is l in 1000 is, $10^{-3}$, given that $\nabla_{heat}$ =14.8 and =139 and K=197.6 $hr^{-1}$. Find the holding time for the batch sterilization operation.**

**Solution:**

Medium = 40$m^2$

Initial count – $N_o$=$5\times10^{12}m^{-3}$

$$\nabla_{heat} = 14.8, \nabla_{cool} = 13.9, K = 197.6 m^{-1}$$

Initial count present for 40$m^3$=$5\times10^2n^{-3}\times40m^3$

$=2\times10^{14}$

$$\therefore \nabla_{total} = \ln\frac{n_o}{n} = \ln\left(\frac{2\times10^{14}}{10^{-3}}\right) = 39.8$$

$$\nabla_{total} = \nabla_{heat} \,\&\, \nabla_{hold} \,\&\, \nabla_{cool}$$

$$\nabla_{hold} = \nabla_{total} - \nabla_{heat} - \nabla_{cool}$$

=39.8-14.8-13.9

=11.1

$$t_{nd} = \frac{\nabla_{hold}}{197.6hr^{-1}}$$

$$= \frac{11.1}{197.6hr^{-1}}$$

$$= 0.056hr$$

$$t_{nd} = 3.37\,min$$

**6. Explain is detailed about continuous sterilization process.**

Continuous sterilization particularly a high temperature, short exposure time process, can significantly reduce damage of medium ingredients while achieving high levels of cell distinction.

Typical equipment configurations for continuous sterilization are shown in below figure.

(a) continuous stream injection with flash coding

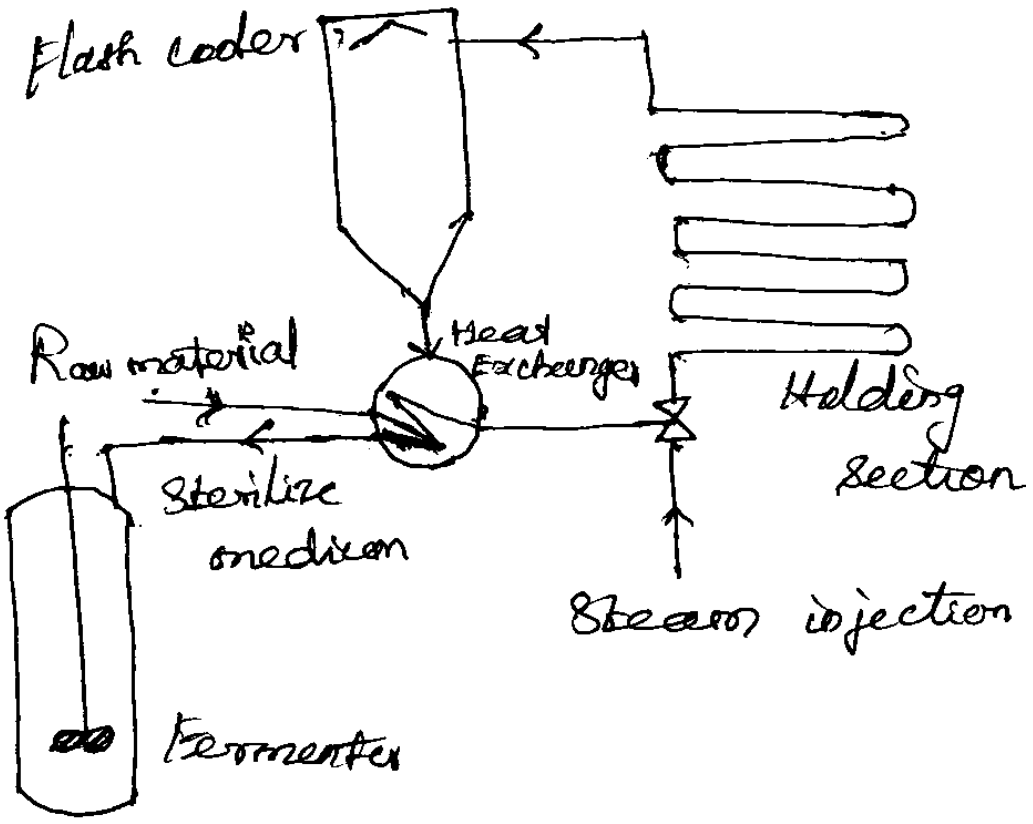



**Fig.**

**(b) Heat transfer using heat exchangers**

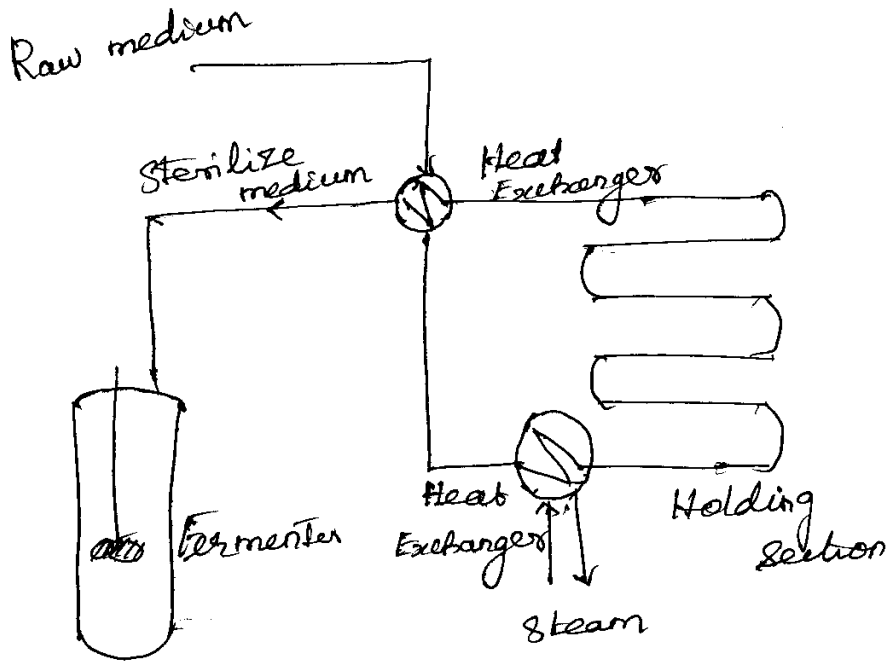



**Fig.**

In continuous steam injection with flash cooling types, raw material entering the systems first preheated by net sterile medium in the heat exchanger; this economises on steam require rates for heating or code the sterile medium. Steam is directly injected into the medium steam is directly injected into the medium as it flows through a pipe; the liquid temperatures raises almost incautiously to the sterilization. The time of exposure of this temperature depends on the length of the pipe in the holding section of the sterilizer.

The length of the holding period is dictated by the length of the coil and the flow rate of the medium, after sterilization, the medium is cooled instantly by passing of through an expansion value into a vacuum chamber, further cooling takes place in the heat exchanger where residual head is used to the heat the incoming medicine.

In heat transfer using heat exchanger type, raw material is pre heated with hot, sterile medium in heat exchanger, then brought to the sterilization temperature by further heat exchange with steam. The sterilization temperature is maintained is the holding section before being reduced to the fermentation temperature by heat exchange with incoming medicine.

Variation of temperature with time in the continuous sterilization as follows.

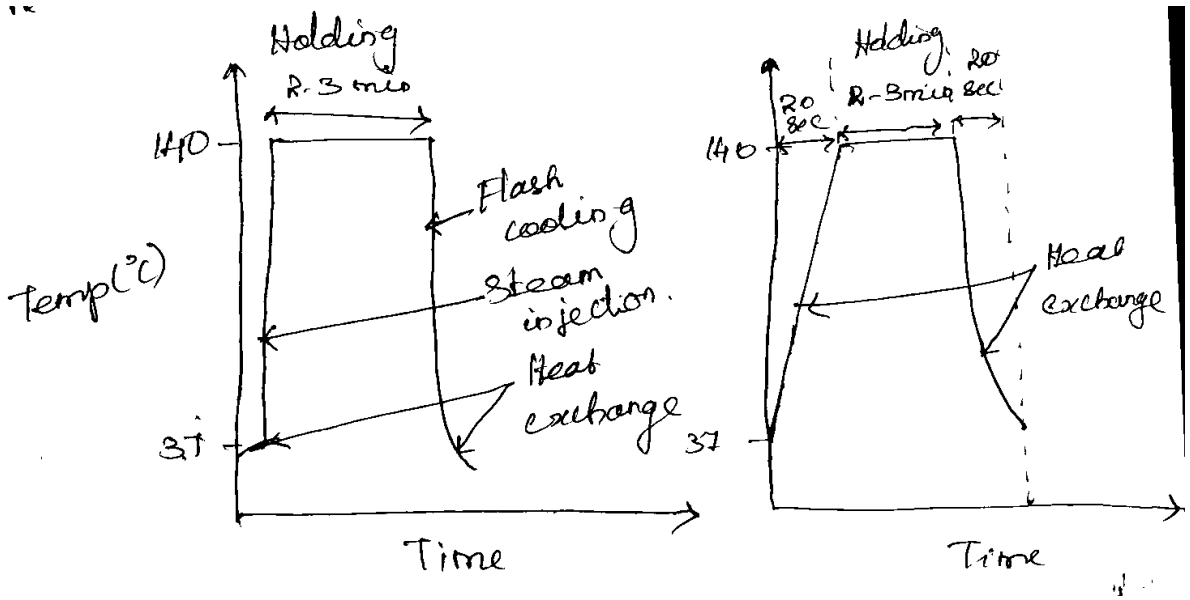



**Fig.**

The rate of heating and cooling in continuous sterilization are much more rapid than in batch; thus in design of continuous sterilizers, contributions to all death outside of the holding period are ignored generally.

An important factor variable affecting performance of continuous sterilizers is the nature of fluid flow in the system ideally, all fluid entering the equipment at a particular instant should spend the same time in the sterilizer and exit the system at the same time; unless this occurs we connect fully control the time spent in the sterilizer by all fluid element. No mixing should to occur in the tubes; if third nearer the entrance of the pipe mixes with fluid head of it, there is a risk that contaminants will be transferred to the outlet of the sterilizer. The types of flow in pipes where there is neither mixing for variation in fluid velocity is called plug flow.
Plug flow is approached is pipes at turbulent Reynolds numbers above about $2\times10^4$; operation at high Reynolds numbers minimizes fluid mixing and velocity variation.

Derivation from plug flow behavior is characterized by the degree of axial dispersion in the system. Axial dispersion is a critical factor affecting design of continuous sterilizers.

**7. Medium at a flow rate of $2m^3h^{-1}$ is to be sterilized by heat exchange with steam in a continuous sterilizer. The liquid contains bacterial spores at a concentration of $5.7\times10^{12}m^{-3}$, the activation energy and arrhenius constant for thermal destruction of these contaminants are 283 KJ $gmol^{-1}$ and $5.7\times10^{39}h^{-1}$, respectively. A contamination risk of one organism surviving every 60 days operation is considered acceptable. The sterilizer pipe has an inner dia of 0.1m; the length of the holding section is 24m. the density of the medium is $1000kg/m^3$ and viscosity $3.6kgm^{-1}$ $h^{-1}$. what sterilizing temperature is required?**

**Solution:**

The desired level of cell destruction is evaluated using a bases of 60 days ignoring any cell death is the heating and cooling sections, the number of cells entering she holding section over 60 days is,

$$M_1 = 2m^3h^{-1}\left(5\times10^{12}m^{-3}\right)\left|\frac{24h}{1d}\right|(60d)$$

$$=1.44\times10^{16}$$

$N_2$, the acceptable number of cells leaving during this period is 1. therefore,

$$\frac{N_2}{N_1} = \frac{1}{1.44\times10^{16}} = 6.9\times10^{-17}$$

The linear velocity is in the sterilizes is equal to the volumetric flow rate divided by the cross sectional area of the pipe:

$$u = \frac{2m^3h^{-1}}{\pi\left(\frac{0.1m}{2}\right)^2} = 254.6mh^{-1}$$

To calculate Pe we must first determine $D_z$ using standard axial dispersion coefficient correlation graph.

$$Re = \frac{Dup}{\mu} = \frac{(0.1m)\left(254.6mh^{-1}\right)\left(1000kgm^{-3}\right)}{3.6kgm^{-1}h^{-1}}$$

$$=7.07\times10^3$$

Fore Re= $7.07\times10^3$ the corresponding
Dz/uD value from standard graph = 0.65

$$Dz=0.65(254.6mh^{-1})(0.lm)$$
$$=16.6m^2h^{-1}$$

$$\Rightarrow Pe = \frac{UL}{Dz} = \frac{\left(254.6mh^{-1}\right)(24m)}{16.6m^2h^{-1}} = 368$$

We can determine the value of $K_d$ for the desired level of cell destination Da corresponding to $N_2/N_1=6.9\times10^{-17}$ and Pe=368 is about 42 from std graph.

$$k_d = \frac{uDa}{L} = \frac{254.6mh^{-1}(42)}{24m} = 445.6h^{-1}$$

The sterilization temperature can be evaluated after by the following equation.

$$\ln\frac{kd}{A} = \frac{-Ed}{RT}$$

$$T = \frac{\left(\frac{-Ed}{R}\right)}{\ln\left(kd/A\right)}$$

$$= \frac{\left(\frac{-283\times10^{3}Jgmol^{-1}}{8.3144JK^{-1}gmol^{-1}}\right)}{\ln\left(\frac{445\text{-}6h^{-1}}{5-7\times10^{39}h^{-1}}\right)}$$

$$\rceil = 398.4k$$

(or)

$$\rceil = 125^{o}C$$

**8. Explain the kinetics of sterilization of air using filters.**

Aerobic fermentation require the continuous addition of considerable quantities of sterile air. Although it is possible to sterilize air by heat treatment, the most commonly used sterilization process is filtration.

Filtration is the most common method for sterilizing air in large scale bioprocesses; heat sterilization of gases is economically impractical. Depth filters consisting of compacted beds or pads of fibrous material such as glass wool have been used widely in the fermentation industry. Distance between the fibers in depth filters are typically 2-10pm, about 10 times greater than the dimensions of the bacteria and spares is be removed.

Depth filters do not perform well if there are large fluctuation in flow rate or if the air is wet; liquid condensing in the filter increases the pressure drop, cause channeling of the gas flow, and provides a pathway for organisms to grow through the bed.

Increasingly depth filter replaced for industrial applications by membrane cartridge filters. These filters use steam sterilizable polymeric membranes which are as surface filters trapping

contaminants as on a size. Membrane filter cartridges typically contain a pleated hydrophobic filter with small and uniformly sized spores 0.45 pm or less in diameter.

Filters are also used to sterilize efficient gases leaving fermenter. The concentration of cells in fermenter off gas is several times greater than in air. Contaminant is particularly important when organisms used in fermentation are potentially harmful to plant personal or the environment companies operating fermentation with pathogenic or recombinant strain are required by regulatory authorities to prevent escape of the cells.

If it is assumed that if a particle touches a filter it remain attached is it and that there is a uniform concentration of particles at any given depth in the filter, then each layer of a unit thickness of the filter should reduce the population entering it by the same proportion, which may be expressed mathematically as,

$$dN/dH = -kN$$

where, n- is concentration of particle in the air at a depth , x

H- filter

k-constant

On integrating equation (1) over the length of the filter.

$$\int_{N_o}^{N} \frac{dN}{N} = -K\int_{o}^{x} dH$$

$$N/N_o = e^{-ka} \rightarrow (2)$$

where, $N_o$ – Number of particles entering the filter

N - Number of particles leaving the filter

On taking ln equ (2) becomes

$$\ln\left(N/N_o\right) = -kx \rightarrow (3)$$

The above equation is termed as log penetration relation ship.

The efficiency of the filter is given by the ratio of the number of particles removed to the original number present, thus

$$E = \frac{N_o - N}{N_o} \rightarrow (4)$$

where, E-efficiency of filter

$$\text{Also} \qquad \left(\frac{N_o - N}{N_o}\right) = I - \left(N/N_o\right) \rightarrow (5)$$

equ (5) can be written as

$$\frac{N_o - N}{N_o} = 1 - e^{-kx}$$

$$E = 1 - e^{-kx} \rightarrow (6)$$

The log penetration relationship equation (3) has been used in filter design, by using the concept $X_{90}$, the depth of filter required to remove 90% of the total number of particles entering the filter, thus

If No were 10 and x were $X_{90}$, then N would be 1;

$$\ln\left(1/10\right) = -Kx_{90}$$

or

$$2.303 \log_{10}\left(1/10\right) = -KX_{90}$$

$$2.303(-1) = KX_{90}$$

$$X_{90} = 2.303/K \rightarrow (7)$$

The value of K is affected by the nature of filter material and by the linear velocity of the air passing through the filter.

**9. What meant by sterilization and explain detail?**

It is the destruction of micro organisms by means of heat, chemical agent, radiation and mechanical means – Another approach is to remove the living microorganisms by means of filtration or high speed centrifugation.

Sterilization is of two types:

A physical methods:

a. heat
b. filtration
c. irradiation

B. Chemical methods

**Physical methods**

**(a) By heat**

Fire and boiling water have been used for sterilization and disinfection same the time of the Greeks, and heating is skill one of the most popular ways to destroy microorganism. Sterilization can be done by either using dry heat or moist heat.

**Sterilization by dry heat:**

Certain instruments are best sterilized in the absence of water by dry heat sterilization. The various methods used to sterilize by dry heat are as follows.

**(i) Red heat**

The instrument is held on the flame until it is red heat eq: inoculation loops, spatulas etc.

**(2) Flaming:** - Effective sterilization of instruments can be achieved by flaming in a Bunsen burner. However this method could cause heat burn and may also lead to a force hazard.

**(3) Infra red sterilizer**: It has a cavity where temperature rises to almost 100$^{o}$C. Sterilization is effected by a 2-5 sec expresser at this temperature being well insulate, these sterilizers do not spill out quantities of heat.

**(4) Hot air oven:** It is used for Sterilization of glass ware, forceps, scalpels, scissors and materials such as fat, oils, powers etc. that are impermeable to moisture. The instruments to be sterilized are placed in an oven at 160$^{o}$ to 180$^{o}$ for 2 to 3 hours. Microbial death apparently results from the oxidation of cell constituents and denaturation of protein.

**(ii) Sterilization by moist heat.**

Moist heat, kills the organism by coagulating and denaturing their enzymes and proteins. Moist heat sterilization is usually carried at is an cuts clave generally sterilized by 121$^{o}$C for 15-40 min

**(iii) Filter sterilization:**

Filtration is an excellent way to reduce the microbial population in selections of neat sensitive material. Some of plant growth regulator urea, certain vitamins etc. membranes with pores of about 0.2pm in diameter are used to remove vegetative cells from solutions ranging from 1ml to many liters. The solution is forced through the filter with a vacuum or with pressure from syringe or peristaltic pump.

**Sterilization of uv radiation:**

UV radiation around 260 mm is quite lethal but does not penetrate glass, dirt films, water and other substances very effectively. Because of their disadvantage. Uv radiation is used as a sterilizing agent only in a few specific situations. Uv lamps are sometimes placed on the ceiling of room or in biological safety cabinets to sterilize the air and ay expressed surfaces.

**Chemical methods:**

Chemical disinfectant are also used to sterilize various instruments as well as working table. It will microorganisms are a result of their oxidizing or alkylating abilities

Some of the major antimicrobial chemical agents are phenol and phenolic compounds, alcohol, halogens (iodine, hydrochlorides), detergents, dyes, acids, gaseous chemosterilizers (ethylene oxide, form aldehyde).

Among the technical, moist heat is the most economical and efficient for general sterilization.

## UNIT – IV

## PART – A

**1. What is the role of metabolic stoichiometry in bioprocessing?**

Metabolic stoichiometry has many applications in bio processing; as well as in mass and energy balance, it can be used to compare theoretical and actual product yields, check the consistency of experimental fermentation data, and formulate nutrient medium.

**2. Write the general equation for aerobic cell growth.**

$$C_w H_n O_y N_z + aO_2 + bH_gO_hN_i \rightarrow cCH\propto D_\beta N_\delta + dco_2 + eH_2O$$

where,

$C_w H_n O_y N_z$ - Substrate

HgOh Ni - Nitrogen source

a, b, c, d, e – Stoichiometric coefficients

**3. What is meant by respiratory quotient?**

$$\text{Respiratory quotient (RQ)} = \frac{\text{moles of } CO_2 \text{ produced}}{\text{moles of } O_2 \text{ consumer}}$$

$$= \frac{d}{a}$$

**4. Explain available electron balance.**

Available election balance refers to the number of electrons available for transfer to oxygen on combustion of a substance, to $CO_2$, $H_2O$ and nitrogen containing compounds the number of available elections found in organic material is calculated from the valence of the various elements: 4 for C, 1 for H, - 2 for O, 5 for P and 6 for S.

**5. Define degree of reduction.**

Degree of reduction $\gamma$ is defined as the number of equivalents of available elections in that quantity of material containing 1g atom carbon. Therefore, for substrate $C_w H_n O_y N_z$, the number

of available electrons is (4w + x – 2y – 3z). The degree of reduction for substrate, γs is therefore (4w + x – 2y – 3z) / w.

**6. Write equations for biomass yield and product yield.**

$$\text{Biomass yield, } Y_{xs} = \frac{\text{g cells produced}}{\text{g substrate consumed}}$$

$$\text{Product yield, } Y_{ps} = \frac{\text{g product formed}}{\text{g substrate consumed}}.$$

**7. Name the factors affect biomass yield.**

A large number of factors influences biomass yield, including medium composition, nature of the carbon and nitrogen sources, $p^H$ and temperature. Biomass yield is greater in aerobic than in anaerobic cultures; choice of election acceptor, e.g. $O_2$, nitrate or sulphate, can also have a significant effect.

**8. Define heat of combustion.**

Heat of combustion Δhc is defined as the heat evolved during reaction of a substance with oxygen to yield certain oxidation products such as $CO_2$ gas, $H_2O$ liquid and $N_2$ gas. The standard heat of combustion Δhc is the specific enthalpy change associated with this reaction at standard conditions, usually 25°C and 1 atm pressure.

**9. Fumaric acid is produced from malic acid using the enzyme fumarase. Calculate the standard heat of reaction for the following enzyme transformation:**

$$\underset{\text{(malic acid)}}{C_4H_6O_5} \longrightarrow \underset{\text{(fumaric acid)}}{C_4H_4O_4} + H_2O$$

**Solution:-**

$$\Delta h°c = O \text{ for liquid water}$$

$$\Delta H°_{rxm} = (n\Delta h°c)_{\text{malic acid}} - (n\Delta h°c)_{\text{fumaric acid}}$$

$$(\Delta h°c)_{\text{malic acid}} = -1328.8 \text{ kJ gmol}^{-1}$$

$$(\Delta h°c)_{\text{fumaric acid}} = -1334.0 \text{ kJ g mol}^{-1}$$

Therefore, using a basis of 1 gmol malic acid converted

$$\Delta H^\circ_{rxn} = (1g\ mol)\ \left(-1328.8\ kJ\ gmol^{-1}\right) - (1gmol)$$

$$\left(-1334.0\ kJ\ g\ mol^{-1}\right)$$

$$\Delta H^\circ_{rxm} = 5.2\ kJ$$

As $\Delta H^\circ_{rxn}$ is positive, the reaction is endothermic and heat is absorbed.

**10. Explain theoretical oxygen demand.**

Oxygen demand is an important parameter in bioprocessing as oxygen is often the limiting substrate in aerobic fermentations. Oxygen demand is represented by the stoichiometric coefficient a Oxygen requirement is related directly to the elections available for transfer to oxygen; the oxygen demand can therefore be divided from an appropriate election balance.

**11. Malonic acid and water are initially at 25°C. If 15g malonic acid is dissolved in 5 kg water, how much heat must be added for the solution to remain at 25°C. What is the solution enthalpy relative to the components.**

**Solution:-**

Molecular weight of malonic acid = 104

$\Delta$hm at room temperature = 4.493 Kcal $gmol^{-1}$

$\therefore$ The heat required for the solution to main 25°C is

$$\Delta H = 4.493\ \text{Kcal gmol}^{-1}\ (15g)\left|\frac{1gmol}{104g}\right|$$

$$= 0.648\ \text{Kcal}$$

Relative to H = 0 for water and malonic acid at 25°C, the enthalpy of the solution at 25°C is 0.648 Kcal.

**12. Name the factors affecting cellular oxygen demand.**

Many factors influence oxygen demand, the melt important of these are cell species, culture growth phase, and nature of the carbon source in the medium. In batch culture, rate of oxygen uptake varies with time.

**13. Write the methods for the measurement of $K_La$.**

(i) Oxygen balance method – this technique is based on the equation for gas liquid mass transfer.

(ii) Dynamic method – it is based on the unsteady state mass balance for oxygen. The main advantage of the dynamic method over the steady state technique is the low cost of the equipment needed.

**14. Define $K_La$.**

$No_2 = K_La$ – Volumetric oxygen transfer coefficient

$C^*$ - Saturated dissolved oxygen concentration
$C_L$ – actual dissolved oxygen concentration
$No_2$- rate of oxygen transfer

**15. Define maintenance coefficient.**

A maintenance coefficient is used to describe the specific rate of substrate uptake for cellular maintenance, or

$$m = \frac{[ds/dt]_m}{X}$$

However, during the stationary phase were little external substrate is available, endogeneous metabolism of biomass components is used for maintenance energy.

**16. Write equation for total rate of heat evolution in a batch process.**

$$Q_{GR} = V_L \ \mu_{net} \times 1/Y_H$$

where, $V_L$ – liquid volume (l)
X - Cell concentration (gμ)
$1/Y_H$ - metabolic heat evolved per gram of cell mass produced (kJ/g cells)

## PART – B

**1. Write short note on cell growth and product formation stoichiometry.**

Cell growth and products formation are complex processes reflecting the overall kinetics and stoichiometry of the thousands of infracellular reaction that can be observed with in cell for many process calculation use whish to compare potential substrates in terms of cell mass yield, or product yield or evolution of heat.

A variety of metabolic end products is released into the growth medium or accumulated intracellularly. The pertinenx stoichiometry for product formation may be classified usefully into four classes.

1. The main product appears as a result of primary energy metabolism. Example, ethanol production during anaerobic growth of years.
2. The main products arise indirectly from energy metabolism. Example, citric acid formation during aerobic mold cultivation.
3. The product is a secondary metabolite Example, penicillin production in aerated mold culture.
4. Biotransformations. The product is obtained from substrate through one or more reactions catalyzed by enzyme in the cells. example, steroid hydroxylation

Class I process have a relatively simple stoichiometric description product appears in relatively constant proportion as all mass alarm later and substrate is consumed. Here, the processes of substrate utilization cell mass synthesis and product formation are linked is a simple, single chemical reaction.

$$a'CH_2O_y + b^1O_2 + C'HlO_mN_n \rightarrow$$

$$CH_2O_PN_s + d'H_2O + e'CO_2 + f'CH_2O_w \rightarrow (1)$$

A corresponding yield faster may be writer, taking into account the are of molar units in the yield faster parameter. Thus, if the substrate is glucose which contains six carbon atoms per molecule, the number of glucose molecule used when reaction (1) occurs is a'/6. Similarly, if the product contain $n_P$ carbon atoms per molecule, f'/$n_\phi$ product molecular are formed. Thus the molar yield faster $Y_P/s$ may be written

$$Y_{P/s} = \frac{f'}{a'}\frac{n_s}{n_p} \rightarrow (2)$$

Here, $n_s$ – number of carbon atoms in a substrate molecule.

For class 2 situations, products formation is not necessarily proportional to substrate utilization or cell mass increase. Representation of the stoichiometry in such a case required an independent reaction equation for product formation.

$dCH_xO_y$(coefficient)$H_2O$+½ ($\gamma_s$-Z$\gamma_P$)$NnD^t$→
$dCH_yO_w$+d(1-Z)$CO_2$+$a^1Y_2$($\gamma_s$-z$\gamma_\theta$)($NADH+H^t$)→3a
$\varepsilon_P$d(ADp+Pi)→$\varepsilon_P$d(ATP)+$H_2O$→3b

Here, z denotes the carbon fraction of substitute used for product formation, which is found is the product. The number of ASPs generated by product formation is denoted $\varepsilon_P$, this coefficient may be negative.

The stoichiometric descriptions appropriate for classes S&4 cases depend on the particular substrates and products involved. Product formation in these cases is typically completely uncoupled from cell growth.

Consider an anaerobic fermentation of glucose to ethanol as an initial example. The best possible case is utilization of all substrate for product formation alone assuming that cell. Growth is negligible, then equ (1) becomes

$CH_2O$ → ⅓$CO_2$+⅔ $CH_3$ $O_o$5.

showing that at most ⅔ of the substrate caution used can appear in the product.

**2. Explain about the degree of reduction of substrate and biomass in microbial growth.**

The degree of reduction, γ, for organic compounds may be defined as the number of equivalents of available electrons per gram atom C. The available electrons are those that would be transferred to oxygen upon oxidation of a compound is $CO_2$, $H_2O$, and $NH_3$. The degrees of reduction for some key elements are C=4, H=1, N=-3, O=-2, P=5 and S=6. The degree of reduction of any element in a compound is equal to the valance of this element. For example, 4 is the valence of carbon in $CO_2$ and -3 is the valance of in $NH_3$.

Methane ($CH_4$): 1(4)+4(1)=8,γ=8/1=8
Glucose($C_6H_{12}O_6$): 6(4)+12(1)+6(-2)=24,γ=24/6=4
Ethanol($C_2H_5OH$):2(4)+6(1)+1(-2)=12,γ=12/2=6

A high degree of reduction indicate a low degree of oxidation. That is

$\gamma_{CH4} > \gamma_{EtOH} > \gamma_{glucose}$.

Consider the aerobic production of a single extra cellular product.

$$\underset{\text{Sab}}{CH_mO_n} + aO_2 + bNH_3 \rightarrow C\underset{\text{Bio}}{CH_2O_\beta}\ N_S + d\underset{\text{pn}}{CH_2OyN_z} + CH_2O + fCO_2 \rightarrow (1)$$

The degrees of reduction of substrate, biomass and product are

$\gamma_s$=4+m-2n → (2)
$\gamma_b$=4+α-2p.3s →(3)
$\gamma_p$=4+x-2y-3z →(4)
Note that for $CO_2$, $H_2O$ and $NH_3$ the degree of reduction is zero.

Equation (1) can lead to elemental balances on C,H,O and N, an available electron balance, an energy balance, and a total mass balance. Of threes equations, only five will be independent. To all the equations are written, then the extra equations are be used to check the consistency of an experimental data set. Because the amount of water formed or used in such reactions is difficult is determine and water is present in great excess the hydrogen and oxygen balances are difficult o use. For such a data set, we would typically choose a carbon, a nitrogen and an available electron balance. Thus,

C+d+F=1 →(5)
cSrdz =b →(6)
C$\gamma_b$-1d$\gamma_p$=$\gamma_s$-4a →(7)

Wish partial experimental data, it is possible to solve this set of equations. Measurements of RQ and a yield coefficient would allow the calculation of the remaining coefficients. It should be noted that the coefficient, C, is $Y_{x/s}$ and d is $Y_{p/s}$.

An energy balance for aerobic growth is

$Q_o$ c$\gamma_b$+$Q_o$d$\gamma_p$=$Q_o\gamma_s$-$Q_o$4q →(8)

If $Q_o$, the heat evolved per equivalent of available electrons transferred to oxygen, is consistent Equation (8) is net independent of equ (7).

Equation (7) and (8) also allow estimate of the fractional allocation of available electrons or energy fro an organic substrate Equation(7) can be rewritten as,

$$1 = \frac{C\gamma_b}{\gamma_s} + \frac{d\gamma_p}{\gamma_s} + \frac{4a}{\gamma_s} \rightarrow (9)$$

$$1 = \varepsilon_b + \varepsilon_p + \varepsilon \rightarrow (10)$$

where ε is the fraction of available electrons is the organic substrate that is transferred is oxygen, $\varepsilon_b$ is the fraction of available electrons that is incorporated into mass, and $\varepsilon_p$ is the fraction of available electrons that is incorporated into extracellular products.

**3. Assume that experimental measurements for a certain organism have shown that alls can convert two thirds of the substrate carbon (alkane or glucose) to biomass.**

**a) Calculate the stoichiometric coefficients for the following biological reactions.**

**Hexadecane:** $\mathbf{C_{16}H_{34}+aO_2+bNH_3 \rightarrow C(C_{44}H_{7.3}N_{0.86}O_{1.2})+dH_2O+eCO_2}$

**Glucose:** $\mathbf{C_6H_{12}O_6+aO_2+bNH_3 \rightarrow C(C_{44}H_{7.3}N_{0.86}O_{1.2})+dH_2O+2CO_2}$

**b) Calculate the yield coefficients $Y_{x/s}$, $Y_{x/o2}$ for both reactions. Comment on the differences.**
**Solution:**

a) for hexadecane

amount of carbons in 1mols of substrate = 16(12)=192g
amount of carbon converted to biomass = 192(2/s)=128g

Then, 128=(4.4)(12); C=2.42

Amount of carbon converted to $CO_2$=192-128=64g

64=e(12)
e=5.33

The nitrogen balance yields,

146=c(0.86)(14)
b=(2.42)(0.86)=2.085

The hydrogen balance is,

84(i)+36=7.3C-12d
d=12.43

The oxygen balance yields.

2a((6)=1.2c(16)+2e(16)+d(16)

a=12.427

For glucose,

Amount of carbon is 1 mole of substrate = 72g

Amount of carbon converted to biomass= 72(2/3)=48g

Then, 48=4.4c(12)

C=0.909

Amount of carbon converted to $CO_2$=72-48=24g

24=12e$\Rightarrow$e=2

The nitrogen balance yields,

146=0.86c914)

b=0.782

The hydrogen balance is,

12+36=7.3C+2d

d=3.854

The oxygen balance yields,

6(16)+2(16)a=1.2(16)C+2(16)e+16d

a=1.473

b) For hexadecane

$$Y_{x/s} = \frac{2.42(MW)_{biomats}}{(MW)_{substrate}}$$

$$Y_{x/s} = \frac{2.42(91.34)}{226} = 0.98 gdwcells/g_{substrate}$$

$$y_{x/o2} = \frac{2.42(MW)_{biomats}}{12.43(MW)O_2}$$

$$y_{x/o2} = \frac{2.42(91.34)}{(12.43)(32)} = 0.557 gdwcells/gO_2$$

For glucose,

$$Y_{x/s} = \frac{0.909(91.34)}{180} = 0.461 \text{ gdwcells} / g_{substrate}$$

$$y_{x/o2} = \frac{0.909(91.34)}{(1.473)(32)} = 1.76 \text{gdw cells} / gO_2$$

the growth yield on more reduced substrate (hexadecane) is higher than that on partially oxidized substrate (glucose) assuming that two thirds of all the entering carbons incorporated in cellular substrates.

**4. Estimate the theoretical growth and product yield coefficients for ethanol formation by S-cerevisiae as described by the following overall reactions.**

**$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$**

**Solution:**

Since $Y_{xA\text{-}IP} \approx 10.5$ gdw / md ATP and since glycolysis yields 2ATP/mol of glucose in yease,

$$Y_{x/3} = 10.5 \text{ gdw/molATP} 2 \frac{\text{moles ATP}}{180 \text{ g glucose}}$$

or

$$y_{x/s} \approx 0.117 \text{gdw} / \text{g glucose}$$

For compute conversion of glucose 60 ethand by the years pathway, the maximal yield would be,

$$Y_{p/3} = \frac{2(46)}{180} = 0.51 \text{g ethand/g glucose}$$

while for $CO_2$ the maximum yields is

$$Y_{CO2/s} = \frac{2(44)}{180}$$

=0.49 g ethanol/g glucose

**5. Write short note on elemented balance in cell growth.**

A material balance on biological earleons can easily be written when the completions of substrates, products, and cellular material are known. Usually, electron proton balances are required in addition to elemented balance is data determine the stoichiometric coefficients in bioreactions. Accurate determination of the completion of cellular material is a major problem. Variations inc cellular, completion with different types of organisms are calculated.

A typical cellular completion can be represented as $CH_{1.8}\ O_{0.5}\ N_{0.2}$. One mole of biological material is defined as the amount containing gram atom of carbon, such as $CH_2O_\beta\ N_s$.

Consider the following simplified biological conversion is which no extracellular products other that $H_2O$ and $CO_2$ are produced.

$$CH_m\ O_n + aO_2 + bNH_3 \rightarrow cCH_\alpha\ O_\beta\ N_g + dH_2O + eCO_2 \quad \rightarrow (a)$$

Where $CH_mO_n$ represents 1 mole of carbohydrate and $CH_\alpha\ O_\beta\ N_g$ stands for 1 mole of cellular material. Simple elemental balance on $C_iH_1O$ and N yield the following equations.

C : 1 = c + e
H : m + 3b = Cαt2d
O : n+2a = cβ + dt 2e $\rightarrow$ (1)
N : b = cδ

The respiratory quotient (RQ| is

$$RQ = e/a$$

Equation (1) & (2) constitute five equations for five unknowns a,b,c,d and e. With a measured value of RQ, these equations can be solved to determine the stoichiometric coefficients.

The chemical formula for cell composition and the stoichiometric coefficients in equation

(a) in general change as a function of cell environment.

**6. Production of single cell protein form hexadecane is described by the following reaction equation**

**$C_{16}H_{34} + aO_2 + bNH_3 \rightarrow cCH_{1.66}\ O_{0.27}\ N_{0.20} + dco_2 + eH_2O$**

**where $CH_{1.66}\ O_{0.27}\ N_{0.20}$ represents the biomass 16 RQ – 0.43 determine the stoichiometric coefficients.**

**Solution:-**

C balance : 16 = c + d → (1)
H balance : 34 + 3b = 1.66 c 12 e → (2)
O balance : 2a = 0.27 c+ 2d + e → (3)
N balance : b = 0.20 C → (4)
Rcd : 0.43 = d/a → (5)

Solve this set of simultaneous equations

From (1) d = 16-C → (6)
From (5) a = d/0.43 = 2.32 d → (7)

Combining equation (6) & equation (7) gives an expression for a in terms of c only,

a = 2.326 (16-C)
a = 37.22 – 2.326 C → (8)

Substituting equation (4) into equation (2) gives

34 + 3 (0.20 C) = 1.66c+2e
34 = 1.06 C + 2e
e = 17-0.53 C → (9)

Substituting (8), (6) and (9) into equation (3) →

$$2(37.22 - 2.326C) = 0.27C + 2(16 - C) + (17 - 0.53C)$$

$$25.44 = 2.39\ C$$

$$C = 10.64$$

Using the value of C in equation 8, 4, 6 & 9 gives,

a = 12.48
b = 2.13
d = 5.37
e = 11.36

**7. Write short note on electron balances in stoichiometry of microbial growth.**

Available elecrion refers to the number of election available for transfer to oxygen on combustion of a substance is $CO_2$, $H_2O$ and Nitrogen containing compounds. The number of available elections found in organic material is calculated from the valence of the various elements. 4 for C, 1 for H, -2 for O, 5 for P, and 6 for S. The number of available election for N depends on the reference state : -3 if ammonia is the reference, 0 for molecular nitrogen N2, and 5 for nitrate. The reference state for cell growth is usually chosen to be the same as the nitrogen source in the medium. In the following discussion it will be assumed for convenience that ammonia is used as nitrogen source: this can easily be changed if other nitrogen sources are employed.

Degree of reduction, $\gamma$ is defined as the number of equivalents of available electrons in that quantity of material containing. 1 g atom carbon Therefore for substrate $C_wH_xO_yN_z$, the number of available electrons is (4w+x-2y-3z). The degree of reduction for the substrate, $\gamma_s$ is therefore, (4w+x-2y-3z)/w. Degrees of reduction relative to $NH_3$ and $N_2$ for several biological compounds. Degree of reduction for $CO_2$, $H_2O$ & $NH_3$ is zero.

Electrons available for transfer to oxygen are conserved during metabolism. In a balanced growth equation, number of available electrons is conserved by virtue of the fast that the amounts of each chemical element are conserved. If ammonia as nitrogen source, the available electron balance written as,

$$\boxed{w\gamma_s - 4a = c\gamma_B}$$

where $\gamma_s$ & $\gamma_B$ are the degrees of reduction of substrate and biomass respectively.

**8. Write short note on yield coefficients of biomass and product stoichiometry.**

**<u>Biomass yield:-</u>**

As cells grow there is a linear relationship between the amount of biomass produced and the amount of substrate consumed. This relationship is expressed quantitatively using the biomass yield, $Y_{xs}$;

$$Y_{xs} = \frac{\text{g cells produced}}{\text{g substrates consumed}} \qquad \rightarrow (1)$$

A large number of factors influences biomass yield, including medium composition, nature of the carbon and nitrogen source (p.r) and temperature biomass yield is greater in aerobic than in anaerobic cultures.

When $Y_{xs}$ is constant throughout growth, its experimentally determined value can be used to determine the stoichiometric coefficient ε can be expressed as,

$$Y_{xs} = \frac{C(\text{Mw cells})}{(\text{Mw substrate})}$$

Where Mw is molecular weight and Mw cells means the biomass formula weight plus anyu residual ash.

One complication with real cultures is that some fraction of 1 substrate consumed is always used for maintenance activities such as maintenance of membrane potential and internal $p^H$, turnover of cellular components and cell motility. These metabolic functions require substrate but not necessarily produce cell biomass, $CO_2$ and $H_2O$.

It is important to account for maintenance when experimental information is used to complete stiochiometric equation; maintenance requirements and the difference between observed and true yields.

**Product Stoichiometry:-**

Consider formation of an extra cellular product $C_jH_kO_lN_m$ during growth. The general produced formation equation

$$C_wH_xO_yN_z + aO_2 + bH_gO_nN_i \rightarrow cCH_\alpha O_\beta N_\delta + dCo_2 + eH_2O + fC_jH_kO_iN_m \qquad \rightarrow (1)$$

Where f is the stoichiometric coefficient for product. Product synthesis introduces one extra unknown stoichiometric coefficient is the equation; thus an additional relationship between coefficients is required. This is usually provided as another experimentally determined yield coefficient, the product yield from substrate, $Y_{ps}$,

$$Y_{PS} = \frac{\text{g Product formed}}{\text{g substrate consumed}} = \frac{f(\text{Mw product})}{(\text{Mw substrate})} \qquad \rightarrow (2)$$

As mentioned above with regard to biomass yields, we must be sure that the experimental system used to measure $Y_{Ps}$ conform to equation (1) & (2) does not held if product formation is not directly linked with growth; accordingly it cannot be applied for secondary metabolite production such as penicillin fermentation.

**9. The chemical reaction equation for respiration of glucose is**

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

**Candida utilis cells convert glucose to $CO_2$ and $H_2O$ during growth. The cell composition is $CH_{1.84}O_{0.55}N_{0.2}$ plus 5% ash. Yield of biomass from substrate is 0.5 $gg^{-1}$. Ammonia is used as nitrogen source.**

a) **What is the oxygen demand with growth compared to that without?**
b) **C. utilis is also able to grow with ethanol as substrate, producing cells of the same composition above. On a mass basis, how does the maximum possible biomass yield from ethanol compare with the maximum possible yield from glucose?**

**Solution:-**

Molecular weights glucose = 180
ethanol = 46

Mw Biomass is (25.44 & ash)

Since ask accounts for 5% of the total weight, 95% of the total = 25.44

$\therefore$ MW biomass = 25.44/0.95 – 26.78

$\gamma_s$ for glucose = 4.00

$\gamma_s$ for ethanol = 6.00

$\gamma_B = (4 \times 1 + 1 \times 1.84 - 2x0.55 - 3x\ 0.2)$

$= 4.14$

For glucose w = 6

ethanol w = 2

a) $Y_{xs} = 0.5\ gg^{-1}$ converting this mass yield to a molar yield.

$$Y_{xs} = \frac{0.5g\ biomass}{g\ glucose}\left|\frac{180g\ glucose}{1gmol\ glucose}\right|.\left|\frac{1gmol\ biomass}{26.76\ g\ biomass}\right|$$

$$Y_{ys} = 3.36\frac{gmol\ biomass}{gmol\ glucose} = c.$$

Oxygen demand is calculated from

$$a = \tfrac{1}{4}\left(w\gamma_s - c\gamma_B - Fjy_p\right)$$

In the absence of Product formation

$$a = \tfrac{1}{4}\left[6(4.00) - 3.36(4.14)\right]$$

$$= 2.52$$

∴ The oxygen demand for glucose respiration with growth is 2.5 gmol $O_2$ gmols glucose consumed. By comparison with the chemical reaction equation for respiration, this is only about 42% that required in the absence of growth.

b) Maximum possible biomass yield is calculated from

$$C_{mass} = \frac{w\gamma_s}{\gamma_B}$$

$$= \frac{6(4.00)}{4.14} = 5.80$$

Converting this is a mass basis.

$$Y_{xs\ mass} = \frac{5.80\text{g biomass}}{\text{gmol glucose}}\left|\frac{1\text{gmol glucose}}{180\text{g glucose}}\right|.\left|\frac{26.78\text{g biomass}}{1\text{gmol biomass}}\right|$$

$$Y_{ys\ mass} = 0.86\frac{\text{g biomass}}{\text{g glucose}}$$

For Ethanol

$$C_{mass} = \frac{2(6.00)}{4.14} = 2.90$$

and

$$Y_{xs\ mass} = \frac{2.90\text{ gmol biomass}}{\text{gmol ethanol}}\left|\frac{1\text{gmol ethanol}}{46\text{g ethanol}}\right|.\left|\frac{26.78\text{ g biomass}}{1\text{g biomass}}\right|$$

$$Y_{xs\ mass} = 1.69\frac{\text{g biomass}}{\text{g ethanol}}$$

∴ On a mass basis, the maximum possible amount of biomass produced per gram ethanol, consumed is roughly twice that per gram glucose consumed.

**10. Explain thermodynamic efficiency of microbial growth.**

Biochemical reactions in cells do not occur in isolation but are linked in a complex array of metabolic transformations. Catabolic and anabolic reactions takes place at the same time, so that energy released in one reaction is used to other energy requiring processes. Cells are chemical energy quite efficiently.

A microscopic view of cell growth is represented by the equation

$$C_wH_xO_yN_z + aO_2 + bHgO_n + N_i \rightarrow cCH_\alpha O_\beta N_\delta + dCO_2 + eH_2O \qquad \rightarrow (1)$$

Where, a,b,c,d,e are stoichiometric coefficients, $C_wH_xO_yN_z$ is the substrate, $H_gO_hN_i$ is the Nitrogen source, and $CH_\alpha O_\beta N_\delta$ is dry biomass. Once the stoichiometric coefficients are determined equation (1) can be used as the reaction equation in energy-balance calculations.

Heat of reaction for cell growth can be estimated using microbial stiochiometry and the concept of available electrons. It has been found empirically that the energy content of organic compounds is related to degree of reduction as follows:-

$$\boxed{\Delta h°c = -q\gamma xc}$$

Where, $\Delta h°c$ is the molar heat of combustion at standard conditions, q is the heat evolved for mole of available electrons transferred to oxygen during combustion, $\gamma$ is the degree of reduction of the compound defined with respect to $N_2$ and xc is the number of carbon atoms in the molecular formula. The coefficient q relating $\Delta h°c$ and $\gamma$ is relatively constant for a large number of compounds.

Patel and Erickson assigned a value of 111KJ $gmul^{-1}$ is q; in another analysis Rods determined value of 115 $kJgmol^{-1}$. The correlation found by Rods is based on analysis of several chemical and biochemical compounds including biomass.

**11. Explain about heat, generation stoichiometry during aerobic cultures.**

Cells use chemical energy quite efficiently but, like any real process, some of the energy in the substrates is released as heat. Metabolic heat generation dictates cooling requirements for bioreactors which harbor cells. Cellular heat production is primarily the result of energy and growth metabolism. Consequently, it is responsible to expect an approximately proportional relationship between heat generated and energy substrate utilized.

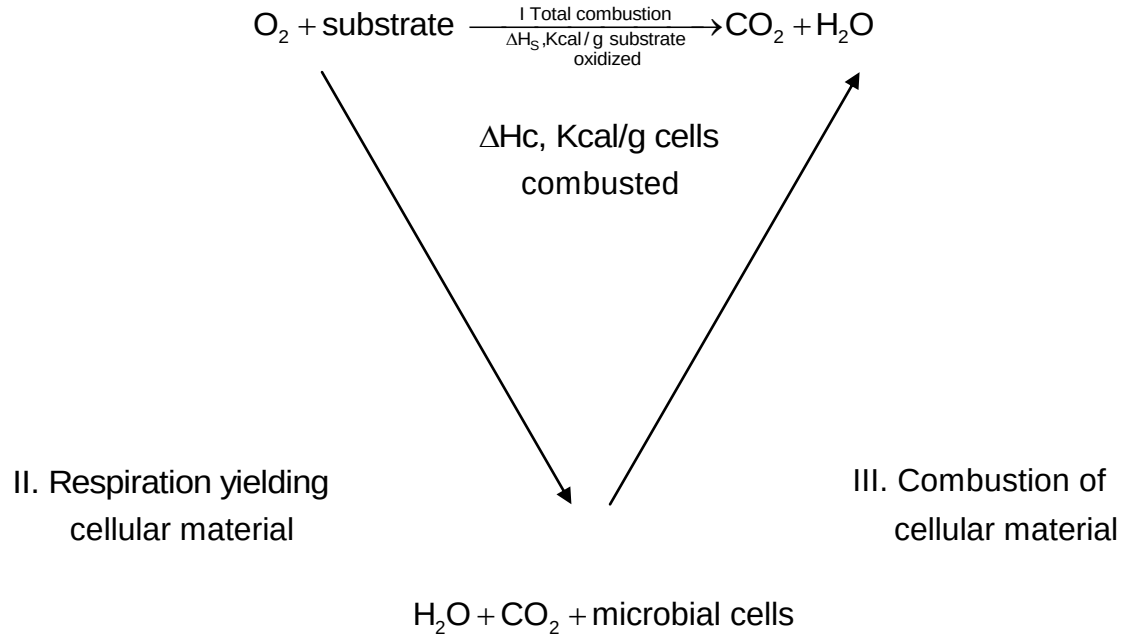
Accordingly, we define a yield factor $Y_\Delta$ analogous to the earlier yields coefficients. If $Y_s$ is grams of cell mass produced per gram substrate consumed and $\Delta H_s$ and $\Delta Hc$ are the heats of combustion of substrate and of cell mass material respectively we can write,

$$Y_\Delta\left(\text{g cell /Kcal}\right)=\frac{Y_s\left(\text{g cell / g substrate}\right)}{\left(\Delta Hs - Y_S\Delta Hc\right)\left(\text{Kcal / g substrate}\right)}$$

This arises from approximate energy balance over two pathways as shown in figure for aerobic growth. Provided that the Predominant oxidant is oxygen, the heat generation $\Delta Hs$ per gram of substrate completely oxidized minus $Y_s\Delta Hc$, the heat obtained by combustion of cells grown from the same amount of substrate, will reasonably approximate the heat generation per gram of substrate consumed in the fermentation with produces cells, $H_2O$ and $CO_2$.

The accuracy of the assumption underlying in the figure is exemplified by data for yeast growing on n-paraffins by experiment $\Delta Hs$ & $\Delta Hc$ were found to be 11.4 & 4.7 Kcal/g respectively, giving

$$\frac{1}{Y_\Delta}=\frac{11{,}400}{Ys}-4700$$




In the absence of experimental data, the heat of combustion of cells can be estimated based upon the following empirical observation; the energy obtained by transferring electrons from a compound which has reductance degree $\gamma_s$ to a compound such as $CO_2$ or $CH_4$, which has zero

reductance degree is given by K. $\gamma_s$, where the value of Ko is in the range 26-30 Kcal/(electron equivalent). Since molecular oxygen $O_2$ accepts four electrons during respiration, this corresponds to 104 – 124 Kcal/mol $O_2$ consumed.

As an example, we shall estimate the heat of combustion of pseudomonas fluorescenes growing in glucose medium. First, a reaction for cell combustion is written the measured cell composition, assuming the combustion products are $CO_2$, $H_2O$ & $N_2$:

$$\underset{\text{Cells}}{CH_{1.66}N_{0.20}O_{.227}} + 1.28O_2 \rightarrow \underset{+0.83\ H_2O}{CO_2 + 0.10N_2} \qquad \longrightarrow (i)$$

Assuming a heat of combustion of 104 Kcal/mole $O_2$, the heat released by combustion of bacteria is as follows,

$$\frac{(1.28\text{mol } O_2)(104\ \text{Kcal/mol } O_2)}{\left[12 + (1.66)(1) + (0.20)(14) + (0.27)(16)\right]\text{g}} = \frac{6.41\,\text{Kcal}}{\text{g}} \qquad \longrightarrow (2)$$

However, cell dry weight measured experimentally includes ash. If the cells dry matter contains 10% ash, then the heat of cell combustion is

$$\Delta H_c \square (0.90)\frac{6.41\,\text{Kcal}}{\text{g}} = 5.8\frac{\text{Kcal}}{\text{g dry cell mass}} \longrightarrow (3)$$

The value of $Y_\Delta$ will depend both upon the particular microbial species and upon the substrate consumed.

## UNIT – V

## PART – A

**1. Name the modes of fermentation operations.**

Three are three different modes of operation is generally carried out

(1) Batch process
(2) Continues process
(3) Fed batch process

**2. Define batch Process.**

Batch cellular is a closed calumet system which contains, initial limited amount of nutrient. The inoculated culture will pass through a number of phases. They are

(1) Lay phase
(2) Logarithmic phase
(3) Desperation phase
(4) Stationary phase
(5) Deash phase

**3. Define continues process.**

In continuous process fresh media is continuously added and product is continuously removed. As a result, cells continuous by receive fresh medicine and product and waste products and cell are continuously removed for processing.

**4. Explain fed batch process.**

It is the most common types of process used in industry, in this mode of operation fresh media is continuous or sometimes periodically added to the vessel bet unlike a continuous reactor, there is no continuous removal. The ferment or is emptied or partially emptied when reactor is full or fermentation is finished.

**5. What are the models proposed for growth kinetics?**

1) Monad model
2) Logistic model & modified logistic model,

**6. Name the model for producer formation kinetics.**

1) Leudeking piret model
2) Logistic incorporated leudeking-piret model
3) Modified logistic incorporated leudeking piret model

**7. Define moned model kinetics.**

Moned model relates the specific growth rate μ and an essential substrate concentration and was described by equation $\mu = \frac{\mu_{mas} S}{ks + s}$

Where, μ = specific growth rate
$\mu_{max}$ = specific growth rate
$k_S$ = Monod constant

**8. Define Leudeking piret model.**

The kinetics of cellulose protein production was described by leudeking piret model which states that the product formation rate ($d_x/dt$) varies linearly with hash the instantaneous cell mass concentration (x) and growth rate as

$$\frac{dp}{dt} = \alpha \frac{dx}{dt} + \beta x$$

where, α & β empherical constants that may vary with fermentation conditions.

**9. Explain logistic model.**

Under optimal growth conditions and when the inhibitory effects of substrates and product play no role, the rate of cell growth kinetics is given by

$$\frac{dx}{dt} = \mu_o x$$

where $\mu_o$ is constant defined as the initial specific growth rate equation implies that x increases with time regardless of substrate availability.

**10. What is meant by fixed volume fed hatch operation?**

In this type of fed batch, the limiting substrate is fed without diluting the culture the culture volume can also be maintained practically constant by feeding the growth limiting

substrate in undiluted form, for example, are very concentrated liquid or gas-Alternatively the substrate can be added by dialyses or in a phats synthetic culture, radiation can be the growth limiting factor without affecting the culture volume.

**11. What is meant by variable volume fed batch mode operation?**

As the name implies, a variable volume fed batch is one in which the volume changes with the fermentation time due to the substrate feed. They way this volume changes it is dependent on the requirements, limitations and objectives of the operators.

The fed can be provided according to one of the following option.

(1) The same medicine used in the batch made is added
(2) The solution of the limiting substrate at the same concentration as that in the initial medium is added, and
(3) A very concentrated solution of the limiting is added at a rate less than (1), (2) and (3).

**12. Write the advantages of fed batch process.**

(i) Production of high cell densities dell to extension of working time.
(ii) Controlled conditions in the provision of substrate during the fermentation, particularly regarding the concentration of specific substrates as for (ex) the carbon source.
(iii) Control over the production of by products.
(iv) Allows the replacement of water loss by evaporation.

**13. Define yield feature (Y).**

If is a measure of the efficiency of conversion of any one substrate into biomass and if can be used to products the substrate concentration required to produce a certain biomass concentration. Y is net a constant and if will very according to a constant and it will vary according to growth rate, pH, temperature, the limiting substrate and the concentration of the substrate in excess.

**14. What is meant by critical dilution rate?**

The dilution rate at which x equable zero termed as critical dilution rate (Derit) and is given by the equations.

$$\text{Derit} = \frac{\mu_{max}}{(K_s + SR)}$$

**15. What is meant by chemostat?**

In a chemostat the liquid volume is kept constant by setting the inlet and outlet flow rates equal; the dilution rate is therefore constant and steady state is achieved by concentrations in the chemostat adjusting themselves to the feed rate.

**16. What is meant by turbid stat?**

In a turbidostat the liquid volume is kept constant by setting the out left flow rate equal to the inlet flow rates, however, the inlet flow rate is adjusted to keep the biomass concentration constant. Thus, in a turbiodostat the dilution rate adjust to its steady state value corresponding to the set biomass concentration.

**17. Define residence time.**

$$\tau = 1/D = V/t$$

where, $\tau$ - residence time
D – dilution rate
V – volume of the reactor
F – Flow rate

**18. A strain of Azotobacter vinelandii is culture d in a 15m$^3$ stirred fermenter for alginate production. Under current operating conditions $k_s a$ is 0.175$^{-1}$. oxygen solubility in the broth is approximately sx10$^{-3}$ kgm$^3$. The specific rate of oxygen uptake is 12.5 5mmol/g$^{-h-1}$ what is the maximum possible cell concentration?**

**Solution:**

$$x_{onan} = \frac{k,aC^* AL}{qo}$$

$$= \frac{\left(0.175^{-1}\right)\left(8\times10^{-3}\text{kgm}^{-3}\right)}{\frac{12.5\text{mmol}}{\text{gh}}\left|\frac{\text{lh}}{3600\text{s}}\right|.\left|\frac{\text{lgmol}}{1000\text{mmol}}\right|.\left|\frac{32\text{g}}{1\text{gmol}}\right|\left|\frac{1\text{kg}}{1000\text{kg}}\right|}$$

$$X_{max} = 1.2\times10^{s}\text{gm}^{-3}$$

$$= 12\text{gl}^{-1}.$$

**19. Define doubling time.**

The time required to double a cell is called doubling time.

$$td = \frac{\ln 2}{\mu}$$

where, td - doubling time
μ - growth rate

**20. Name alternate equation for monad equation.**

1) Blackman equation, $\mu g = \mu_m$

$$\mu g = \frac{\mu_m}{2ks} s$$

2) Tessier equation, $\mu g = \mu_m (1-e^{-ks})$

3) Moser equation, $$\mu_g = \frac{\mu_m S^n}{k_s + s^n} = \mu_m \left(HK_s S^{-n}\right)^{-1}$$

4) Contois equation, $$\mu_g = \frac{\mu_m S}{K_{sx} X + S}$$

## PART – B

**1. Explain in detail about batch cultivation kinetics.**

**Batch Processes**

Batch culture is a closed culture system which contains initial, limited amount of nutrient. The inoculated culture will pass through a number of phases. They are

i) Lag phase
ii) Logarithmic or exponential phase
iii) Deceleration phase
iv) Stationary phase
v) Death phase

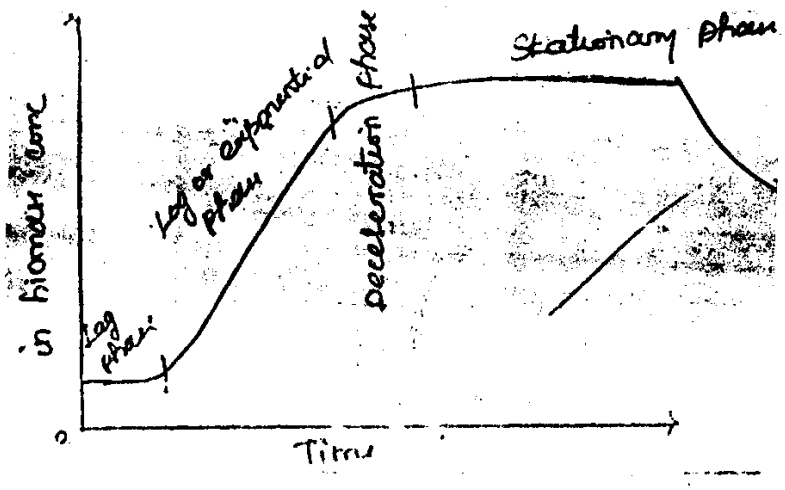



**Figure:**

After inoculation there is a period during which it appears that no growth takes place; this period is referred to as the lag phase. In a commercial processes the length of the lag phase should be reduced as much as possible.

Following a period during which the growth rate of the cells gradually increases the cells grow at a constant, maximum ratio and this period is known as log or exponential phase. This phase may be described by the following equation.

$$\text{log phase} \Rightarrow) \; dx/dt = \mu_x \rightarrow (1)$$

where, x - is the concentration of microbial
t – is time in hours biomass
μ - is specific growth role in $hr^{-1}$

On integration equ (1) gives:-

$$Xt = x_{oe}^{\mu t} \rightarrow (2)$$

Where $x_o$ – the original biomass concentration
$X_t$ – the biomass concentration after the time interval, t hour
E – the base of the oat real logarithm

On taking natural logarithms equ(2) becomes:

$$L_{nxt} = l_{nxo} + \mu t \rightarrow (3)$$

A plat of the natural logarithm of biomass concentration against time should yields straight line, the slope which gives the specific growth rate value, μ.

In the case of filamentous fungi have a growth unit which is replicated at a constant rate and is composed of the apex of the hypha and a short length of supporting hypa. So that the total length of the mycelium and the number of tips increased exponentially at approximately the same rate.

Hence the rate of increase in hyphal mass, total length and number of tips is dictated by the specific growth rate and,

$$dx/dt = \mu x$$
$$dh/dt = \mu H$$
$$dA/dt = \mu A$$

where H is total hyphal length and A is the number of growth tips.

After the log phase period the growing rats of the culture decrease until growth ceases. The cessation of growth may be due to the depletion of same essential nutrient in the medium, the accumulation of some autotoxic product of the organism in the medium or a combination of two this phase is referred as stationary phase.

The nature of the limitation of growth may be explored by growing the organism in the presence of a range of substrate concs and plotting the biomass concentration at stationary phase against the initial substrate concentration as shown in fig.

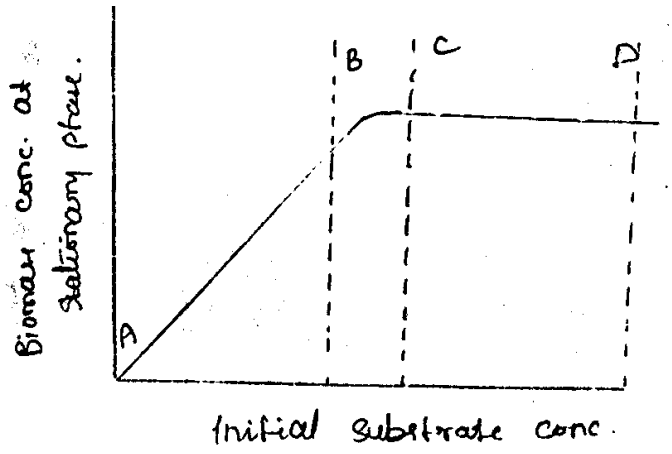



**Figure: Initial substrate conc.**

The zone A to B an increase in initial substrate concentration gives a proportional increase in the biomass produced at stationary phase.

This may be described by the equation

$$x = y(SR - S) \quad \rightarrow (4)$$

where x – is the concentration of biomass produced
y – is the yield face or (g biomass produced $g^{-1}$ substrate consumed)
SR – is the initial substrate concentration
S – is the residual substrate concentration

Over the Zone A to B, S equals is zero at the point of cessation of growth. This equ(4) may be used to predict the biomass which may be

**Produced**

Over the zone c to α an increase in the initial substrate concentration does not give a proportional increase in biomass. This may be due to either the exhaustion of another substrate or the accumulation of toxic products.

**Yield factor (y):**

It is a measure of the efficiency of conversion of any the substrate into biomass and it can be used to predict the substrate concentration required to produce a certain biomass concentration.

Y is not a constant and it will vary according to growth rate, pH, temperature, the limiting substrate and the concentration of the substrate in excess.

The decrease in growth ratio and the cessation of growth, due to the depletion of substrate, may be described by the relation ship between μ and the residual growth limiting substrate in equ(5) and the following fig.

$$\mu = \mu_{max} S/(ks+s) \rightarrow (5)$$

where s - is the residual substrate concentration

ks – substrate utilization constant, numerically equal to substrate concentration when μ is half $\mu_{max}$ and is the measure of the affinity of the organism for its substrate.

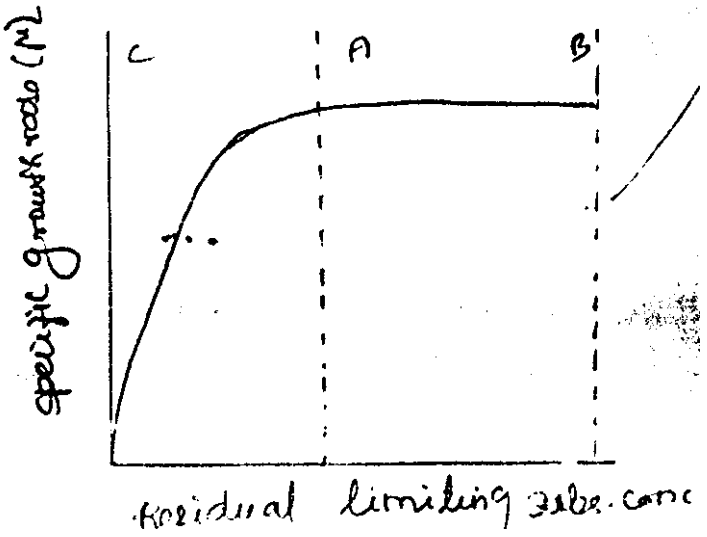



**Figure: Residual limiting subs.conc**

The zone A to B is equivalent to the exponential phase in batch culture where substrate concentration is in excess and growth is at $\mu_{max}$.

The zone c to A is equivalent to the deceteration phase of batch culture where the growth of the organism has resulted in the depletion of substrate to a growth limiting concentration which will not support $\mu_{max}$.

In the exponential phase secondary metabolites were synthesized. Pirt (1975) has discussed the kinetics of product formation by microbial cultures in terms of growth linked products and non growth linked products. Growth linked may be considered equivalent to primary metabolites which are synthesized by growing cells.

The formation of a growth linked product may be described by the equation.

$$dp/dt = q_{p^x} \rightarrow (6)$$

where p – is the concentration of product
qp – is the specific rate of products formation
(my product $g^{-1}$ biomass $h^{-1}$)

Also, product formation is related to biomass production by the equation.

$$dp/dx = 1/p/x \quad \rightarrow (7)$$

where Y p/x = is the yield of product in terms biomass (g product $g^{-1}$ biomass)
Multiply equ (7) by dx/dt, then

$$\frac{dx}{dt} \times d_p/dx = Y\,p/x\; dx/dt$$

$$d\,p/dt = Y\,p/x\; dx/dt$$

But dx/dt = μx and therefore from (1)

$$d\,p/dt = Y\,p/0l\,\mu x$$

and $dp/dt = qp.x$

and therefore,

$$q_p.x = Y\,p/0l\,\mu.x$$

$$\boxed{qp = Y\,p/x\,.\mu} \rightarrow (8)$$

Equation (8) is seen that when products formation of growth associated the specific rate of product formation increases with specific growth rate. Thus, productivity in batch culture will be greater at $\mu_{max}$ and improved products output will be achieved by increasing both μ and biomass concentration. Thus increased productivity in batch culture should be associated with an increase in biomass.

**2. Explain in detail about continuous mode of operation.**

**Continuous Culture:**

Exponential growth is batch culture may be prolonged by the addition of fresh medium to the vessel. Provided that the medium has been designed such that growth is substrate limited (i.e by some component of the medium) and not dozen limited, exponential growth will proceed until the additional substrate is exhausted. This exercise may be repeated until the vessel is full. If an overflow device were fitted to the fermenter such that the added medium displaced an equal volume of culture from the vessel then continuous production of cells could be achieved.

The flow of medium into the vessel is related to the volume of the vessel by the form dilution rate $\propto$, defined as.

$$\propto = F/V \qquad \rightarrow (9)$$

where F – is the flow rate ($dm^3h^{-1}$)
V – is the volume ($dm^3$)

Thus $\propto$ is expressed in the units $h\propto^{-1}$

The Net change in cell concentration over a time period may be expressed as

$$dx/dt = \text{growth} - \text{output}$$
$$\text{or} \quad dx/dt = \mu_x - D_x \quad \rightarrow (10)$$

Under steady-state concentration remains constant, thus

dx/dt = o and

$$\mu x = Dx$$
$$\text{and} \quad \mu = D \quad \rightarrow (11)$$

Thus under steady state conditions specific growth rate is controlled by dilution rate which is an experimental value.

The growth of the cells in a continuous culture of this type is controlled by the availability of the growth limiting chemical component of the medium and, thus the system is called as a chemostat. The mechanism expressed by the expression

$$\mu = \mu_{max} S/(k_s + S)$$

At steady state, $\mu = D$ and therefore

$$D = \mu_{max}\, S/(K_s+S)$$

Where s is the steady state concentration of substrate in the chemostat and

$$S = K_S D/(\mu_{max}-D) \rightarrow (12)$$

This equation products that the substrate concentration is determined by the dilution rate.

If substrate is depleted below the level that supports the growth rate dictated by the dilution rate, the following sequences of events takes place.

(i) The growth rate of the cells will be left than the dilution rate and they will be washed out of the vessel at a rate greater than they are being produced, resulting in a decrease in biomass concentration.
(ii) The substrate concentration in the vessel will rise because fewer cells are left in the vessel to consume it.
(iii) The increased substrate concentration is the vessel will result in the cells growing at a rate greater than the dilution rate and biomass concentration will increase.
(iv) The steady state will be reestablished

The concentration of cells in the chemostat at steady state is described by the equation.

$$\bar{x} = Y(SR - \bar{S}) \qquad \rightarrow (13)$$

where $\bar{x}$ - is the steady state cell concentration in the chemostat.

By combining equ(12) and (13), then

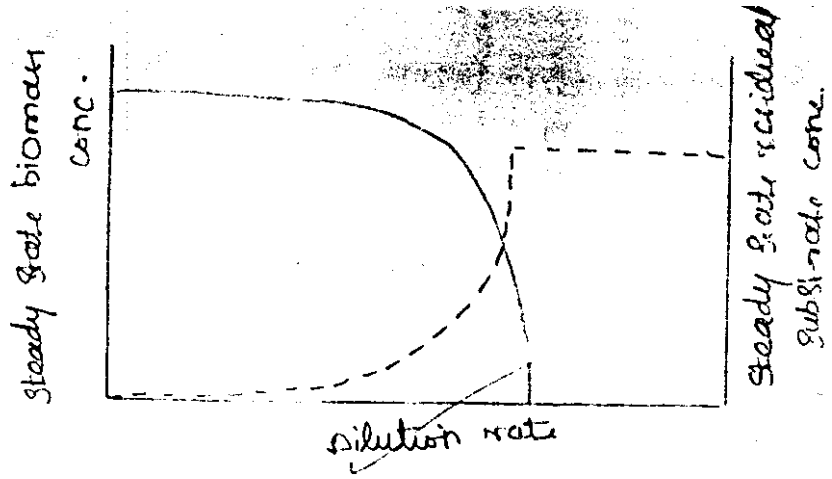
$$\bar{x} = Y\left[SR - \{K_S D/(\mu_{max} - D)\}\right] \qquad \rightarrow (14)$$

Thus, at steady state biomass concentration is determined by the operational variables SR and D. If SR increased, $\bar{x}$ will increase but, $\bar{s}$ the residual substrate concentration in the chemostat, will remain same.

If D is increased, μ will increase (M=D) and the residual substrate concentrations at the new steady state would have inversed to support the elevated growth rate. Thus less substrate will be available to be converted into biomass, resulting in a lower steady state value.

The following figure illustrates the continuous culture behavior of a hypothetical bacterium with a lows value for the limiting substrate concentration with increasing dilution rate, the residual substrate concentration increases only slightly until D approaches $\mu_{max}$ when s increases significantly. The dilution rate raise at which x equals zero is termed the critical dilution rate (Dorit) and is given by the equation.

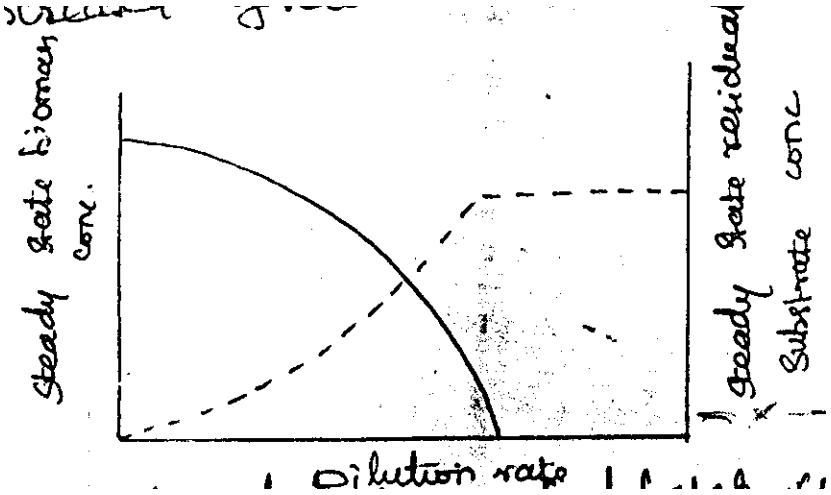
$$\text{Dorit} = \mu_{max}{}^{SR} / (ks + SR)$$




(_____) steady state biomass conc.
(_ _ _ _ _) Steady state $\bar{s}$ conc.

**Figure:**

Following fig. illustrated the continuous culture behaviour of a hypothetical bacterium with a high ks for the limiting substrate compared with the initial limiting substrate concentration. With increasing dilution rate the residual substrate concentration increases significantly to support the increased growth rate.




(_____) steady state biomass conc.

(_ _ _ _ _) Steady state $\bar{s}$ conc.

**Figure: Dilution rate**

**3. Explain in detail about fed batch cultivations Process.**

**FEO BATCH CULTURE**

Yoshida of al (1973) introduced the term fed batch culture to describe batch culture which are fed continuously or sequentially with medium, without the removal of culture fluid A fed batch mode culture is established initially in batch maintenance then fed according to one of the following feed strategies:

(i) The same medium used to established the batch culture is added, resulting in an increase in volume

(ii) A solution of the limiting substrate at the same concentration as that in the critical medium is added, resulting in an increase in volume.

(iii) A concentrated solution of the limiting substrate is added at a rate case than in (1) and (2) resulting in an increase in volume.

(iv) A very concentrated solution of the limiting substrate is added at a rate lese than in (1), ® and ® resulting, in an insignificant increase in volume.

Fed batch systems employing strategies (1) and (2) are described as variable volume, where as system employing strategy (4) is described as fixed volume. The use of strategy (3) gives a culture intermediate between the two extremes of variable and fixed volume.

**Variable volume fed batch culture**

Consider a batch culture in which growth is limited by the concentration of one substrate, the biomass at any point in time will be described by the equation.

$$x_t = x_o + Y(S_R - R) \rightarrow (1)$$

where $x_t$ = is the biomass concentration after time, t, hours.

$X_o$ – is the inocluam concentration,

The final biomass concentration produced when s=o may be described as $x_{max}$ and provided that $x_o$ is small compared with $x_{max}$.

$$X_{max} \approx Y\ SR \qquad \rightarrow (2)$$

If, at the time when x = $x_{max}$, a medium feed is started such that the dilution rate is les than $\mu_{max}$, virtually all the substrate will be consumed as fast as it enter the culture thus;

$$FSR = \mu(X/Y) \qquad \rightarrow (3)$$

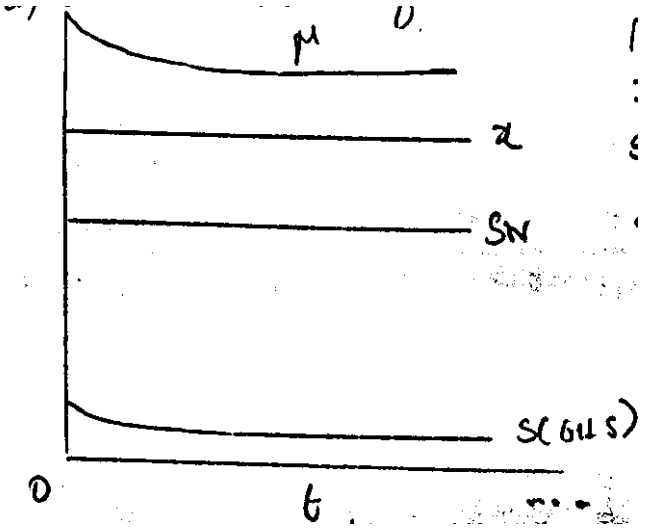
where F – is the flow rate of the medium feed

x – is the total biomass in culture, described by x = xV, where V is the volume of the culture in the vessel time t.

The total biomass in the culture (x) increases with time, cell concentrations (x) remain virtually constant, that is dm/dt =O and therefore μ=D. This situation is sermed a quasi steady state. The dilution rate will be given by the expression.

$$D = F/(V_o + F_t) \qquad \rightarrow (4)$$

Where $V_o$ – is original volume. Thus according to monod kinetics residual substrate should decrease as D decreases resulting in an increase in the cell concentration. The quasi steady state is illustrated in the fig. The major difference between the steady state of a chemostats and the quasi steady state of a fed batch culture is that μ is constant in the chemostat but decreases in the fed batch.




μ - specific growth rate
x - biomass concen
s(GLS) – growth limiting substrar
$S_N$ - any other subtrate than S(GLS)

**Figure:**

The change in product concentration in variable volume fed batch culture is the same way as for continuous culture.

$$Dp/dt = q_P x - DP \rightarrow (5)$$

Thus product concentration changes according is the balance between production rate and dilution by the feed.

**Fixed volume fed batch culture.**

Consider a batch culture in which the growth of the process organism has depleted the limiting substrate to a limiting level. If the limiting substrate is then added in a concentrated feed such that the broth volume remains almost constant. Then

$$dx/dt = GY$$

where G – is the substrate feed rate (g $dm^{-3}$ $l^{-1}$) and Y is the yield factor
But $dx/dt = \mu_x$, thus substituting for dx/dt in equation gives:

$\mu_x = Gy$ and thus

$\mu = GY/x \rightarrow (6)$

However, dx/dt may net be equated to zero, as in the case of variable volume fed batch, because the biomass concentration as well as the total amount of biomass in the fermenter, will increase with semi, Biomass concentration is given by the equation.

$$x_t = x_{oa} + GY_t \quad \rightarrow(7)$$

where $x_t$ = is the biomass after operating in fed batch for t hours

and $x_{oa}$ – is the biomass concentration at the onset of fed batch culture.

As biomass increases then the specific growth rate will decline according equation (6). The behavior of a fixed volume fed batch culture is illustrated by the following figure. From which it may be seen that μ declines the limiting substrate concentration remains virtually constant, biomass increases and the concentration of the non-limiting nutrients declincs.

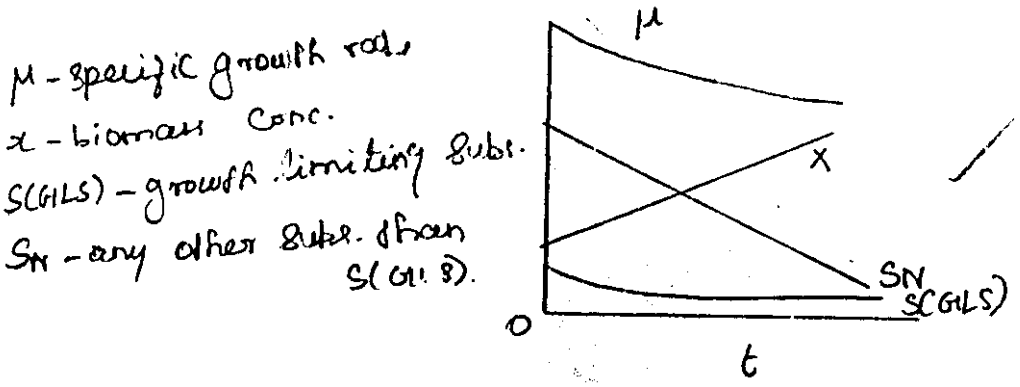



**Figure:**

Pird (1970\9) described the product balance in a fixed volume fed batch system as

$$dp/dt = q_{px},$$

but substituting for x from equ (7)

$$d_p/dt = q_p (x_{oa} + GYt)$$

If ap is strictly growth rate related then product concentration will rise linearly are for biomass. However if qp is constant then the rate as growth rate declines ie the time progresses and x increases. Thus relation ship is should in the following fig. If $q_p$ is related to μ in a complex manner then the product concentration will very according to that relationship. As in the case of

variable volume fed batch the feed profile would be optimized according to the relationship between $q_{po}$ and $\mu$.

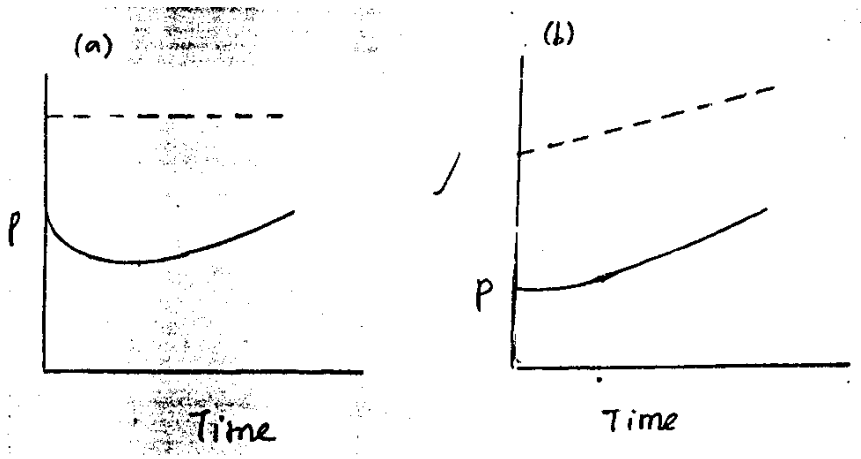



**Figure:**

a) Variable volume fed batch culture
b) Fixed volume fed batch culture

$q_P$ is growth related (----------)

$q_P$ is non growth related (— )

**4. Explain monod kinetics model for microbial growth kinetics.**

**Monod Growth Kinetics**

Monod growth kinetics is applied to the substrate limited growth. Monod growth kinetics is given by the following equation

$$\mu_g = \frac{P_{max}.c_s}{k_s + c_s}$$

$\mu_g$ – g specific growth rate ($h^{-1}$)
$\mu_{max}$ – Maximum growth rate ($h^{-1}$)
$C_s$ – Substrate concentration
$K_s$ – Saturation constant or half velocity constant.

Monod growth kinetics can be solved by two methods.

1. Rapid equilibrium method
2. Quasi steady state method.

**Rapid equilibrium method**

$S + C_R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C_P \xrightarrow{L_3} P + 2C$ Resting

$C_R$ – cell Resting

$C_P$ – CEll Pregnent

P-Product

S-Substrate

**Assumption**

1. $\left[(T) = \left[C_p\right] + \left[C_R\right] \quad \left[C_R\right] = \left[C_T\right] - \left[C_P\right]\left[C_T\right]\right]$ = Total cell

2. $M_g = k_3\left[C_P\right] \Rightarrow \left[C_P\right] = \dfrac{\mu_g}{K_3}$

3. $\mu_{max} = K_3\left[(C_T)\right]$

$$\frac{K_2}{K_1} = \frac{\left[C_R[S]\right]}{\left[C_P\right]}, \quad \left[C_P\right]\frac{K_2}{K_1} = \left[C_R\right][S]$$

$\left[C_P\right]\dfrac{K_2}{K_1} = (C_T)[S] - \left[C_P\right][S]$ $\otimes$ by s

$$C_P\left[\frac{K_2}{K_1} + [S]\right] = (C_T)[S]$$

$$\left[C_P\right] = \left[C_T\right][S] / [S] + K_2/K_1$$

$$\mu_g = \frac{K_3\left[C_T\right][S]}{[S] + K_2/K_1}$$

$$\mu_g = \frac{\mu_{max}[S]}{[s] + K_2/K_1}$$

$$\boxed{\mu_g = \frac{\mu_{max}.[s]}{[s] + K_s}}$$

(ii) The quasi steady state Assumption

$$\frac{d(C_P)}{dt} = K_1[S][C_R] - K_2[C_P] - K_3[C_P]$$

By quasi steady state assumption

$$d[C_P]/dt = o$$

$$K_1[s][C_R] = [K_2 + K_3][C_P]$$

$$[C_P] = \frac{K_1[S][C_R]}{K_2 + K_3}$$

$$[C_P] = K_T \frac{[[C_T] - [C_P]][S]}{K_2 - K_3}$$

$$[C_P]K_g = [C_T][S][C_P][S]$$

$$[C_P][K_S + S] = [C_T][S] \quad \boxed{\because K_S = \frac{K_T K_3}{K_1}}$$

$$[C_P] = \frac{[C_T][S]}{KS + [S]}$$

$$\mu_g = \frac{K_3[C_T][S]}{[K_S] + [S]}$$

$$\mu_g = \frac{\mu_{max}[s]}{[K_s] + [s]}$$

Double reciprocal plot

$$\frac{1}{\mu_g} = \frac{K_s + [S]}{\mu_{max}[s]}$$

$$\frac{1}{\mu_g} = \frac{K_s}{\mu_{max}[s]} + \frac{1}{\mu_{max}}$$

from graph,

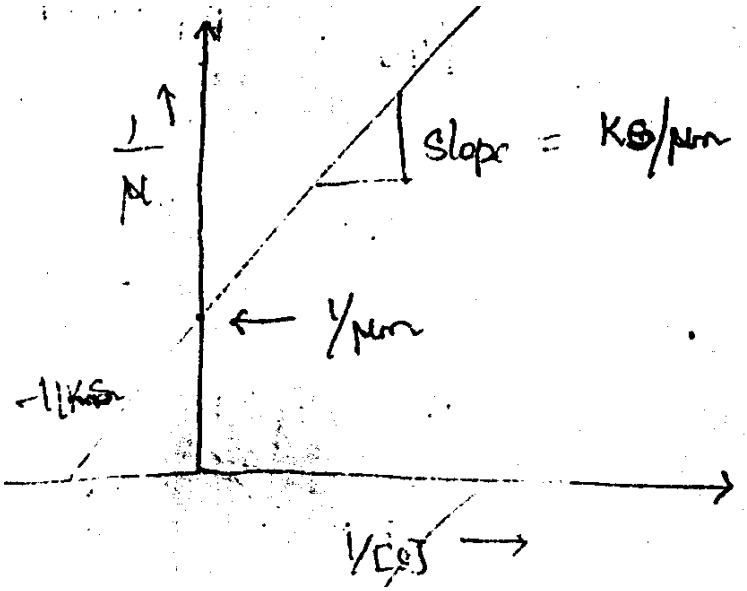



**Figure:**

**5. Explain simple unstructured kinetic models for microbial growth.**

As shown in Fig. the relationship of specific growth rate to substrate concentration often assumes the form of saturation kinetics. Here we assume that a single chemical species.s. is growth-rate limiting (i.e., and increase in S inflaences growth rate, which changes in other nutrient concentrations have no effect). These kinetics are similar to the Langmuir-Hinshelwood (or Hougen-Watson) kinetics in traditional chemical kinetics of Michaelis-Menten kinetics for enzyme reactions. When applied to cellular systems, these kinetics can be described by the Monod equation.

$$\mu_s = \frac{\mu 3}{K. + S}$$

where μ, is the maximum specific growth rate when S >> K. If endogenous metabolism important, then = $\mu_g$. The constant K, is known as the saturation constant or half-velocity constant and is equal to the concentration of the rate-limiting substrate when the specific rate of growth is equal to one-half of the maximum. That is, $K_s$, = S when $\mu_g = \mu_{max}$. In general, $\mu_g$ = for S >> K, and $\mu_g=(\mu_g/K)$ S for S << K. The Monod equation is semiempirical; it derives from the premise that a single enzyme system with Michalis-Menten kinetics is responsible for uptake of S, and the amount of that enzyme or its catalytic activity is sufficiently low to be growth-rate limiting.

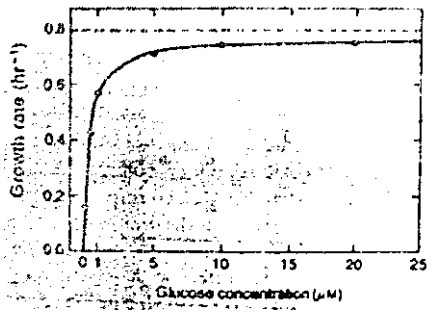



**Figure: Effect of nutrient concentration on the specific growth rate of E.coil (With permission, from R.Y.Stanier. M.Doudoroff, and E.A.Adelberg. The Microbial World, 5th ed. Pearson Education, Upper Saddle River.NU.1986.p.192).**

This simple premise is rarely. If ever, tree: however, the Monod equation empirically fits a wide range of data satisfactorily: and is the most commonly applied unstructured, no segregated model of microbial growth.

The Model equation describes substrate-limited growth only when growth is slow and population density is low. Under these chemistances, environmental conditions can be related simply to S. If the consumption of a can energy substrate is rapid, then the release of toxie waste products is more likely (due to energy-spiting reactions). At high population levels, the buildup of toxic metabolic by products becomes more important: The following rate expressions have been proposed for rapidly growing dense cultures:

$$\mu_g = \frac{\mu_g S}{K_0 S_0 + S}$$

$$\mu_g = \frac{\mu_g S}{K_o + K_o S_o + S}$$

where $S_o$ is the initial concentration of the substrate and $K_s$ is dimensionies..

Other equations have been proposed to describe the substrate-limited growth phase. Depending on the shape of μ-S curve, one of these equations may be more plausible than the others. The following equations are alternatives to the Monod equation:

Blackman equation: $\mu_g = \mu_g$ iff$S \geq 2K$,

$$\mu_g = \frac{\mu_g}{2A} S, \quad \text{iff} S, < 2K,$$

Tessier equation: $\mu_g = \mu_g\left(1-e^{-xs}\right)$

Moser equation: $\mu_s = \frac{\mu_g S}{K_s + S} = \mu\left(1+K_g S^{-g}\right)^{-1}$

Contois equation $\mu_g = \frac{\mu_g S}{K_{sx} X + S}$

Although the Blackman equation often fits the data better than the Monod equation, the discontinuity in the Blackman equation is troublesome in many applications. The Tessier equation has two constants ($\mu_g K$), and the Moser equation has three constants ($\mu_g K_g$), the Monod equation when n=1. The Contois equation has a saturation constant proportional to cell concentration that describes substrate-limited growth at thigh cell densities. According to this equation, the specific growth rate decreases with decreasing substrate concentrations and eventually becomes inversely proportional to the cell concentration in the medium (i.e., $\mu_g \propto X^{-1}$).

These equations can be described by a single differential equation as

$$\frac{d\upsilon}{ds} = K\upsilon * \left(1-\upsilon\right)^{>}$$

| | a | b | k |
|---|---|---|---|
| Monod | 0 | 2 | 1/K |
| Tessier | 0 | 1 | 1/K |
| Moser | 1-1/4 | 1-1/4 | n/K |
| Contios | 0 | 2 | $1/K_o$ |

where $\upsilon = \mu/\mu_g.S$ is the rate-limiting substrate concentration, and K, d and b are constants. The values of these constants are different for each equation and are listed in Table.

The correct rate form to use in the case where more than one substrate is potentially growth-rate limiting is an unresolved question. However, under most circumstances the noninteractive approach work best:

$$\mu_g = \mu_g\left(S_1\right) or \mu_g\left(S_2\right) or .... \mu_g\left(S_n\right)$$

where the lowest value of $\mu_g$ ($S_1$) is used.

**6. Explain substrate and product inhibition kinetic of cell growth & product formation.**

Models with growth inhibitors. At high concentration of substrate or product and in the presence of inhibitory substances in the medium, growth becomes limited and growth rate depends on inhibiter concentration. The inhibition pattern of microbial growth is analogous to enzyme inhibition. If a single-substrate enzyme-catalyzed reaction is the rate-limiting step in microbial growth then kinetic constants in the rate expression are biologically meaningful. Often the underlying mechanism is complicated, and kinetic constants do not have biological meanings and are retained from experimental data by curve fitting.

1. **Substrate inhibition:** At high substrate concentrations, microbial growth rate is inhibited by the substrate. As in enzyme kinetics, substrate inhibition of growth may be competitive or noncompetitive. If a single-substrate enzyme-catalyzed reaction is the rate-limiting step in microbial growth, then inhibition of enzyme activity results in inhibition of microbial growth by the same pattern.

   Noncompetitive substrate inhibition: $\mu_g = \dfrac{\mu_g}{\left(1+\dfrac{K_g}{S}\right)\left(1+\dfrac{S}{K_1}\right)}$

   Or if $K_1$ >>, then: $\mu_g = \dfrac{\mu_g S}{K_g + S + S^2/K_1}$

   For competitive substrate inhibition: $\mu_g = \dfrac{\mu_g S}{K_s\left(1+\dfrac{S}{K_1}\right)+S}$

Note that differs from and $K_1$ in and differ. Substrate inhibition may be activated by slow, intermitted addition of the substrate to the growth medium.

2. **Product inhibitions:** High concentrations of product can be inhibitory for microbial growth. Product inhibition may be competitive or noncompetitive, and in some cases when the underlying mechanism is not known, the inhibited growth rate is approximated to exponential or linear decay expressions.

   Competitive product inhibition: $\mu_g = \dfrac{\mu_m s}{K_s\left(1+\dfrac{P}{K_p}\right)+S}$

   Noncompetitive product inhibition: $\mu_g = \dfrac{\mu_m}{\left(1+\dfrac{K_g}{S}\right)\left(1+\dfrac{P}{K_p}\right)}$

Ethanol fermentation from glucose by yeasts is a good example of noncompetitive product inhibition, and ethanol is inhibitor at concentrations above about 5%. Other rate expressions used for ethanol inhibition are

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_s}{S}\right)}\left(1-\frac{P}{P_s}\right)^n$$

where $P_n$ is the product concentration at which growth stops, or

$$\mu_g = \frac{\mu_g}{\left(1+\frac{K_g}{S}\right)} e^{-P/K_g}$$

where $K_P$ is the product inhibited constant.

3. **Inhibition by toxic compounds:** The following rate expressions are used for competitive, noncompetitive, and uncompetitive inhibition of growth in analogy to enzyme inhibition.

Competitive inhibition: $\mu_g = \frac{\mu_m S}{K_g\left(1+\frac{1}{K_1}\right)+S}$

Noncompetitive inhibition: $\mu_g = \frac{\mu_m}{\left(1+\frac{K_g}{S}\right)\left(1+\frac{1}{K_1}\right)}$

Uncompetitive inhibition: $\mu_g = \frac{\mu_m S}{\left(\frac{K_g}{\left(1+\frac{1}{1}\right)}+S\right)\left(1+\frac{1}{K_1}\right)}$

In some cases, the presence of toxic compounds in the medium results in the inactivation of cells or death. The net specific rate expression in the presence of death has the following form:

$$\mu_g = \frac{\mu_m S}{K_s + S} - k_g$$

where $K_d$ is the death-rate constant ($h^{-1}$).

**7. Explain the growth kinetics of growth of filamentous organisms.**

**Growth models for filamentous organism.** Filament ions organizes such as molds often form microbial pellets at high cell densities in suspension culture. Cells growing inside pellets may be subject to diffusion limitations. The growth molds should include the simultaneous diffusion and consumptions of nutrients within the pellet at large pellet size. This problem is the same we face in modeling the behavior of bacteria or yeasts entrapped in spherical gel particles.

Alternatively, filamentous cells can grow on the surface of a moist solid. Salt growth is usually a complicated process, involving not only growth kinetics but the diffusion of nutrients and toxic metabolic by products. However, for an isolated colony growing on a rich medium, we can ignore some of these complications.

In the absence of mass-transfer limitations, it has been observed that the radius of a microbial pellet in a submerge culture or of a mold colony growing on an agar surface increases linearly with time.

$$\frac{dr}{dt} = k_p = \text{constnat}$$

La terms of growth rate of a mold colony, can be expressed as

$$\frac{dM}{dt} = \rho 4\pi R^2 \frac{dR}{dt} = j_p 4\pi R^2 \rho$$

or

$$\frac{dM}{dt} = \gamma M^{2/3}$$

where $\gamma = k_r(36\pi\rho)^{1/3}$. Integration yields

$$M = \left( M_o^{1/3} + \frac{\gamma t}{3} \right)^3 = \left( \frac{\gamma t}{3} \right)^3$$

The initial homass. $M_{is}$ is usually very small compared to M and therefore M varies with 1. This has been supported by experimental data.

A complete analysis of filamentous organism growth should also consider the kinetics of pellet formation. This occurs due to agglomeration of spores and subsequent growth or by outgrowth from an individual spore. Research to date has shown that many properties of the organism and its growth environment interact to influence pellet formation. Because the

mechanism are complicated and not sufficiently well understood or documented, general kinetic models for pellet formation have not yet been developed.

When considering growth of existing bellets, the model outlined above must be viewed as a crude approximation of reality pellet size, morphologes and internal structure, all of which are expected to influence pellet kinetics, are determined by interactions among agitation intensity, pellet concentration, organism prosperities and medium composition.

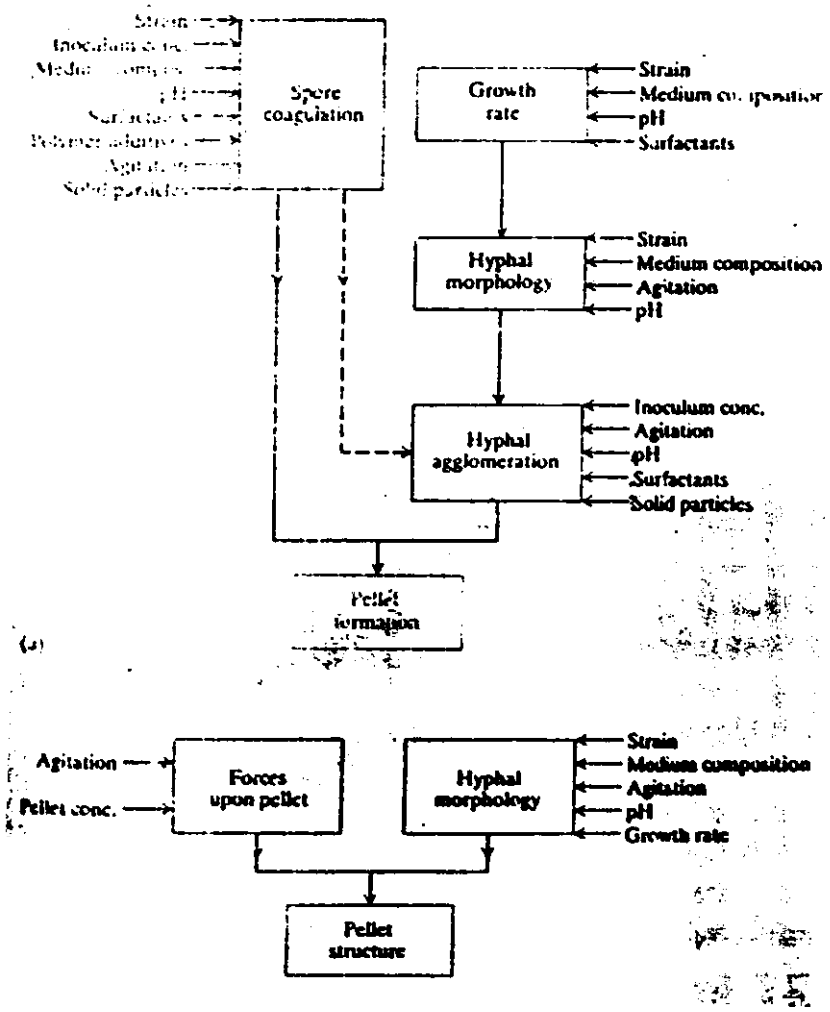



**Figure: Summary of the factors which interact to determine (a) pellet formation and (b) pellet structure during cultivation of mycelial organism (Reprinted by permission from B. Metz and N.W. P Kossen. Biotechnology. Review: The Growth of Molds in the Form of Pellets-A Literature Review. Biotechnical Bioeng-vol.192.p.781.1977).**

**8. A plasmid containing strain of E.coli is asked to produced recombinant protein in a 250 l fermenter. The probability of plasmid less per generation is 0.005. The specific growth rate of plasmid free cells is 1.4h$^{-1}$, the specific growth rate of plasmid bearing cells is 1.2h$^{-1}$. Estimate the fraction of plasmid bearing cells after his growth if the incolumn contain only cells with plasmid?**

**Solution:**

The number of generation of plasmid carrying cells in 1sh is calculated from

$$n = \frac{\mu^x t}{\ln 2}$$
$$= \frac{1.2h^{-1} \times 18h}{\ln^2}$$
$$n = 31$$

substituting this in following equation

$$F = \frac{1-\alpha-P}{1-\alpha-2^n(\alpha+P-1)}$$

where, P=0.005, $\alpha$=1.4h$^{-1}$ $P/_{1.2h^{-1}} = 1.17$

$$\Rightarrow t = \frac{1-1.17-0.005}{1-1.17-2^{31(1.17+0.005-1)} \times 0.005} = 0.45$$

$\therefore$ after 18h only 45% of the cells contains plasmid.

**9. A mouse-mouse hybridoma cell line is used produced monodonal antibody. Growth in batch cellular is monitored with the following results.**

| Time(d) | Cell concentration (cell $m/^{-1}_{x10} - 6$ |
|---|---|
| 0.0 | 0.45 |
| 0.2 | 0.52 |
| 0.5 | 0.65 |
| 1.0 | 0.81 |
| 1.5 | 1.22 |
| 2.0 | 1.77 |
| 2.5 | 2.13 |

| | |
|---|---|
| 2.5 | 3.55 |
| 3.0 | 4.02 |
| 3.5 | 3.77 |
| 4.0 | 2.20 |
| 4.5 | |

(a) Determine the specific growth rate during the growth phase.
(b) What is the culture doubling time?

**Solution:**

(a) The data was platted a, a semi leg graph

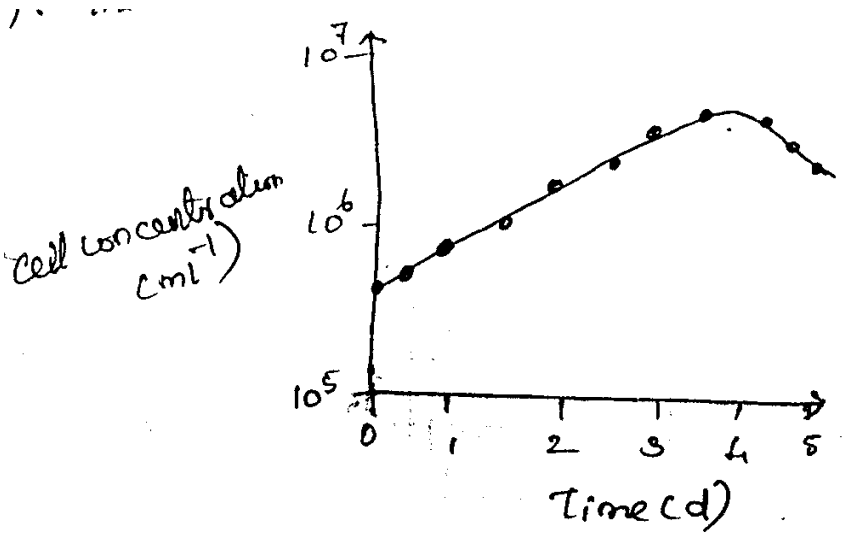



**Figure:**

From the graph, slope = 0.67

$\therefore \quad \mu = 0.67\ d^{-1}$

(b)
$$t_{\partial} = \frac{\ln 2}{\mu}$$
$$= \frac{\ln 2}{0.67} = 1.0d$$

This doubling time applies only during the growth phase of the culture.

**10. A new strain of yeast is bearing considered for biomass production. The following data were obtained using a chemostat. An influent substrate concentration of 800 mg/l and an excel of oxygen were used at a pH of 5.5 and 7.35°C. Closing the following data, calculated.**

$\mu_m, k_s, Y^m k/s\ k_d \quad \alpha$ ms,assuming $\mu_{net} = \dfrac{\mu_m s}{(k_s + s)} - k_d$

| Dilution rate ($h^{-1}$) D | Carbon substrate conc.(mg/l) | Cell concentration ($m_g$/l) |
|---|---|---|
| 0.1 | 16.7 | 366 |
| 0.2 | 33.5 | 407 |
| 0.3 | 59.4 | 408 |
| 0.4 | 101 | 404 |
| 0.5 | 169 | 371 |
| 0.6 | 298 | 299 |
| 0.7 | 702 | 59 |

**Solution:**

The first step is plet

$$\frac{1}{Y_{x/s}^{AP}} V_s \, 1/\infty$$

$Y_{x/s}^{AP}$ is calculated fro X/($s_o$-S).

The intercept is $1/Y_{x/s}^m = 1.58(\text{or}) Y_{x/s}^m = 0.633 g\ x/g$

The slope is 0.06 g s/g x-h

$$m_s = k_a / Y_{x/s}^m$$

$$k_d = m_s Y_{x/s}^m$$

$$= 0.06 gs/gx - h \text{ xo.633 g x/g S}$$

$$\boxed{k_d = 0.A38 h^{-1}}$$

The second step,

$$D = \mu_g = k_d = \frac{\mu_m s}{k_s + S} - k_d$$

$$\frac{1}{D + k_d} = \frac{1}{\mu_m} + \frac{k_s}{\mu_m}\frac{1}{S}$$

$$\Rightarrow \text{so plat } \frac{1}{D+k_d} \; V_s \; 1/S$$

then we get, intercept = 1.25 h (or) $\mu_m = 0.sh^{-1}$

slope = 100 h/($m_g$/l)

$$k_s/\mu_m = 100(\text{or})$$

$$\boxed{K_s = 80 mg/l}$$

**11. The specific growth rate for inhibited growth in a chemostat is given by the following equation.**

$$\mu_g = \frac{\mu_m S}{k_s + s + IK \; S/K_I}$$

where, $S_o$ = 10g/l ks=1 g/l I = 0.05 g/l

$X_o$ = O $K_g$ = 0.01 g/l $\mu_m = 0.5h^{-1}$

$$Y^m_{k/s} = 0.1 \; \frac{g \;\; cell}{g \; subs}$$

$$k_d = O.$$

a) Determine X and S as a function of ∝ when I =O.
b) Wish inhibiter added to a chemostat, determine the effluent substrate concentration and X as a function of ∝.
c) Determine the cell productivity, Dx as a function of detection rate.

**Solution:**

a) $S = \frac{K_s \infty}{\mu_m - \infty} = \frac{\infty}{0.5 - \infty}$

$X = Y^{\mu}_{x/s}(s_0 - s) = 0.1\left(10 - \infty/0.5 - \infty\right)$

b) In the presence of inhibitor

$$\mu_g = \frac{\mu_m s}{k_s\left(1+\frac{I}{K_I}\right)+S} = \infty$$

$$S = K_s \frac{\left(1+\frac{I}{K_I}\right)\infty}{\mu_m - \infty} = \frac{I\left(1+\frac{0.05}{0.01}\right)\infty}{0.5-\infty}$$

$$S = \frac{6\infty}{0.5-\infty}$$

$$X = Y^{\mu}_{x/s}\left(s_0 - s\right) = 0.1\left(10 - \frac{6\infty}{0.5\infty}\right)$$

c) $Pr_x = \infty X = \infty Y_{x/s}\left(s_0 - s\right)$

$$= 0.1\infty\left(10 - \frac{6\infty}{0.5-\infty}\right).$$

November- December 2010

Part-A(10×2=20 marks)

1.What is biotransformation?. Give example?
2. Name the upstream and downstream process involved in a fermentation process?
3. Differentiate synthetic media from complex media with a suitable example.
4. What is the role of precursors in fermentation? Give two examples of precursors.
5. An unsterile broth contains initially $1 \times 10^{10}$ viable cells. Find the Del factor of sterilization ($N_t = 10^{-3}$)
6. What is accepted risk of contamination in sterilization?
7. What is degree of reduction?. Calculate the degree of reduction of glucose($C_6H_{12}O_6$) and glycerol ($C_3H_8O_3$).
8. What is meant by maintainance coefficient of microbial growth?
9. Differentiate the fed batch and continuous mode of fermentor operation?
10. Write the Leuderking Piret model for product formation.

Part-B (5×16=80 marks)

11. (a) (i) What are the general requirements of fermentation process? Name some of the major products of bioprocessing?

(ii) Give the schematic representation of typical fermentation process

Or

(c) Explain in detail the various parameters that have to be monitored during the fermentation process

12 (a) (i) Describe the medium composition taking an example of production of bioproducts in lab scale.

(ii) Explain in detail the various carbon and nitrogen sources used in fermentation media

Or

(b) (i)Write the advantages of medium optimization methods over classical design method?

(ii)Describe in detail the Plackett Burman method of medium optimization?

13 (a) (i) Derive the thermal death kinetics of microorganism

(iii) Write a note on filter sterilization of liquid media

0r

(c) Explain the continuous sterilization process in detail

14 (a) assume that experimental measurements for a certain organism have shown that cells can convert two-third (wt/wt) of the substrate carbon (glucose or alkane) to biomass.

(i) Calculate the stoichimetric coefficients for the following biological reaction

$C_{16}H_{34} + aO_2 + bNH_3 \dashrightarrow c(C_{4.4}H_{7.3}N_{0.86}O_{1.2}) + dH_2O + eCo_2$

$C_6H_{12}O_6 + aO_2 + bNH_3 \dashrightarrow c(C_{4.4}H_{7.3}N_{0.86}O_{1.2}) + dH_2O + eCo_2$

(ii) Calculate the yield coefficient $Y_{X/S}$ (g dw cell/ g substrate), $Y_{X/O2}$ (g dw cell/ g $O_2$) for both the reactions

Or

(b) Write short notes on the following:

(i) Oxygen consumption in aerobic culture
(ii) Heat evolution in aerobic culture
(iii) Yield coefficient of biomass and product
(iv) Respiratory quotient

15(a).Give the material balance equation for CSTR and explain it with a neat schematic diagram.

Or

(b)Explain briefly about the following;

(i)Growth model for filamentous organisms
(ii)Substrate inhibited and product inhibited cell growth.

# CBT372-CANCER BIOLOGY

## UNIT – I

## PART – A

**1. What is apoptosis?**

Cell suicide mediated by a group of protein degrading enzymes called caspases; involves a programmed series of events that leads to the dismantling of the internal content of the cell.

**2. What is checkpoint?**

The pathway that monitors conditions within the cell and transiently halts the cell cycle if conditions are not suitable for continuing.

**3. What is malignant tumor?**

Tumor that can invade neighboring tissues and spread through a blood stream to other parts of the body.

**4. What is cell transformation?**

The process of cell change in which a cell loses its ability to control its rate of division, and thus becomes a tumor is called cell transformation.

**5. What are the Ideal characters of the cancer cell?**

1. Immortality
2. Loss of contact inhibition
3. Loss of anchorage dependence
4. Lower serum requirement
5. Reduced requirement for growth factors
6. Invasion and metastasis
7. Reduced cellular adhesion
8. Cell migration
9. Angiogenesis

**6. What are the control points present in the cell cycle?**

1. DNA Replication check point

2. DNA Damage check point'
3. Spindle check point
4. Restriction point

**7. What is benign tumor?**

The cell becomes abnormal and grows, it produces a mass of reproducing cells characteristic of a tumor. Some times, all the cells of this growing mass stay together, never moving elsewhere in the body. Such a tumor is called benign.

**8. Why is cancer predominantly a disease of the elderly?**

Cancer seems to result from as accumulation of mutations that alter a particular cell in specific ways, providing all the characteristics necessary to begin tumor formation. A cell accumulates mutations over the course of its lifetime and inches closer to becoming tumorous. Clearly, the longer a human lives and the greater the incidence of mutation, the more likely tumor formation occurs.

**9. What is Growth factor? And its role in cell cycle.**

The cells of multi-cellular animals do not normally divide unless they are stimulated to do so by an appropriate signaling protein is known as a Growth Factor.

**10. Explain Platelet – derived growth factor (PDGF).**

A protein produced by blood platelets that stimulates the proliferation of connective tissue cells and smooth muscle cells. Another growth factor in blood serum called Epidermal Growth Factor (EGF), is also widely distributed in tissues.

**11. Write the important steps involved in Apoptosis.**

As a cell begins to undergo apoptosis, its cytoplasm shrinks and bubble – like protrusions of cytoplasm forms at the cell surface. The Nucleus and other cellular structures then disintegrate and the entire cell breaks apart, forming small apoptic bodies that are engulfed by neighboring phagocytic cells. The two main routes for triggering apoptosis are the external pathway, external molecules binds to death receptors on the outer surface of the targeted cell. The activated death receptors then trigger the caspase cascade. In the internal pathway, damaged DNA triggers accumulation of the P53 protein, which stimulates the production of death – promoting proteins that alter the permeability of mitochondrial membranes. This event leads to the release of group of mitochondrial proteins, including cytochromes, that activates the caspase cascade.

**12. Give example of Tumor suppressor Gene and the Pathway which affected.**

| **Gate Keeper Genes** | **Pathway Affected** |
|---|---|
| APC | Wnt signaling |
| CDKN2A | Rb and P53 signalling |
| PTEN | PI 3K – Akt signaling |
| RB | Restriction point control |
| SMAD4 | TGEB – Smad signaling |
| TGFB receptor | TGFB – Smad signaling |
| P53 | DNA Damage Response |
| **Care taker Genes** | |
| ATM | DNA damage response |
| BRCA1, BRCA2 | Double-strand break repairs |
| MSH2 – MSH6 | |
| $PMS_1$ – $PMS_2$, MLH1 | DNA mismatch repairs |
| XPA, XPB, XPC, XPD | DNA excision repairs |
| XPE, XPF, XPG. XPV | Translation synthesis |

**13. What is the role of Rb protein in cell cycle control?**

In its normal, dephosphorylate state, the Rb protein binds to the $E_2F$ transcription factor. This binding prevent, $E_2F$ from activating the transcription of genes coding for proteins required for DNA replication, which are needed before the cell can pass through the restriction point and into 'S' phase. In cells that have been stimulated by growth factors, signaling pathways such as the Ras-MAPK pathway trigger the production of Cdk-cyclin complexes that catalyze Rb phosphorylation. The phosphorylated Rb can no longer bind to $E_2F$, which allows $E_2F$ to activate gene transcription and trigger the onset of S phase. At the time of subsequent mitosis, the phosphate groups are removed from Rb so that it can once again inhibit $E_2F$.

**14. What is the role of ubiquitin in Targetting proteins for Degradation?**

A common mechanism for targeting proteins for destruction involves tagging them with a small protein called ubiquitin ligases, each of which attaches ubiquitin molecules to a specific set of target proteins. MdM2 is one example of such a ubiquitin ligase. Proteins that have been linked to ubiquitin are degraded by proteosomes, the cell's main protein – degrading apparatus. ATP provides energy required by this pathway at two different points.

**15. What is the role of P53 protein in DNA Damage?**

Damaged DNA activates the ATM protein kinase, leading of phosphorylation of the P53 protein. Phosphorylation stabilizer P53 by blocking its interaction with MdM2, a protein that would otherwise mark P53 for degradation by attaching it to ubiquitin. When the interaction between P53 and MDM2 is blocked by P53 for phosphorylation, the phosphorylated P53 protein accumulates and triggers two events. The P53 protein binds to DNA and activates transcription of the gene coding for the P21 protein, a cdk inhibitor. The resulting inhibition of cdk-cyclin prevents phosphorylation of the Rb protein, leading to cell cycle arrest at the restriction point. When the DNA damage cannot be repaired, P53 activates genes coding for a group of proteins involved in triggering cell death by apoptosis. A key protein is Puma, which promotes apoptosis by binding to, and blocking the action of the apoptosis Inhibitor $Bcl_2$.

**16. Which protein destruct the Rb and P53 protein by virus?**

Mutating the P53 gene is not the only mechanism for distrupting P53 function; the P53 protein can also be targeted directly by certain viruses. For example, Human papilloma virus – whose E7 oncoprotein inactivates the Rb protein – produces another molecule called the E6 oncoprotein, which binds to and targets the P53 protein for destruction. The ability of human papilloma virus to cause cancer is therefore linked to its capacity to block the action of proteins produced by both the RB and P53 Tumor suppressor genes.

**17. How is Wnt pathway produce cancer?**

The Wnt Pathway is turned on by signalling molecules called Wnt proteins, which bind to and activate cell surface Wnt receptors. The activated receptors stimulate a group of proteins that inhibit the axin – APC –GSK3 destruction complex and thereby prevent the degradation of β -catanin. The accumulating β-catanin then enters the nucleus and interacts with transcription factors that activate a variety of genes, including some that stimulate cell proliferation.

**18. Define Carcinomas.**

Carcinomas are tumors made up principally of epithelial cells of ectodermal or endodermal origin. Cancers of the external epithelia (56% of the total) include those of the skin, large intestine, lung, stomach and cervix. These epithelia are in immediate contact with the external environment. Cancers of internal epithelia (36% of the total) which line the various glands of the body, include cancers of the breast, prostate, ovary, thyroid and bladder. The solid tumors in nerve tissue, which is of ectodermal origin, are also examples of carcinoma.

**19. How the Receptors that Activate Tyrosine Kinases?**

A number of receptors do not have intrinsic enzymatic activity but stimulate associated tyrosine kinases. Important examples of this type of receptor include the cytokine and interferon receptors that associate constitutively with members of the Jak family of tyrosine kinases and the multichain immune recognition receptors that activate SFK and Syk family tyrosine kinases. The kinases appear to be inactive in the absence of ligand, but, as happens in receptors with intrinsic tyrosine kinase activity, signaling is initiated by ligand-stimulated heterodimerization and conformational changes of the receptors.

**20. Write the role of Serine-Threonine Kinase Receptors.**

The TGF-β family of receptors are transmembrane proteins with intrinsic serine-threonine kinase activity.[15] TGF-β ligands are dimers that lead to oligomerization of type I and type II receptors. The type I and type II receptors are homologous but distinctly regulated. The type II receptors seem to be constitutively active but do not normally phosphorylate substrates, whereas the type I receptors are normally inactive. On ligand-mediated dimerization of the type I and type II receptors, the active type II receptor phosphorylates the type I receptor and converts it to an active kinase. Subsequent signal propagation is dependent on the kinase activity of the type I receptor and the phosphorylation of downstream substrates.

**21. What is Tumour suppressor gene? Give two examples.**

Tumour suppressor genes or anti oncogenes, as these names indicate, suppress tumours caused by cancer – causing oncogenes. Tumour suppressor gene block tumour development by regulating genes controlling cell growth. The presence of one or more normal copies of TSG is sufficient to prevent the expression of the cancer phenotype.

Examples: RB, P53, WT1, VHL, BRCA 1, BRCA 2.

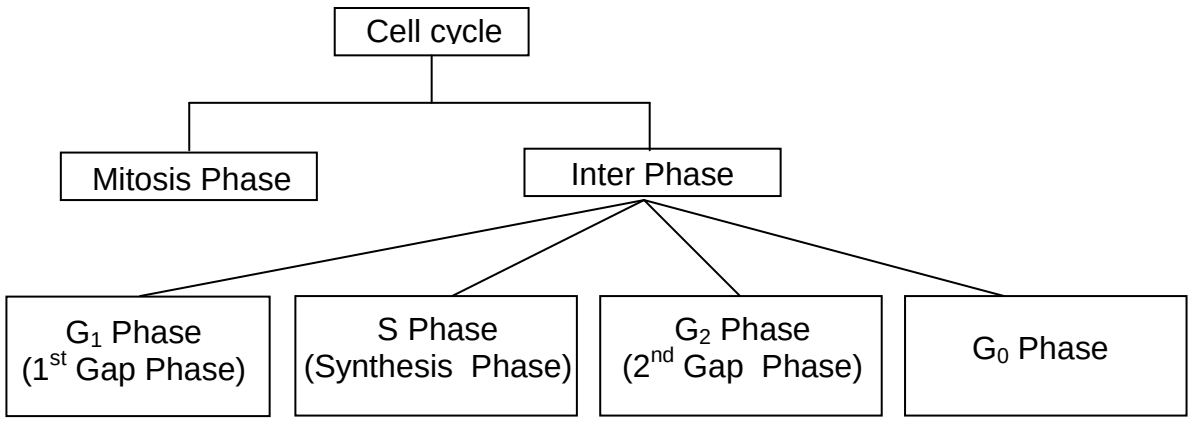
**22. What are the phytochemicals used in the treatment of cancer?**

a) Lycopene - It is a natural red pigment derived mainly from tomatoes and tomato based sause. It is potent antioxidant nd it is responsible for reducing cancer risk.
b) Isothiocyanate: sources-cabbage,cauliflower,broccoli
c) Epigallocatechin gallate: sources: Green Tea
d) Isoflavones: Anti estrogen- Breast cancer Source: Soya bean derived foods
e) Reveretal: Anti oxidant. Source: Red grapes and red wines
f) Sulfides: Source: Garlic-diallyl sulphide

## PART – B

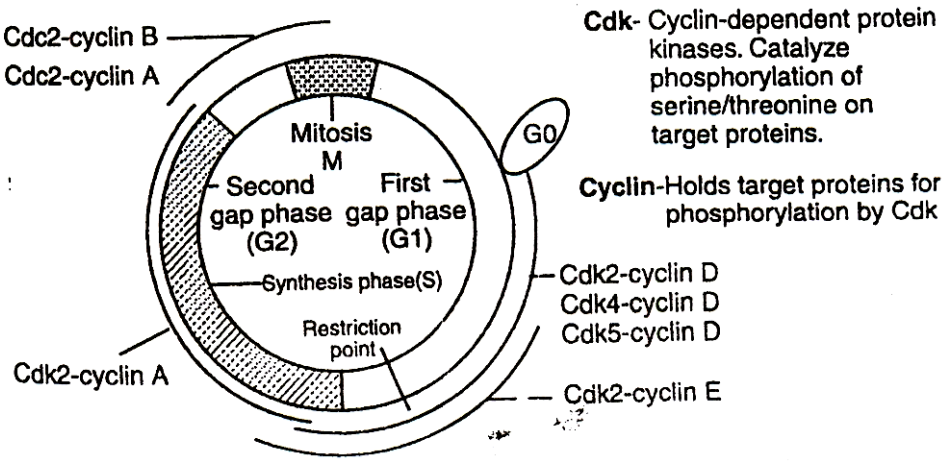
**1. What is cell cycle and its relation with cancer?**

**Modulation of cell cycle:-**




These phases are of relatively fixed duration in cell cycle.

$G_0$ Phase – Shunt of variable duration, determines time variation in the cell cycle.




**Figure**

Mammalian Cell Cycle and activity of Cdk – cyclin during the cycle.

Cdk - Cyclin – dependent protein kinases.
Catalyses phosphorylation of serine / threonine on target proteins.

Cyclin - Holds target proteins for phosphorylation by Cdk.

**Cell Cycle Molecules:**

1. Cdks - Catalyses phosphorylation ofser/thr.
2. Cyclins - Helds target proteins.

Proteins encoded by Tumor suppressor gene that regulate cell cycle.

1. Rb encoded or Rb genes and P53. Rb gene responsible for rare childhood tumour of the eye called Retinoblastoma.
2. P53 – encoded byP53 genes, controls different function

Cell cycle is important in the growth and proliferation of cells.

Rb proteins –

- Regulating the progression of cell cycle from the $G_1$ to S Phase.
- The Rb Protein controls the transcription of genes required for progression of cell cycle from $G_1$ to S.

(a) During $G_1$ ($E_2F$) Transcription factor binds to Rb Proteins.
(b) Activation of Cdk leads to phosphorylation of Rb, Resulting is release of $E_2F$
(c) Active $E_2F$ can now bind to regulatory sequence in DNA.
(d) Transcription factor $E_2F$ activates gene expression leading to production of DNA synthesis enzyme.
(e) This allows $\boxed{G_1} \rightarrow \boxed{S}$ progression of cell cycle from $G_1$ to S Phase.

In all of the tumour suppression genes, the P53 gene has the most important role in human carcinogenesis. It is an Antitumour gene that is associated with 50% of all Human cancers.

- P53 is not required for normal cell division.
- If the DNA is damaged during $G_1$ by mutagens. Eg. X-ray irradiation.
  The level of the P53 protein expressed rises.
  This arrests progression of the cycle through $G_1$ – Apoptosis.
- Inactivation of both alleles of P53 gene results in non production of the P53 protein.
- So either death of the cell due to mitotic failure or continuous proliferation with damaged chromosomes.
- Latter cells become genetically unstable and go on accumulating mutations.
- Leads to formation of malignant growth and tumours.

**2. How the Mutation those cause changes in signal molecules?**

**Polypeptide growth factors:**

Variety of cell signals molecules controls the cell growth with differentiation
Rita Levi Montaleini discovered 1st Growth Factor in 1950.

Nerve Growth Factor (NGF) – regulates the development and survival of Neurons.

**Platelet derived Growth Factors (PDGF)**

- It affects only one cell type, Fibroblast and causes them to grow and divide.
- PDGF is synthesized in megakaryolytes. It is then packaged into platelet alpha granules and released from platelets activated by thrombin, at sites of injury of blood vessels.
- PDGF is believed to diffuse to the luminal surface of the wall of the blood vessel and initially biological functions on cellular substrates of the wall.
- PDGF is purified as two proteins 1. PDGF I 2. PDGF II
- They have the same immunological reactivity and equal mitogenic activity and affinity for cell surface receptors.
- The SIS gene codes for a polypeptide chain of platelet – derived growth factor.
- PDGF-I is about 7% carbohydrates.
  It is very basic protein has about 16 cysteine half residues which are linked by disulphide bonds.
  It is hetro dimers.
- PDGF – II is about 4% carbohydrate.
- PDGF – II is homo dimer.

**<u>Activities induced by PDGF:</u>**

- When PDGF binds to its cell surface receptor, activation of tyrosine kinases is induced.
- Serine / threonine kinase activities are also stimulated in cells activated by PDGF.
- A series of highly specific but diverse events are initiated in target cells when PDGF bind to its receptor.
  (i) Ion fluxes are influenced, stimulating influx of $Na^+$ and efflux of $H^+$. There is a transient decrease in intra cellular pH followed by rise. The $Na^+/K^+$ activity also increase raising intracellular $K^+$.
  (ii) $Ca^+$ is released from intracellular stores within 15 seconds of stimulation.
  (iii) PDGF stimulates amino Acid transport and protein synthesis.
  (iv) PDGF also modulates expression of receptors for other ligands.
  (v) PDGF induces competence for cell division.

- Several genes are activated shortly after stimulation of cells by PDGF. These include KC, JE, C – CYC and C –FOS.
- The int-2 onco gene codes for a protein related to a fibroblast growth factor (FGF).

**EPIDERMAL GROWTH FACTOR:**

- The erb B gene codes for transmembrane receptor with tyrosine protein kinase activity.
- It is derived from the gene for the EGF receptors.
- The EGF is a small mitogenic preotein that stimulates the multiplication of cells carrying specific membrane – associated EGF receptors.

**3. Tumour Suppressor Gene:**

| S.No. | Gene | Protein | Function / Features of protein |
|---|---|---|---|
| 1. | RB | PRB | Transcriptional regulation |
| 2. | P53 | P53 | Transcription factor |
| 3. | WT1 | WT1 | Transcriptional regulation |
| 4. | VHL | VHL | Interacts with splicing factors. |
| 5. | INK 4A/MTS1 | INK4A P16 | Interacts with elongation factors involved in cell cycle regulation as inhibitor of cyclin D: CDK complex. |
| 6. | BRCA 1 | | Zinc finger protein. |
| 7. | BRCA 2 | | Function not known |
| 8. | DPC 4 | | Transcriptional response to TGEB stimulation |
| 9. | NF1 | | GTPase-activating protein that acts on RAS protein. |
| 10. | NF2 | | |
| 11. | TSC2 | | |
| 12. | APC | | |
| 13. | PTC | | |
| 14. | DCC | | |
| 15. | FHIT | | |

**The P53 Gene:-**

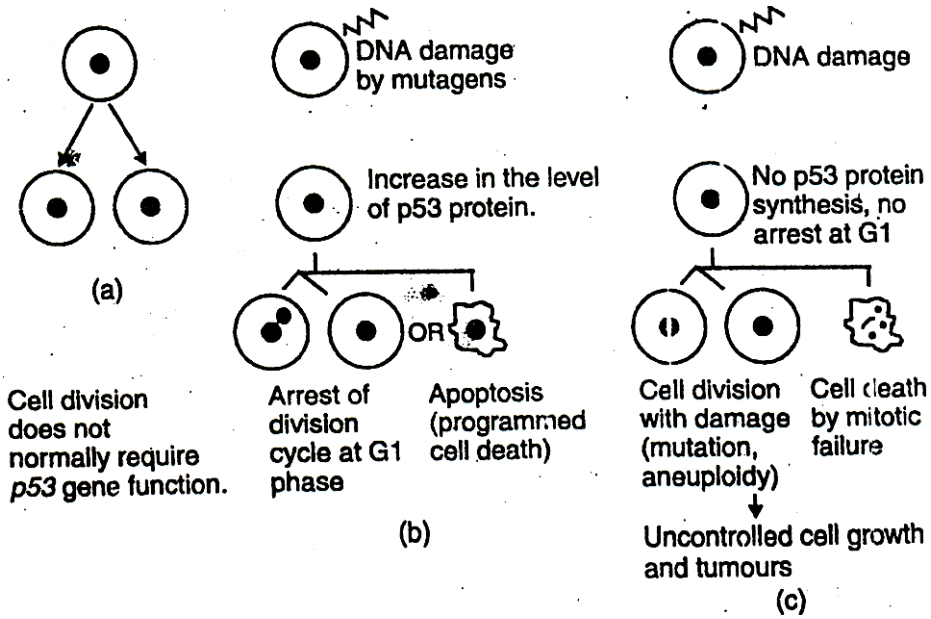
- The P53 gene is located at chromosome band 17P 13-1 in the human genome.
- The gene is about 10 kb long.
- It consists of 11 exons of which the first is non coding and located 6 – 10 kb away from the other 10 exons.
- Gene in the normal condition keeps the cell normal.

- If the Gene is absent or mutated or complexed with other molecules, cell becomes cancer.
- Scientist Vogelstein identified that P53 gene is involved in 80% colorectal cancers.
- Subsequently involved in more than 60% of human cancers.
- The Active (wild type) P53 protein is a transcriptional regulator that is activated in response to DNA Damage.
- It positively regulates the transcription of the gene.
- The P53 protein normally responds in several ways to DNA damage in the cell.

(i) It halts the cell cycle until the DNA damage is repaired. This it does by acting as a transcription factor and stimulating the synthesis of a 21 KDa protein that blocks cyclin – dependent protein kinases (Cdks) from interacting with cyclin proteins. This block functions in preventing the progression of the cell cycle, giving the cell time to repair DNA damage. Therefore the errors are not passed on to the daughter cells.

(ii) The P53 protein can also trigger damaged cells to undergo apoptosis if the repair process fails. This is further step to halt inheritance of genetic abnormalities.
The P53 genes thus act as a link between the cell cycle and apoptosis.

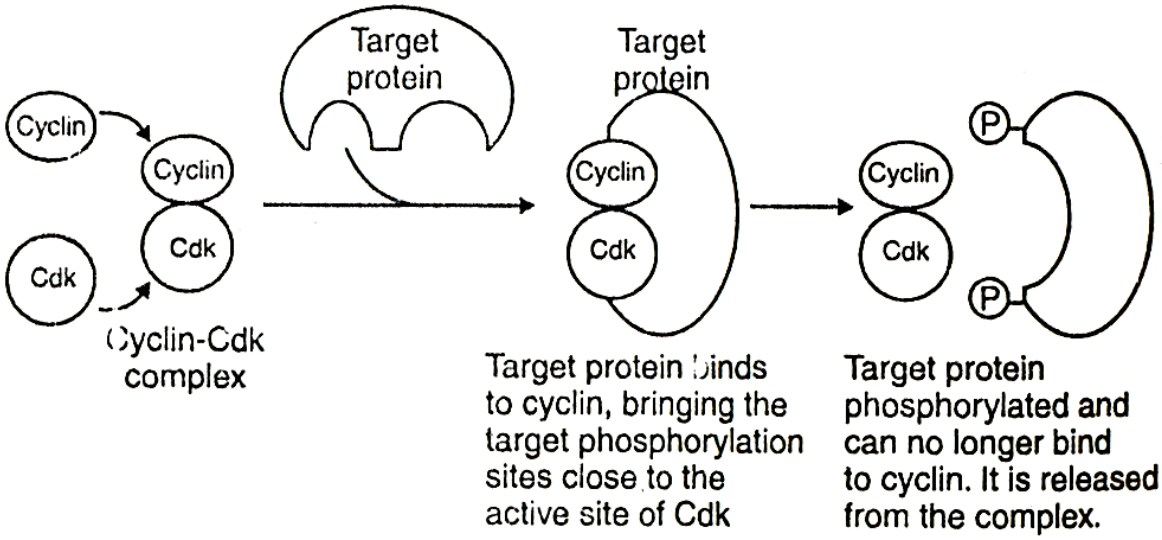
(iii) The P53 protein also functions in stimulating the DNA repair mechanism. Thus the P53 protein can suppress the formation of cancer cells by these 3 mechanisms.



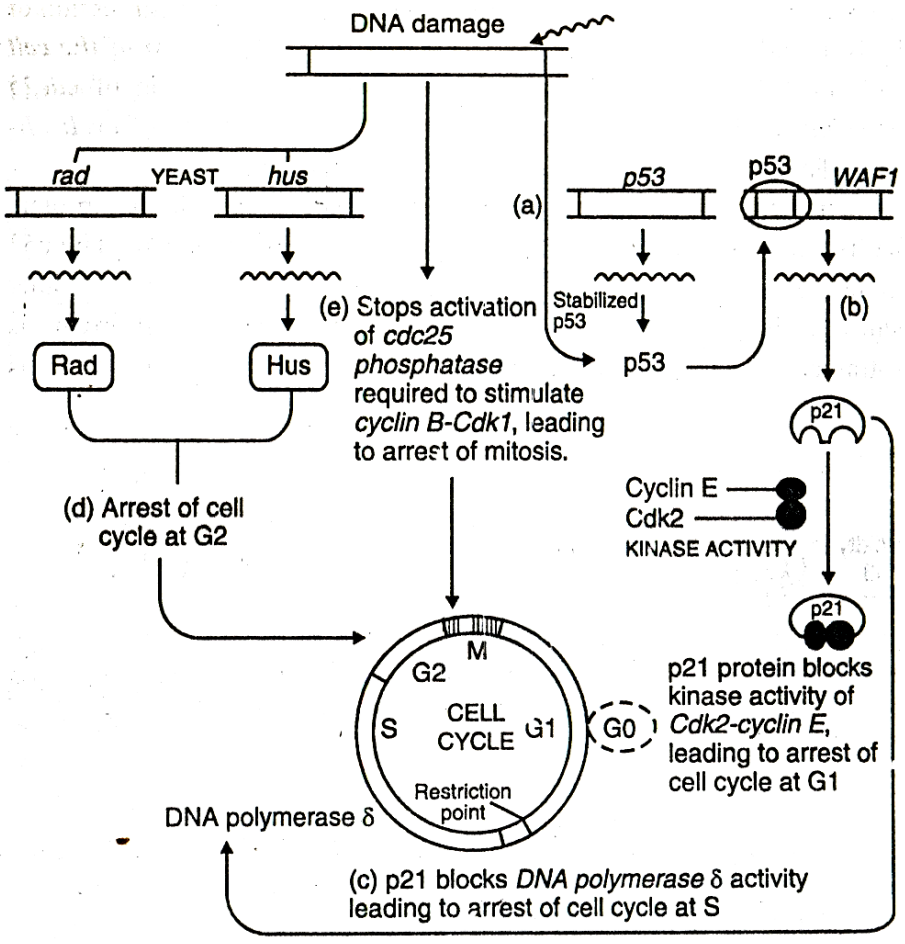

**Figure**

**<u>Function of P53 protein and cell cycle:</u>**

1. $G_0$, $G_1$, S, $G_2$, M. Phases
2. Cyclin – A, B, C, D, E
3. Cyclin dependent protein kinases.




**DNA Damage and Inhibitory control of cell cycle progression:**




**P53 Protein:**

- Human P53 protein is a phospho protein of 393 Amino Acids.
- It probably exists as a tetramer of four subunits.
- The protein appears to give 3 structural domains.

(i) An N-terminal domain of the First 75 – 80 domains of amino acids which are very acidic.
(ii) Amino Acids 75 – 150 domain of prolin rich and Hydrophobic Amino Acids.
(iii) C-terminal domain (amino acid 319 – 393) which contains many basic amino acids.
(iv) The P53 protein has been conserved during evolution.
(v) P53 is considered to be the guardian of the genome.

**P53 gene and cancer therapy**

(i) Radiation is commonly used to treat cancer. The rays do not damage the cell enough to kill it, but activate theP53 gene if present in the cell.
(ii) Chemotherapy:
Some, chemotherapeutic agents also work by activating the P53 gene.
(iii) Tamoxifen, which is used in the chemotherapy of breast cancer, activates the P53 gene, which in turn activates the cell's suicide mechanism.
(iv) Gene therapy:
The P53 gene has been used as a target for gene therapy.

**Characteristic of Tumour Suppressor Gene or Anti Oncogene:-**

- Tumour Suppressor Gene (TSG) or anti oncogene are genes that can suppress or inhibit the proliferation of cells in tumour.

**I. Function of the mutant allele**

- In TSG, the mutations results in loss of function, and thus act in a recessive manner to a wild type.
- Tumour suppressor genes usually impose some constraint on the cell cycle or cell growth, thereby ensuring Normal division. Loss of function of the genes removes this constraint, resulting in unregulated growth or cancer.
- Tumour suppressor genes give rise to tumours by loss of function; they are also known as recessive oncogenes.

**II. Number of Mutation required to produce cancer**

TSG require 2 independent mutational events to cause abnormal growth regulation. Mutation takes place in one allele, making the locus heterozygous. This is followed by mutation is $2^{nd}$ allele, resulting in loss of heterozygosity or reduction to homozygosity at that locus.In this process both wild type alleles are eliminated in the locus.

**III. Inheritance of mutant allele through the germ line**

TSG frequently have an inherited or familial form. For most of TSGs there is an associated familial cancer syndrome caused by the inheritance of the mutant allele of that gene.

**IV. Somatic mutation**

Somatic mutations result in the sporadic form of the disease which is not transmitted to the next generation.

**V. Tissue Specificity**

TSG show much more tissue specificity in causing tumours, especially in the inherited forms of the disease.

**VI. Tumours Suppressor genes**

**<u>The retinoblastoma gene (RB)</u>**

- The human RB gene is some 200 Kb in length and located at 13 q 14.
- It consists of 27 exons.
- A 70 bp promoter region is located 70 bp upstream of first exon. The promoter is rich in G + C bases and contains consensus sequences for transcription factors E2F-1, ATF and SP-1. These sequences are of functional importance.
- Tumours contain inactive RB with deletions or point mutations in the promoter.
- The RB gene produces an RNA transcript in all cells and tissues of the body.
- RB gene was the first tumour suppressor gene.
- Inactivation of RB gene leads to

    1. retinoblastoma
    2. small cell lung carcinoma
    3. breast and bladder carcinoma
    4. In Mice – pituitary endocrine tumours.

**The RB protein and cell cycle regulation**

1. Phosphorylation of RB
2. Binding of unphosphorylated RB to proteins during $G_1$.
3. Phosphorylation of RB and Release of E2F.
4. Further plhosphorylation of RB in S and $G_2$ phase.
5. Association of RB with proteins inthe M phase.

**4. What are the different forms of cancer?**

**1. Benign and malignant tumours**

- Tumours are commonly classified as benign and malignant.
- Abnormal and persistant cell division that remains localized at the spot of origin results so called benign tumours.
- The cells of benign tumours closely resemble normal cells, and may function as Normal Cells.
- Benign tumours are confined to the spot of origin.
- Thus benign liver tumours remain in the liver and benign intestinal tumours in the intestine.
- Benign tumours are usually enclosed in fibrous capsule.
- Some times cause medical complications.
  Eg. Brain tumour that cause pressure on a vital centre (or)
  Glandular tumour that result in excessive secretion of biologically active substance as hormones.

**Malignant tumours** are characterized by their properties of invasiveness and spread. Tumour cells may be carried by the blood stream or the lymphatic stream or by direct penetration, to other parts of the body where they induce secondary (metastatic) tumours. Such invasive cancers ultimately result in the death of the organism, and are therefore said to be malignant. Malignant tumours usually contain less differentiated cells than benign tumour cells.

Malignant tumours usually contain less differentiated.

- The properties of the cells often change over time. Thus liver cells may lack certain characteristic enzymes, and may ultimately reach a state where they lack most liver functions.
- Cancer cells show several chromosomal structural abnormalities, as well as abnormal and unstable numbers of chromosomes.
- It is possible for benign tumours to become malignant.

| Tissue of Origin | Name of Tumour |
|---|---|
| Skin | Squamous cell carcinoma |
| Lung | Pulmonary adeno carcinoma |
| Breast | Mammary adeno carcinoma |
| Stomach | Gastric adeno carcinoma |
| Colon | Colon adeno carcinoma |
| Uterus | Uterine endometrial carcinoma |

| | |
|---|---|
| Prostate | Prostatic adeno carcinoma |
| Ovary | Ovarian adeno carcinoma |
| Pancreas | Pancreatic adeno carcinoma |
| Urinary Bladder | Urinary bladder adeno carcinoma |
| Liver | Hepato carcinoma |
| | |
| **Sarcomas** | |
| | |
| Bone | Osteo sarcoma |
| Cartilage | Chondra sarcoma |
| Fat | Lipo sarcoma |
| Smooth muscle | Leiomyo sarcoma |
| Skeletal muscle | Rhabdomyo sarcoma |
| Connective tissue | Fibro sarcoma |
| | |
| **Leukemias** | |
| | |
| RBC | Erythrocytic leukemia |
| Bone marrow cells | Myeloma or myelocytic leukemia |
| WBC | Lymphoma or lymphocytes leukemia |

**Types of cancer based on site of formation**

- Cancer is not one single disease but a complex of many diseases.
- About 200 distinct types of cancer have been recognized.
- Cancer can be grouped into four main types based on the site of the cancer.

a. carcinomas
b. sarcomas
c. lymphomas
d. leukemias

A & B are solid tumours
C & D are liquid tumours,

**1. Carcinomas** are tumours made up principally of epithelial cells of ectodermal or endodermal origin. Cancer of the external epithelia (56% of the total) includes those of the skin, large intestine, lung, stomach and cervix. These epithelia are immediate contact with the external environment.

- Cancers of internal epithelia (36% of total) which line the various glands of the body, include cancers of the breast, prostate, ovary, thyroid and bladder.

- The said tumours in nerve tissue, which is of ectodermal origin, are also example of carcinoma.

**2. Sarcomas** are made up principally of connective tissue cells, which are of mesodermal origin. They develop in various supporting tissues of the body and are solid tumours growing from connective tissue, cartilage, bone and muscle. Although they accound for most of the cancer studied in laboratory animals, they constitute only about 2% of human cancers.

**3. Lymphomas** are cancers in which there is excessive production of Lymphocytes by the lymph nodes and the spleen. They include conditions such as lympho sarcoma and Hodgkin's disease. Lymphoma constitutes 5% of human cancers.

**4. Leukemias** are Neoplastic growth of leukocyte. They are characterized by excessive production of leukocytes in the blood. They constitute about 4% of human cancers.

**5. What is Receptors?**

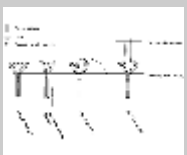
The plasma membrane of eukaryotic cells serves to insulate the cell from the outside environment, but this barrier must be breached to signal to the cell. Signals transverse the plasma membrane either by activating by transmembrane receptors or by using ligands that are membrane permeable. Cells are exquisitely sensitive to most ligands. The affinity of receptors for ligands generally is in the picomolar to nanomolar range, and very few receptors have to be occupied to transmit a signal. It has been estimated that activation of ten T-cell receptors is sufficient to send a maximal signal. Cytokine-responsive cells may express only a few hundred receptors on the cell surface. Given the small number of receptors that are activated, amplification of most signals is necessary for the cell to respond. A requirement for signal amplification also allows opposing signals to affect signal strength more efficiently. As a result of ligand binding, receptors undergo conformational changes or oligomerization, or both, and the intrinsic activity of the receptor or of associated proteins is stimulated. Receptors may bind and respond to more than one ligand [e.g., the epidermal growth factor (EGF) receptor binds to transforming growth factor (TGF)-α, EGF, heparin-binding EGF (HB-EGF), betacellulin, epiregulin, epigen, and amphiregulin]. The stimulation of most receptors leads to the activation of several downstream pathways that either function cooperatively to activate a common target or stimulate distinct targets. Generally, some of the pathways activated are counter regulatory and serve to attenuate the signal. Receptors may also activate other receptors. A well-studied example is the activation of the EGF receptor by G protein–coupled receptors (GPCR), which occurs as a result of protease cleavage and activation of HB-EGF.

**6. Write briefly about the Receptor Tyrosine Kinases.**

Receptor tyrosine kinases are transmembrane proteins that have an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. The ligands for these receptors are proteins or peptides. Most receptor tyrosine kinases are monomeric, but the insulin receptor families are heterotetramers in which the subunits are linked by disulfide bonds. Receptor tyrosine kinases have been divided into six classes, primarily on the basis of the sequence of extracytoplasmic domain. Examples of tyrosine kinase receptors include the insulin receptor, the platelet-derived growth factor (PDGF) receptor, the EGF receptor family, and the fibroblast growth factor (FGF) receptor family.

Activation of receptor tyrosine kinases generally requires tyrosine phosphorylation of the receptor. In the case of the insulin receptor, an insulin-stimulated conformational change activates the kinase. Most other tyrosine kinases are activated by oligomerization, which brings the kinase domains into close proximity so that they cross-phosphorylate. Auto- or transphosphorylation of a tyrosine in the activation loop of the kinase domain locks the kinase into a high-activity conformation, stimulating phosphorylation of other sites on the receptor, as well as other substrates.

Ligands stimulate receptor oligomerization in a variety of ways. Some ligands, such as PDGF, are dimeric, so that the ligand is able to bind two receptors simultaneously. Other ligands, such as growth hormone, are monomeric but have two receptor-binding sites that allow them to induce receptor dimerization. FGFs are also monomeric but have only a single receptor-binding site. FGF molecules bind to heparin sulfate proteoglycans, which concentrates FGF and facilitates dimerization of the FGF receptor. EGF is also monomeric, but binding of EGF to the receptor changes the receptor conformation and promotes interaction with a second ligand/receptor dimer leading to activation. Ligands not only stimulate receptor-dependent signaling, but also some ligand-receptor interactions result in signaling by the ligand. Ephrins are ligands for the protein tyrosine kinase EPH receptors. Ephrins are expressed on the surface of adjacent cells, and interaction of EPH receptors and ephrins activates the tyrosine kinase activity of the receptor and leads to stimulation of signaling by the ligand in the adjacent cell.



View Figure

Figure 3.1-1
Dimerization of tyrosine kinase receptors. Most tyrosine kinase receptors are activated by ligand-induced dimerization. Some ligands, such as platelet-derived growth factor (PDGF), are dimeric and induce dimerization using the two receptor-binding domains. Other ligands, such as growth hormone, contain two receptor-binding domains in the same molecule. The fibroblast growth factors (FGFs) rely on proteoglycans to aid the formation of ligand dimers. Some ligands, such as the ephrins (EPHs), are present on nearby cells and, when the cells come into contact, bind to the receptors and promote clustering.

Studies of the EGF receptor family illustrate some important concepts in signal transduction. The EGF-signaling pathways involve four known receptors (EGF receptor, erbB2, erbB3, and erbB4) and many ligands. EGF stimulates homodimerization of the EGF receptor, but, under certain conditions, heterodimerization with other family members also occurs. The same ligand activates different signaling pathways, depending on the subgroups of EGF receptor family members expressed in a cell. For example, HB-EGF–like growth factor stimulates mitogenesis but not chemotaxis when it activates the EGF receptor but is a mitogen and chemotactic factor when it activates Erb4. One study also suggests that different ligands binding to the same receptors activate distinct downstream-signaling pathways. These findings suggest that different ligands may cause distinct conformational changes that lead to the phosphorylation of different sets of tyrosine residues on the receptor and could lead also to phosphorylation of distinct sets of substrates.

**7. Write the role of Serine-Threonine Kinase Receptors.**

The TGF-β family of receptors are transmembrane proteins with intrinsic serine-threonine kinase activity.[15] TGF-β ligands are dimers that lead to oligomerization of type I and type II receptors. The type I and type II receptors are homologous but distinctly regulated. The type II receptors seem to be constitutively active but do not normally phosphorylate substrates, whereas the type I receptors are normally inactive. On ligand-mediated dimerization of the type I and type II receptors, the active type II receptor phosphorylates the type I receptor and converts it to an active kinase. Subsequent signal propagation is dependent on the kinase activity of the type I receptor and the phosphorylation of downstream substrates.

**8. Write the Role of Receptor Phosphotyrosine Phosphatases.**

Receptor protein tyrosine phosphatases (RPTPs) have an extracellular domain, a single transmembrane-spanning domain, and cytoplasmic catalytic domains. The extracellular domains of some receptor tyrosine phosphatases contain fibronectin and immunoglobulin repeats, suggesting that some of these receptors may recognize adhesion molecules as ligands. Several RPTPs are capable of homotypic interaction, but no true ligands are yet known for RPTPs. Most receptor tyrosine phosphatases have two catalytic domains, and both are active in at least some receptors. Functional and structural evidence suggests that the phosphatase activity of some of these receptors is inhibited by dimerization. These receptors may be constitutively active as tyrosine phosphatases but lose that activity after ligand binding. Inhibition of tyrosine phosphatase activity would enhance signals emanating from tyrosine kinases. RPTPs do not always function in strict opposition to tyrosine kinases, however. For example, CD45 is necessary for signaling by the B-cell receptor, which also requires tyrosine kinase activity.[17]

**9. Write role of G Protein–Coupled Receptors.**

GPCRs are by far the most numerous receptors. Almost 700 GPCRs are present in the human genome. The number of GPCRs is so high because they encode the light, smell, and taste receptors, all of which require great diversity. These receptors have seven membrane-spanning domains: The N-terminus and three of the loops are extracellular, whereas the other three loops and the C-terminus are cytoplasmic. A wide variety of ligands bind GPCRs, including proteins and peptides, lipids, amino acids, and nucleotides. No common binding domain exists for all ligands, and interactions of ligands with GPCRs are fairly distinct.[20] In the case of the thrombin receptor, thrombin cleaves the N-terminus of the receptor, freeing a new N-terminus that self-associates with the ligand pocket, leading to activation. Amines and eicosanoids bind to the transmembrane domains of their GPCRs, whereas peptide ligands bind to the transmembrane domains and the extracellular loops of their GPCRs. Neurotransmitters and some peptide hormones require the N-terminus for binding and activation.

GPCRs basally are kept in an inactive conformation by intramolecular bonds involving residues in the transmembrane or juxtamembrane regions. In the inactive state, the receptor is bound to a heterotrimeric G protein, which is also inactive. Agonist binding results in a conformational change that stimulates the guanine nucleotide exchange activity of the receptor. Exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the αsubunit of the heterotrimeric G proteins initiates signaling. Ultimately, GPCRs stimulate the same downstream pathways as other receptor types, including protein tyrosine and serine kinases, phospholipases (PLs A, C, and D), and ion channels. Certain GPCRs also activate receptor tyrosine kinases. As mentioned earlier in the section Signals, GPCR-dependent cleavage of HB-EGF stimulates the EGF receptor, which is necessary for the GPCR to activate the mitogen-activated kinase (MAP kinase) pathway.

**10. Short notes on Notch Family of Receptors.**

The Notch receptor has a large extracellular domain, a single transmembrane domain, and a cytoplasmic domain. Ligands for the Notch receptor are proteins expressed on the surface of adjacent cells, and activation results in two separate proteolytic cleavages of Notch. Initial cleavage by ADAM family proteases removes the extracellular domain and causes endocytosis. Subsequent proteolysis by the preselinin protease family releases the cytoplasmic region of Notch as a soluble signal. This fragment moves to the nucleus, where it complexes with the transcriptional repressor CBF1, relieving its inhibitory effects and stimulating transcription.

**11. What is the role of Guanylate Cyclases?**

Cyclic nucleotides are important second messengers and allosteric regulators of enzyme activities. The synthesis of cyclic adenosine monophosphate (cAMP) by adenylate cyclase is regulated principally by heterotrimeric G proteins, but the synthesis of cyclic guanosine

monophosphate is regulated directly by ligands. Plasma membrane guanylate cyclases are receptors for atrial natriuretic hormone. Nitrous oxide binds to soluble guanylate cyclases. Both stimuli increase cyclic guanosine monophosphate levels.

**12. Write about Tumor Necrosis Factor Receptor Family.**

The tumor necrosis factor family of receptors has a conserved cysteine-rich region in the extracellular domain, a transmembrane domain, and a domain called the *death domain* in the cytoplasmic tail. The receptors undergo oligomerization after ligand binding, which is necessary for signaling. These receptors are distinct in several respects. Stimulation of the receptor leads to recruitment of cytoplasmic proteins that bind to each other and the receptor through death domains, activating a protease, caspase 8, that initiates apoptosis. Under some conditions, however, tumor necrosis factor receptors (TNFRs) stimulate antiapoptotic signals. This family of receptors also includes "decoys" or receptors that are missing all or part of the cytoplasmic tail and thus cannot transmit a signal. This feature provides a unique mechanism for inhibiting and further regulating signaling. A second class of TNFRs lack death domains but bind to TNFR-associated factors.

**13. What is Nuclear Receptors?**

Some ligands diffuse into the cell and bind to receptors either in the cytoplasm or the nucleus. These ligands include steroids, eicosanoids, retinoids, and thyroid hormone. The receptors for these ligands are transcription factors that have DNA- and ligand-binding domains. The unliganded receptor is bound to heat-shock proteins, which are released after ligand binding. Release from the chaperone complex and ligand binding allow the DNA-binding domain to contact DNA and the receptors to regulate transcription directly.

**14. What is Adhesion Receptors?**

Cell adherence either to the extracellular matrix or to other cells is mediated by receptors that function mechanically and stimulate intracellular signaling pathways, primarily through tyrosine kinases.[25] Integrins, which mediate adherence to extracellular matrix, are composed of heterodimers of αand βsubunits and bind to an arginine, glycine, aspartate, or leucine aspartate valine motif found in matrix molecules. Activation of integrin signaling involves binding to ligand and clustering of integrins. Structural studies have shown that inactive integrins adopt a conformation that inhibits ligand binding, and the intracellular regions are also hindered from binding to effector molecules.[26] Binding of ligand opens the intracellular regions so that they bind to the molecules required to transmit integrin-dependent signals. Similarly, modification of the intracellular region, such as phosphorylation, affects the conformation of the extracellular region to favor ligand binding. Integrin signaling is necessary for cell movement, but, in contrast to other

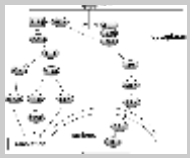
pathways, adherence in nonmotile cells provides a continuous signal. This signal appears to be necessary for survival of most cells. The ability to circumvent the requirement for adherence-dependent survival plays a major role in the development of human cancers by allowing tumor survival in inappropriate locations

**15. Describe the Regulation of the Cell Cycle.**

To preserve organismic function and integrity, the cell cycle must be regulated at a number of levels. These include entry into and exit from proliferation mode, coordination of cell-cycle events, and specialized responses that increase the probability of surviving a variety of environmental and internally generated insults.

**Quiescence and Differentiation**

The most fundamental aspect of cell-cycle control is the regulation of entry and exit. For mammalian cells, the decision to enter or exit the proliferative mode is based on environmental signals such as mitogens, growth factors, hormones, and cell-cell contact, as well as on internal differentiation programs. If the state of cell-cycle exit is reversible, it is referred to as *quiescence*. If it is in the context of terminal differentiation, cell-cycle exit may merely be one component of a differentiation program. Although cells entering quiescence and postmitotic differentiation differ from each other in many respects, from the perspective of cell-cycle control, they have much in common. First, cell-cycle exit is usually associated, at least initially, with an accumulation of $G_1$/S cdk inhibitors. Members of the INK4 family, targeting cdk4 and cdk6 and members of the Cip/Kip family, as well as the Rb-related protein p130, all targeting cdk2, are up-regulated. This causes accumulation of cells in $G_1$, from where cell-cycle exit can occur. Next, or simultaneously, the positive cell-cycle machinery is dismantled by down-regulation of cdks and cyclins, primarily at the transcriptional level. In the case of quiescence, cell-cycle exit is paralleled by a reduced rate of protein synthesis, indicative that cells have entered the resting state. Entry into and exit from quiescence are mediated largely by growth factors and mitogens that interact with cell surface receptors. These in turn are linked to intracellular signaling cascades that up-regulate the rate of protein synthesis as well as the transcription of genes that promote proliferation, such as cdks and cyclins. The two best-characterized signaling pathways in this context are the mitogen-activated protein kinase/extracellular signaling–regulated kinase pathway[25] and the phosphoinositide 3 (PI3) kinase/AKT pathway,[26] shown in **Figure 3.2-4**. Whereas the mitogen-activated protein kinase/extracellular signaling–regulated kinase pathway tends to stimulate expression of genes required for proliferation, the PI3-kinase/AKT pathway primarily stimulates protein synthesis and growth but also affects key cell-cycle regulatory proteins. Just as the presence of growth factors and mitogens stimulates these pathways, promoting cell-cycle entry, their removal shuts down these pathways, promoting quiescence. This is the basis for the reversibility of the quiescent state.



View Figure

Figure 3.2-4
Growth factor (GF)/mitogen stimulation via Ras. Occupancy of many GF receptors (GFR) by ligand depends on the small guanosine triphosphatase transducer Ras. Receptor activation leads to phosphorylation of the receptor cytoplasmic domain. The phosphorylated receptor assembles a complex that includes Ras and its activated nucleotide exchange factor, son of sevenless (SOS), leading to activation of Ras. Activated Ras can then stimulate two important signal transduction pathways: the extracellular signaling–regulated kinase (ERK) pathway and the phosphoinositide 3 kinase (PI3K) pathway. Activated Ras stimulates the protein kinase activity of Raf, activating a protein kinase cascade consisting of Raf, MEK, and ERK. Activated ERK then translocates into the nucleus, where it phosphorylates and activates transcription factors, notably Elk-1. Genes important for growth and division are then transcribed. Activated Ras also stimulates PI3K activity, leading to the accumulation of phosphatidylinositol 3,4,5-triphosphate. This in turn stimulates the protein kinase activity of phosphoinositide-dependent kinase 1 (PDK1), activating a protein kinase cascade consisting of PDK1, AKT, and mTOR. Activation of this signal transduction pathway has the effect of stimulating translation and growth. AKT phosphorylates and inhibits the protein kinase glycogen synthase kinase-3β (GSK3β), thereby activating EIF2B required for translational initiation. mTOR phosphorylates and inhibits the protein phosphatase PP2A, thereby activating EIF4E, also required for translational initiation. Finally, mTOR phosphorylates and activates pp70S6 kinase, which in turn phosphorylates and activates ribosomal subunit S6.

**16. What are diets recommended for cancer patients?**

**Diet and Cancer Etiology**

Cancer is caused by hereditary, genetic, and environmental factors. Studies of cancer incidence among populations migrating to a country with different lifestyle factors have indicated that cancer etiology likely has a large environmental component. Although the contribution of environmental influences differs by cancer type, the incidence of most cancers changes considerably among migrants over time, approaching that of the host country. The age at migration affects the degree of adaptation among first-generation migrants, which indicates that the susceptibility to environmental carcinogenic influences varies with age. Identifying the environmental and lifestyle factors most important to cancer etiology, however, has proven difficult.

Cancer is characterized by an excess of cells beyond the number necessary for normal organ function. Mutation of DNA induces malignant cell transformation. Cancerous cells have escaped their own self-repair mechanisms, which usually protect them from malfunctioning. Given the large number of cells, such failure of self-repair is rare. DNA can be altered by environmental influences to adapt, to change, and to allow natural selection. This potential for change of the DNA makes it also more susceptible to damage.

Environmental influences such as diet may increase the likelihood of DNA mutation but may also protect DNA, either by aiding DNA repair or by supporting apoptosis (the death of a cell) in cells whose DNA is damaged beyond repair.

**Fruits and Vegetables Diet and Cancer Etiology**

Cancer is caused by hereditary, genetic, and environmental factors. Studies of cancer incidence among populations migrating to a country with different lifestyle factors have indicated that cancer etiology likely has a large environmental component. Although the contribution of environmental influences differs by cancer type, the incidence of most cancers changes considerably among migrants over time, approaching that of the host country. The age at migration affects the degree of adaptation among first-generation migrants, which indicates that the susceptibility to environmental carcinogenic influences varies with age. Identifying the environmental and lifestyle factors most important to cancer etiology, however, has proven difficult.

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Two decades ago, Doll and Peto speculated that 35% (range, 10% to 70%) of all cancer deaths in the United States may be preventable by alterations in diet. Fruits and vegetables may be major dietary contributors to cancer prevention, because they are rich in potential anticarcinogenic substances. Fruits and vegetables contain antioxidants and minerals and are good sources of fiber, potassium, carotenoids, vitamin C, folate, and other vitamins. Although fruits and vegetables supply less than 5% of total energy intake in most countries worldwide on a population basis, the concentration of micronutrients in these foods is greater than in most others.

The comprehensive report of the World Cancer Research Fund and the American Institute for Cancer Research entitled *Food, Nutrition and the Prevention of Cancer: A Global Perspective* reached the consensus based on the available evidence that "there is a strong and consistent pattern showing that diets high in vegetables and fruits decrease the risk of many cancers, and perhaps cancer in general." However, with additional evidence accumulating from prospective cohort studies, which had previously been scarce, doubts have been cast on the protective association between fruit and vegetable consumption and cancer.

The majority of available data on the association between fruit and vegetable intake and cancer risk comes from case-control studies. Case-control studies, in which diet is assessed retrospectively in individuals with and without cancer, are prone to recall bias. Participants with cancer may underreport their consumption of foods that are considered "healthy" and overestimate consumption of foods that are considered "unhealthy," as they try to find the reasons for their malignancy.

**17. What are the Micronutrient Components of Fruits and Vegetables used in Cancer?**

**Folate**

Folate is a micronutrient commonly found in fruits and vegetables, particularly oranges, orange juice, asparagus, beets, and peas. Folate may reduce carcinogenesis through various mechanisms: DNA methylation, DNA synthesis, and DNA repair. In the animal model, folate deficiency enhances intestinal carcinogenesis. Folate deficiency is related to incorporation of uracil into human DNA and to an increased frequency of chromosomal breaks. A number of epidemiologic studies indicate that a diet rich in folate may lower risk of colorectal adenoma and colorectal cancer. Because folate intake from dietary sources is generally relatively low, dietary folate is highly susceptible to oxidative destruction by cooking and food processing, and folate is not well absorbed, folate intake from supplements plays an important role. Although the optimal dose of folate supplementation to minimize colorectal cancer risk has not been established, preliminary evidence based on pooled results from nine prospective studies suggests that intake of 400 to 500 μg/d may be required to minimize risk.

Potential interactions between alcohol consumption, folate intake, and methionine intake have been described. Although alcohol consumption has been fairly consistently related to an increase in colorectal cancer, this elevated risk has not been found among individuals with a high intake of folate and methionine. A similar folate-alcohol interaction has been observed for breast cancer risk. Although the incidence of breast cancer has been positively correlated with alcohol intake, the potential detrimental effect of alcohol seems to be eliminated in women with high folate intake.

Genetic susceptibility may explain this effect modification. A polymorphism of the MTHFR gene (cytosine to thymine transition at position 677) may result in a relative deficiency of methionine. Homozygotes for this allele experience the greatest protection from high folate or methionine intake and low alcohol consumption. The evidence is inconclusive, however, due to the present scarcity of data.

**Carotenoids**

Carotenoids, prevalent in fruits and vegetables, are antioxidants, enhance cell-to-cell communication, promote cell differentiation, and modulate immune response. In 1981, Peto and colleagues speculated that β-carotene may be a major player in cancer prevention and encouraged testing of its anticarcinogenic properties. Indeed, subsequent observational studies supported a reduced cancer risk—in particular, of lung cancer—with high intake of carotenoids. Clinical trials randomizing intake of β-carotene supplements, in contrast, have not revealed evidence of a protective effect of β-carotene. In fact, β-carotene was found to increase the risk of lung cancer and total mortality among smokers in the Finnish Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. These adverse affects disappeared during longer periods of follow-up, however. The discrepancy in evidence between epidemiologic studies and randomized clinical trials has been attributed to recall bias in case-control studies and to residual confounding.

**18. What is Cancer Screening?**

Appropriate cancer screening should lead to early detection of asymptomatic or unrecognized disease by the application of acceptable, inexpensive tests or examinations in a large number of persons.[1] The results of a screening test then should be applied expeditiously to separate apparently well persons who probably have disease from those who probably do not. The main objective of cancer screening is to reduce morbidity and mortality from a particular cancer among persons screened.

Several characteristics make particular cancers suitable for screening. These include substantial morbidity and mortality, high prevalence in a detectable preclinical state, possibility of effective and improved treatment because of early detection, and availability of a good screening test with high sensitivity and specificity, low cost, and little inconvenience and discomfort. Only breast, cervical, and colorectal screening have met the rigorous criteria of the U.S. Preventive Services Task Force and have sufficient high-quality evidence to justify population-based screening.

**19. How is Screening Test Evaluated?**

In evaluating a screening test, it is essential to answer questions concerning its ability to accurately predict the presence or absence of disease. If the test is *abnormal*, what are the chances that disease is present? If the test result is *normal*, what are the chances that disease is absent? The

validity of a screening test is measured by whether it correctly classifies those persons who have disease as test-positive and those without it as test-negative.

*Sensitivity* and *specificity* address the validity of screening tests. Sensitivity is the probability of testing positive if the disease is truly present. As sensitivity increases, the number of persons with the disease who are classified as test-negative (false-negative) decreases. Specificity is the probability of screening negative if the disease is truly absent. A highly specific test rarely is positive in the absence of disease and therefore results in a lower proportion of persons without disease who are incorrectly classified as test-positive (false-positive). PV+ is an estimate of test accuracy in predicting presence of disease; PV– is an estimate of the accuracy of the test in predicting absence of disease. Predictive value is a function of sensitivity, specificity, and prevalence of disease. *Accuracy* is a measure of the percent of all results that are true results, whether positive or negative, that is, total correct test results. A screening test that results in many false-positive findings is inefficient and potentially dangerous, because it subjects people to follow-up procedures that are often costly, with a range of risks. Similarly, false-negative results can be life threatening, because they miss cancers that could possibly be identified and treated. Every organized screening program must balance the potential for false-positives and false-negatives when establishing criteria for follow-up.

Sources of bias are of particular importance in evaluating screening programs. People who choose to participate in screening programs (volunteers) are likely to be different from the general population in ways that affect survival; thus, *volunteer bias* is a concern.[1] *Lead-time bias* is defined as the interval between diagnosis of disease at screening and when it would have been detected due to the development of symptoms.[1] If lead-time bias is not taken into account, survival erroneously may appear to be increased among screen-detected cases as compared to unscreened cases. Finally, *length bias* is the overrepresentation among screen-detected cases of those with a long preclinical period (thus, less rapidly fatal), leading to the incorrect conclusion that screening was beneficial.[1]

Another potential outcome is improved quality of life.[5] Unfortunately, few trials have collected such data. These data are being collected as part of the NCI's Prostate, Lung, Colorectal, Ovarian (PLCO) cancer screening trial and the newer NCI-funded trial to compare special computed tomography (CT) for lung cancer screening versus chest x-rays.

In assessing effectiveness of screening technologies, the RCT has been the gold standard. It is the most powerful methodology for demonstrating the value of screening in comparison to an unscreened group. RCTs minimize biases inherent in other designs, especially lead time, length bias,[6] selection bias, and overdiagnosis.[7] Case-control studies also can provide useful information, and at less cost than RCTs. In addition, increasingly sophisticated statistical modeling techniques may be helpful in assessing the impact of screening, especially in situations in which large RCTs

cannot be conducted. The U.S. Preventive Services Task Force,[3] PDQ,[8] and others have rated levels of evidence; the RCT is uniformly regarded as the highest level of evidence.

***Positive and Negative Consequences of Screening***

Every medical activity has positive and negative consequences, and screening is no exception. Potential benefits include improved prognosis for those with screen-detected cancers, the possibility of less radical treatment, reassurance for those with negative test results, and resource savings if treatment costs are reduced because of less radical treatments. The optimal outcome is a reduction in cancer mortality. Potential negative effects of screening include physical, economic, and psychological consequences of false-positives and false-negatives, the potential for overdiagnosis, the potential carcinogenic effects of screening, and the labeling phenomenon.[9–13] The last refers to the fact that telling individuals that they have cancer may change how they see themselves or how others see them. In addition, Baines[14] has noted the largely unexplained increase in cancer among the unscreened group as a negative consequence of screening.

Physicians should engage patients in discussions of the risks and benefits of cancer screening. Because most people overestimate the risks for certain types of cancers (e.g., breast),[15] they may inflate the need for screening and the potential benefits.[16] For some cancers, such as colorectal cancer, people may underestimate their personal susceptibility and may need encouragement to consider screening, with positive as well as negative consequences. In the case of prostate cancer, for which the evidence is still equivocal and population-based screening is not recommended, it is especially important that men understand the limitations of screening and make informed decisions with full understanding of the potential downstream effects of positive and negative test results.[17] Informed decision making increasingly is becoming a paradigm with the potential to be achieved in contemporary clinical practice. Informed decision making occurs when an individual understands the disease or condition being addressed and also comprehends what the clinical service involves, including its benefits, risks, limitations, alternatives, and uncertainties; has considered his or her own preferences, as appropriate; believes he or she has participated in decision making at a level that he or she desires; and makes a decision consistent with his or her preferences.[18] Decision aids are tools used to help patients examine the nature of screening tests and their benefits and limitations.

Austoker[19] outlined topics that should be included when helping patients to make informed decisions about cancer screening. These include the purpose of screening; the likelihood of positive and negative findings and the possibility of false-positive or false-negative results; the uncertainties and risks involved; any significant medical, social, or financial implications of screening; and follow-up plans.

**20. Write about Various Cancer Screening.**

**Skin Cancer Screening**

The incidence of skin cancer has increased worldwide, with U.S. incidence data mirroring this trend.[20] In the United States, the incidence rate for melanoma has increased approximately 4% per year since the early 1970s, with a 162% increase in male melanoma cases and 95% in women.[20] It is unclear whether this increase is due to actual changes in prevalence or is partly a function of increased awareness, with subsequent diagnosis, improved reporting by tumor registries, or both. In 2003, 63,400 new cases of skin cancer and 12,000 deaths were projected, with 54,200 new cases of melanoma (skin) and 7600 deaths.[20] Melanoma now ranks sixth in incidence among cancers in males and seventh in incidence among cancers in females. Approximately 800,000 nonmelanoma skin cancers are diagnosed each year.[20] The United States lags behind many other countries in the creative application of interventions to reduce the incidence of and mortality from melanoma and other skin cancers. Australia, which has the highest reported incidence of melanoma anywhere, has mounted successful population-based programs that have had dramatic effects.

Experts have not agreed about screening guidelines for skin cancer. The U.S. Preventive Services Task Force recommends routine screening for individuals at high risk (e.g., those who have a family or personal history of skin cancer, clinical evidence of precursor lesions, and increased exposure to sunlight). The Task Force neither defines what is meant by routine screening nor reports on the specific recommendations for skin self-examination.[3,94] The ACS recommends a cancer-related checkup, including a skin examination, every 3 years, and more frequently for persons at risk.[72] The NCI also recognizes the benefits of skin cancer screening but offers no specific guidelines for such screening.[95] Only one study demonstrates evidence regarding skin self-examination. Although it showed a decrease in mortality, there were limitations that preclude making recommendations on the basis of this study alone.[96,97]

**Prostate Cancer Screening.**

Prostate cancer is the most commonly diagnosed cancer among men in the United States and is the second leading cause of male cancer deaths. It is estimated that in 2003, 220,900 new cases of prostate cancer were identified, with 28,900 deaths.[20] However, consensus is lacking about recommendations for prostate cancer screening. The controversy has been raised for several reasons. First, there is no definitive evidence that prostate cancer screening results in improved clinical outcomes, especially a reduction in mortality from the disease.[98,99] Second, the rise in the incidence of prostate cancer from 1989 to 1992 (see(http://www.seer.cancer.gov)) was due largely to detection of latent, asymptomatic cases with uncertain clinical relevance, thus putting the value of screening in doubt.

*Screening Tests for Prostate Cancer.*

The two main screening modalities for prostate cancer include digital rectal examination (DRE) and serum prostate-specific antigen (PSA; concentration more than 4 ng/dL) and endorectal (transrectal) ultrasonography (TRUS). The most widely used and oldest technique for detection of prostate cancer is the DRE; ranges in estimates of sensitivity and specificity are reported. Estimates suggest a PPV of 15% to 30% and a sensitivity of approximately 60%.[2] Ultimately, only one in three patients with a positive DRE has prostate cancer.[100] With the development and application of intraluminal (rectal) probes with high resolution, studies have shown that small, nonpalpable malignant lesions of the prostate could be detected.[101] TRUS has fallen short of expectations, with large variation in reports of sensitivity and specificity, both ranging from 41% to 79%.[101,102] Despite this, TRUS is considered an excellent ancillary modality to increase accuracy of biopsy over the digital guidance alone.

PSA is a blood test that allows for earlier detection of many prostate cancers, with sensitivity of up to 80% to 85%. Unfortunately, PSA tests have low specificity, resulting in high rates of false-positives.[2] Interest in the PSA grew in the late 1980s as PSA levels were shown to drop to undetectable levels after prostatectomy.[103,104] However, normal PSA values are found in approximately one-third of men with localized cancers, and PSA levels are often elevated in men with noncancerous conditions, such as benign prostatic hyperplasia.[104,105] Only approximately 7% of prostate cancers detected by screening are microfocal and low grade.[106]

Some investigators have argued that integration of DRE with determination of PSA levels and the use of TRUS in selected cases should improve prostate cancer detection.[104,107] Using age-specific PSA ranges may be a promising strategy for increasing PSA sensitivity.[108] Prostate cancer screening is more controversial in older men; concerns about quality of life outweigh potential benefit of screening.[109] Several reports indicate that men over age 70 years are unlikely to benefit from PSA testing.[100]

**Lung Cancer Screening.**

Lung cancer screening is not recommended on a population basis due to lack of evidence that any available screening procedure, even for smokers, can identify tumors early enough to reduce mortality.[118] This remains a major challenge to research and technology because of the tremendous burden caused by this cancer, including among ex-smokers. In 2003, there were an estimated 171,900 new cases and 157,200 deaths, making it by far the most common killer from cancer in men and in women.[20]

None of four randomized trials conducted during the 1960s and 1970s reduced mortality significantly over no screening.[119–122] The Mayo Lung Project, the primary trial contributing to this evidence, demonstrated that screening with either chest x-rays or chest x-rays plus sputum

cytology lowered the stage at presentation and increased survival, but neither approach had any effect on lung cancer mortality.[122] Although lack of connection between improved survival and the absence of a mortality benefit can be attributed to lead-time and length biases, these studies have been criticized for other methodologic reasons.[123,124] Extended mortality follow-up of participants in the Mayo Lung Project suggested that overdiagnosis, the identification of clinically unimportant lung cancer lesions, may have occurred.[125]

Low-dose CT scanning is a new and potentially efficacious method for early detection of lung cancer.[126] This noninvasive technique, which creates an image of the entire thorax during a single held breath with a low radiation dose, is being offered by an increasing number of imaging facilities for older smokers and former smokers. However, to date, evidence of high sensitivity comes only from observational studies that are susceptible to lead-time and length-time bias. The possibility of overdiagnosis and concerns of harm from CT screening have also been raised,[124] and there is, as yet, insufficient evidence to support mass lung cancer screening with this procedure.[125] Nevertheless, the potential of this technology and the enormous societal burden imposed by lung cancer motivated the NCI to begin a large, randomized controlled trial to assess the effect of low-dose CT screening compared to standard chest x-ray on lung cancer mortality. Other observational studies of low-dose CT screening are ongoing and may add to the evidence produced by the NCI-supported trial, which will take approximately 10 years to produce results.[124] Meanwhile, decisions about use of CT scans for lung cancer screening remain a matter of individual judgment between physicians and patients.[123] Several reports suggest the need for caution in adopting lung CT screening, especially in view of aggressive direct-to-consumer marketing of the procedure.[127,128] In a detailed decision analysis, Mahadevia et al.[128] showed that even if efficacy of helical CT is shown, it is unlikely to be cost effective. Like many tests, helical CT may be able to identify small lesions. However, questions remain about the mortality impact and cost effectiveness. Thus, the ongoing trial is of critical import.[129] Efforts to prevent initiation, especially by youth, and cessation of tobacco use remain the physician's best tool for combating lung cancer.

## UNIT – II

## PART – A

**1. What is carcinogen activation by cytochrome P450?**

Many carcinogens share a similar need for metabolic activation before they can cause cancer. Carcinogens exhibiting such behaviour are more accurately called pre-carcinogens, a term referring to any substance that is capable of causing cancer only after it has been metabolically activated. The activation of pre-carcinogens is generally carried out by liver proteins that are members of the cytochrome P450 enzyme family one function of these liver enzymes is to catalyze the oxidation of ingested foreign chemicals, such as drugs and pollutants, with the aim of making molecules less toxic and easier to excrete from the body. The hydroxylation reaction done in one of several ways in which cytochrome P450 oxidizes foreign chemicals to make them more water soluble, thereby facilitating their excretion in urine. Occasionally, however, oxidation reactions catalyzed by cytochrome P450 accidently convert substances into carcinogens, a phenomenon known as carcinogen activation.

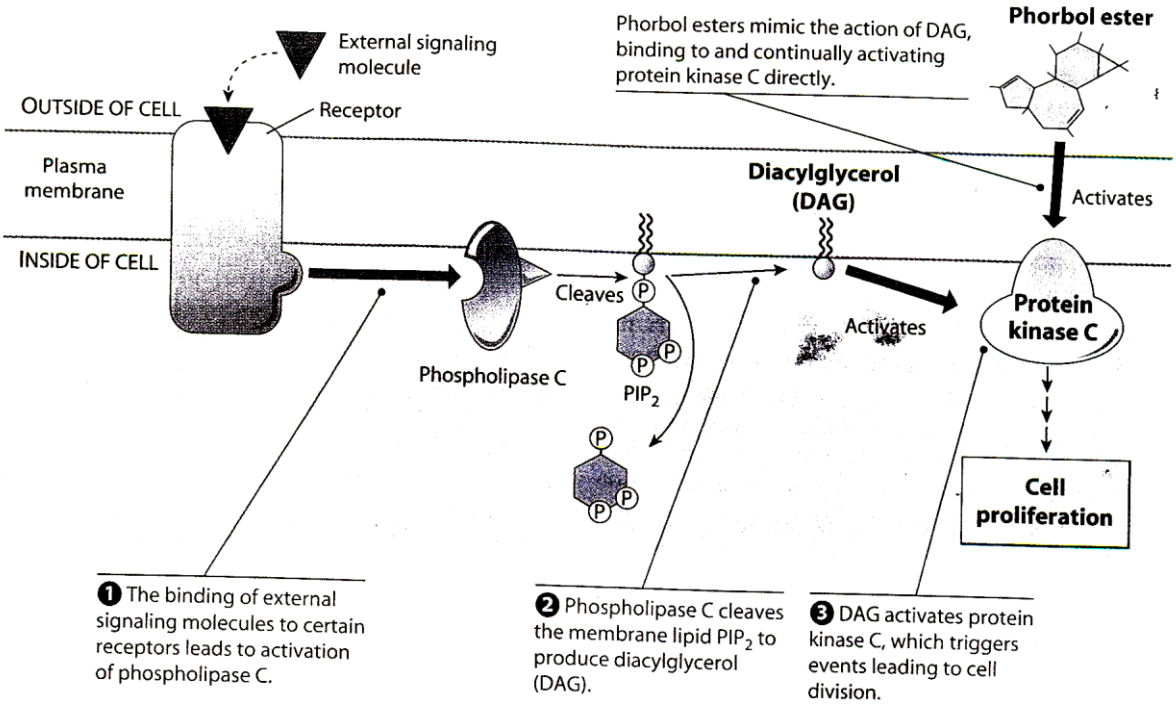
**2. What are different classes of carcinogenic chemicals?**

| | |
|---|---|
| 1. Polycyclic aromatic hydrocarbons | Dimethyl benz (a) anthracene.<br>Benzo (a) Pyrene<br>3-methyl cholanthrene |
| 2. Aromatic amines and Amino Azo compounds. | 2-Naphthylamine Benzidine<br>2-Acetylamino fluorine |
| 3. N-Nitroso compounds | Dimethyl nitro samine methyl nitrosourea |
| 4. Alkylating Agent | Vinyl chloride<br>Sulfur mustard<br>Ethylene Oxide |
| 5. Natural products | Aflatoxin $B_1$<br>Pyrolizidine alkaloid Safrole |
| 6. In organic substances | Asbestos<br>Cadmium sulfide<br>Nickel cadmium<br>Barium chromate |

**3. What is the mechanism of Action of Phorbol esters?**

Phorbol esters activate protein kinase C, a component of a signaling pathway that stimulates cell proliferation. In the normal operation of this pathway, external signaling molecules bind to cell surface receptors whose activation leads to the production of diacyl glycerol (DAG).

The DAG then activates protein kinase C, which triggers events leading to cell division. Phorbol esters mimic the action of DAG, binding to and activating protein kinase C directly.



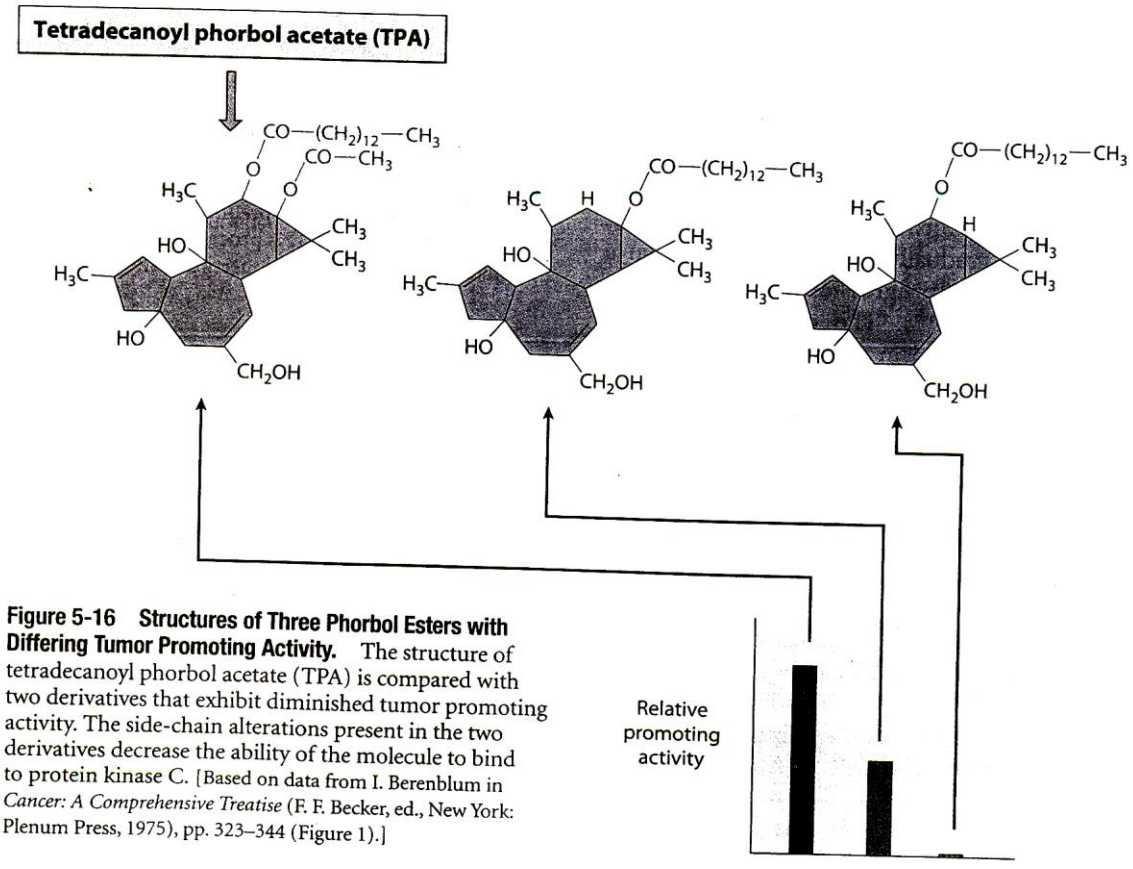




**Figure 5-16 Structures of Three Phorbol Esters with Differing Tumor Promoting Activity.** The structure of tetradecanoyl phorbol acetate (TPA) is compared with two derivatives that exhibit diminished tumor promoting activity. The side-chain alterations present in the two derivatives decrease the ability of the molecule to bind to protein kinase C. [Based on data from I. Berenblum in *Cancer: A Comprehensive Treatise* (F. F. Becker, ed., New York: Plenum Press, 1975), pp. 323–344 (Figure 1).]

**4. Give some example of carcinogens activated by Epoxide formation.**

**Site of DNA modification**

| | |
|---|---|
| 1. Benzo (a) pyrene | $N_2$ of guanine<br>$N_6$ of Adenine |
| 2. Aflatoxin $B_1$ | $N_7$ of guanine |
| 3. Vinyl Chloride | $N_3$ and $N_4$ of cytosine |

**5. Why many carcinogens are Electrophilic molecules?**

DNA, RNA and proteins all have electron – rich atoms, making each a potential target for electrophillic carcinogens of the three, DNA is the prime candidate because the Ames test has shown that most carcinogens cause DNA mutations.

**6. What is the main stage of carcinogenesis?**

Cancer arises by a complex process involving three main stages. The First stage, initiation, is based on DNA mutation. Initiation is followed by a promotion stage in which the initiated cell is stimulated to proliferate. During tumour progression, further mutations and epigenetic changes in gene expression create variant cells exhibiting enhanced growth rates or other aggressive properties that give certain cells a selective advantage such cells tend to outgrow their companions and become the predominant cell population in the tumor. Repeated cycles of this process, called clonal solution, and creates a population of cells whose properties change over time.

**7. What is a chemical carcinogen?**

Chemical carcinogens are a structurally diverse group of substances that include polycyclic hydrocarbons, aromatic amines, N-nitroso compounds, alkylating agents, natural products and inorganic elements. Many of these substances must be metabolically activated by enzymes in the liver before they can cause cancer. Metabolic activation creates highly unstable electrophillic molecules that react directly with DNA, creating DNA adducts that distort the double helix and cause mutations to arise during DNA replication.

**8. How is Naming Tumors?**

| Prefix | Cell Type | Benign Tumor | Malilgnant Tumor |
|---|---|---|---|
| **Tumors of epithelial origin** | | | |
| Adeno | Gland | Adenoma | Adeno carcinoma |
| Basal cell | Basal Cell | Basal Cell adenoma | Basal cell carcinoma |
| Squamous cell | Squamous cell | Kerato carthoma | Squamous cell carcinoma |
| Melans | Pigmented cell | Mole | Melanoma |
| Terato | Multi-potential cell | Teratoma | Terato carcinoma |
| **Tumours of supporting tissue origin** | | | |
| Chondro | Cartillage | Chondroma | ChandraSarcoma |
| Fibro | Fibro blast | Fibroma | Fibro Sarcoma |
| Hemanigio | Blood vessels | Hemangioma | Hemangio Sarcoma |
| Leiomyo | Smooth muscle | Leiomyoma | Leiomyo sarcoma |
| Life | Fat | Lipoma | Lipo sarcoma |
| Meningio | Meninges | Meningioma | Meningio sarcoma |
| Myo | Muscle | Myoma | Myo sarcoma |
| Osteo | Bone | Osteoma | Osteo sarcoma |
| Rhabdomyo | Straiten muscle | Rhabdo myoma | Rhabdomyo sarcoma |
| **Tumors of blood and Lymphatic origin** | | | |
| Lympho | Lymphocyte | | Lymphoma or Lymphocytic Leukemia |
| Erythro | Erythrocyte | | Erythrocytic leukemia |
| Myolo | Bone marrow | | Myelome or myelogenous Leukemia |

**9. What is mean by complete carcinogen?**

Some Chemicals possess both initiating and promoting activites, and can therefore cause cancer by themselves; such chemicals are called complete carcinogens.The ability to function as a complete carcinogen may be dose dependent. For example, certain polycyclic hydrocarbons act as initiating agents at lower doses but are complete carcinogens at higher doses.

**10. Define Mutagen.**

Any agent capable of altering the genetic constitution of a cell by changing the structure of the hereditary material, (DNA) Deoxy ribonucleic acid. Many forms of electromagnetic radiation (eg. Cosmic rays, x-rays, U.V. light) are mutagenic, as are a variety of chemical compounds. The effect of some mutagens are potentiated (increased) or suppressed in some organism by the presence of certain other, non mutagenic substances; oxygen, for example, makes cells more sensitive to the mutagenic effects of x-rays.

**11. Define Teratogens.**

Teratogens refers to any agent that causes a structural abnormality following fetal exposure during pregnancy. Cases of phocomelia in the early 1960's in Germany and Australia lead to the identification of drug thalidomide as a human teratogen. Thalidomide was used to treat morning sickness, resulting in exposure at the stage in development when the embryo is most vulnerable, the first trimester.

**12. What is Focus – Forming Unit?**

A measurement of the concentration of live virus in a given amount of fluid. This is measured by spreading a known amount of the fluid over a layer of cultured cells which are infected by the virus, then counting the number of areas in the culture which look infected.

**13. Define Relative Biological Effectiveness (RBE).**

$$\text{RBE} = \frac{\text{Dose from reference radiation}}{\text{Dose from test radiation DT}}$$

Analysis of the Relative Biological Effectiveness, RBE, is a useful way to compare and contrast the results observed in these studies. The relative biological effectiveness for a given test radiation, is calculated as the dose of a reference radiation, usually x-rays, required to produce the same biological effect as was seen with a test dose DT, of another radiation thus for the same biological end point.

**14. What is Mitotic Index? How is it measured?**

Mitotic Index is a measure for the proliferation status of a cell population. Cell is cell cycle can be identified using antibodies against the nuclear Antigen ki-67. The mitotic index can be worked out from a slide, even with light microscopy.

It is the number of cells containing visible chromosomes divided by the total number of cells in the field of view.

**15. What is SOS response?**

In bacteria, the induction of various proteins involved in DNA repair and DNA Synthesis in response to the presence of high levels of DNA damage as might occur following exposure to a mutagenic agent.

**16. What are the major classes of Carcinogens?**

1) Direct activity Carcinogens: it directly binds to DNA eg. Alkylating agent
2) Procarcinogens: It cause the newolastic conversion of target cells eg.aflotoxin, azo dyes

## PART – B

**1. What is Radiation Carcinogens?**

The common types of mutagenic radiation found in nature are ultra violet, gamma and x-radiation. Radiation induces mutations by damaging DNA. The kinds of DNA damage caused by the three types of radiation mentioned above differ, since the radiations very greatly in energy. The relatively weak ultraviolet radiations vary greatly in energy. The relatively weak ultraviolet radiation cross links adjacent pyrimidires in DNA to form pyrimidine dimmers, usually thymine dimmers. Gamma rays and X-rays have much more energy. They cause DNA damage by ionizing molecules surrounding DNA, especially water, to form free radicals that attack the DNA molecule. This can results in a base change or cause single stranded and double-stranded breaks in the DNA strands (chromosome breaks). The mutagenic events caused by radiation can become carcinogenic events as a result of the cell'S inaccurate attempts to repair the damage, leading to permanent mutations such as base changes and translocations.

Some light-skinned persons, with relatively little defence against ultraviolet radiation in the sun that dark-skinned persons, inherit a condition called xeroderma pigmentation in which the DNA repair mechanism is defective. Therefore, on exposure to the sun they develop several skin cancers, including malignant melanomas which are lethal. The cancers are caused by misrepaired DNA damage rather than unrepaired damage.

**2. Describe briefly about Checkpoints.**

Cells are constantly faced with insults resulting in damage that can threaten their survival. These insults can be generated internally as by-products of metabolism or can originate in the external environment—for example, chemical agents or radiation. As a result, mechanisms have

evolved to remove damaged molecules and make necessary repairs. In instances in which cell-cycle progression would be harmful or catastrophic before repair of damage, further mechanisms have evolved to delay progression pending repair. These are called *cell-cycle checkpoints*. The necessity of checkpoints can be easily envisioned for genotoxic agents. Cells are particularly susceptible to the harmful effects of DNA damage at two points in the cell cycle: S phase and M phase. Unrepaired DNA damage poses a number of problems for cells undergoing DNA replication. Chromosomal lesions present physical barriers to replication forks. Replication that does traverse regions of DNA damage is likely to be error prone, resulting in accumulation of mutations. Likewise, segregation of severely damaged chromosomes at mitosis might lead to loss of genetic information, seriously threatening the survival of daughter cells. Therefore, cells possess mechanisms for preventing DNA replication and mitosis in response to genotoxic stress. Although the scope of this review does not permit a detailed description of all known checkpoints, those thought to be most basic to cell survival are characterized later.

**DNA Damage Checkpoints**

Although DNA damage exists in many forms, ranging from chemical adducts to double-strand breaks, they all pose similar problems for proliferating cells. As stated above, impeded and error-prone DNA replication and loss of genetic material during mitosis are some of the likely consequences in the absence of DNA damage checkpoints. Therefore, cell-cycle progression is blocked at three points: before S-phase entry (the $G_1$ DNA damage checkpoint), during S phase (the intra–S phase DNA damage checkpoint), and before M-phase entry (the $G_2$ DNA damage checkpoint). Although the responses to different types of DNA damage are not identical, they are similar enough to generalize. DNA damage of various forms is first detected by DNA-bound protein complexes that serve as sensors. In mammalian cells, two related atypical protein kinases that share homology with lipid kinases, ATM and ATR, are primary signal transducers that are activated by DNA damage at all points in the cell cycle. A key effector of the $G_1$ and $G_2$ checkpoint responses is a transcription factor known as *p53*. In response to DNA damage, p53 is activated and stabilized leading to increased levels. The principal transcriptional target of p53 in the context of the $G_1$ checkpoint is the Cip/Kip inhibitor $p21^{Cip1}$. The resulting high levels of p21 block cdk2 activity and possibly cdk4 and cdk6 activity, leading to $G_1$ arrest. Another transcriptional target of p53, GADD45, inhibits cdk1, thereby contributing to the $G_2$ DNA damage checkpoint. However, the primary mechanism underlying the $G_2$ DNA damage checkpoint is p53 independent. It involves one of two effector protein kinases known as *chk1* and *chk2* that have the effect of inhibiting CDC25C, which carries out the activating dephosphorylation of cdk1. Therefore, in response to DNA damage, $G_2$ cells accumulate inhibited cyclin B–cdk1 complexes and are incapable of entering into mitosis. The intra–S-phase DNA damage checkpoint response appears to be p53 independent but requires the chk1 or chk2 kinases, or both. A key target is CDC25A, responsible for activating dephosphorylation of cdk2. In response to DNA damage, phosphorylation of CDC25A by chk1 or chk2 leads to its destabilization and the accumulation of

inactive cdk2 complexes phosphorylated on threonine 14 and tyrosine 15. Because ongoing DNA replication requires the activity of cdk2, DNA synthesis ceases until damage is repaired.

**Replication Checkpoint**

Under normal circumstances, DNA replication is complete well before the time when the accumulation and activation of cyclin B–cdk1 would drive cells into mitosis. However, through the action of toxins or the rare but finite probability that the duration of S phase will be excessively long, situations can be encountered in which completion of replication extends beyond the normal time of mitotic induction or replication is blocked entirely. Under such circumstances, it is necessary to delay or block entrance into M phase accordingly, as segregation of incompletely replicated chromosomes would be catastrophic. Although the signaling pathways are somewhat different, the replication checkpoint ultimately functions like the $G_2$ DNA damage checkpoint in that mitotic entry is blocked by inhibiting CDC25C via the action of chk1, thus preventing activation of cdk1.

**Spindle Integrity Checkpoint**

The actual act of division is a dangerous time for a cell. It requires aligning duplicated chromosomes by attaching them via bipolar attachments to the spindle and then separating the chromatids so that each daughter cell gets a full complement. Errors result in aneuploidy, an extremely undesirable outcome. As a result, assembling a mitotic spindle and attaching chromosomes to it are extensively monitored processes. The mechanism of delay at prometaphase or metaphase in response to spindle defects or improper chromosome attachment is referred to as the *spindle integrity checkpoint*. The sensor for this checkpoint consists of a number of proteins that reside at the chromosome kinetochores, sites of spindle microtubule attachment. The target is the essential APC/C cofactor, cdc20. Unattached or improperly attached kinetochores not experiencing an appropriate level of tension indicative of biopolar attachment inhibit cdc20 function. This in turn prevents the ubiquitylation and degradation of the anaphase inhibitor, securin. As a result, cells are prevented from initiating anaphase until all kinetochores are properly attached to a bipolar spindle.

**Restriction Point**

Cells deprived of an essential nutrient or growth factor are blocked from cell-cycle progression at a point in mid $G_1$. Cells that have already passed this point, termed the *restriction point* or *R*, enter into S phase and complete the current cell cycle before arresting in the subsequent $G_1$ interval. In contrast, $G_1$ cells that have not reached the restriction point arrest immediately. The molecular basis for the restriction point has remained elusive. Initially, it was thought that passage through the restriction point was a manifestation of $G_1$ cdk activation.

However, more recent work has indicated that cdk activation occurs after passage through the restriction point. Significantly, most malignant cells do not have a functional restriction point, which presumably helps them evade normal growth control signals.

**3. Write Shortly about *Senescence.***

All normal mammalian cells have a finite proliferative life span. As cells approach the end of their proliferative capacity, they enter a state referred to as *replicative senescence*. Although the reasons for programmed senescence are not known, it has been speculated that restricting cells to a finite number of divisions may be a protective mechanism against malignant growth. Although the rationale for senescence is not known, the mechanism has been largely elucidated, particularly for human cells. It is based on the requirement for a specialized replicase, telomerase, in the replication of the ends of chromosomes known as *telomeres*. Whereas germline cells express telomerase, most if not all somatic cells do not. As a result, because of the topology of telomeres and the requirements of conventional DNA replication, progressive telomere shortening or attrition occurs with each cell cycle. Although linear chromosome ends create a discontinuity, which topologically is indistinguishable from a chromosome break, telomere-specific DNA sequences are shielded from the DNA damage checkpoints. However, when sufficient telomere attrition has removed these protected sequences, cells enter into a chronic checkpoint response, which is the molecular basis for senescence. Senescence is characterized by the accumulation of high levels of cdk inhibitors and ultimately permanent $G_1$ arrest. It should be noted that one of the requirements of malignant transformation of cells is to overcome the senescence barrier so as to provide tumor cells with unlimited proliferative capacity.

**4. How is the Tobacco Smoking influence Cancer Risk?**

Tobacco smoking is the major cause of cancer and accounts for almost 96% of all male lung cancers in whites. Although the risk of lung cancer decreases after smoking cessation, it never returns to that for nonsmokers. Tobacco smoke contains more than 3500 chemicals, of which more than 20 are carcinogens. Specific chemicals in tobacco smoke include PAHs and N-nitrosamines, aromatic amines, ethylene oxide, 1,3-butadiene, and agents that cause oxyradical damage. PAHs and tobacco-specific nitrosamines are considered to be the most potent carcinogens in tobacco smoke. During the last 40 years, although the tar (which contains PAHs) and nicotine content has decreased approximately threefold, there also has been an increase of other carcinogens including tobacco-specific nitrosamines.[43] Today, light cigarettes dominate the market, and, until recently, it was believed that they were less harmful than regular cigarettes. However, because smokers must maintain their nicotine addiction, the smoking of lower-nicotine cigarettes results in having to smoke more (numbers of cigarettes per day and inhalation). Also, the chemical composition of the smoke is different for light and regular cigarettes, yielding more mutagenic tar and different mixtures of carcinogens. As a result, light cigarettes are not less harmful and are considered to be

the reason why lung cancer histology has changed from squamous cell cancers to mostly peripheral adenocarcinomas.

Convincing laboratory animal and human studies have demonstrated a relationship between tobacco smoke constituents, carcinogen-DNA adduct formation, and cancer.[44] Several determinants of tobacco carcinogen exposure and cancer risk are known, including the number of cigarettes smoked per day, years smoked, cigarette type [e.g., tar content (the total dry particulate component of smoke)], and smoking topography (e.g., how much smoke enters the lung, measured by puff volume, number of puffs per cigarette, puff duration, and interpuff interval). However, wide interindividual variation for carcinogen metabolism and DNA adduct formation affects lung cancer risk. The evaluation of cancer risk has more recently focused on biomarkers that can be used to assess different levels of carcinogen exposure, but no single biomarker is sufficient for this.[20] Importantly, not all smokers have the same carcinogenic risks; lung cancer develops in only one in ten heavy smokers, and some heavy smokers live to their 90s. This is thought to be due to genetic polymorphisms relating to DNA carcinogen metabolism, DNA repair, and cell-cycle control.

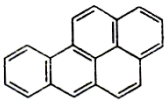
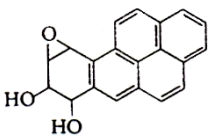
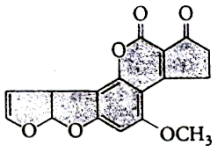
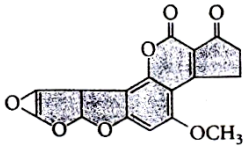
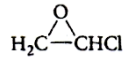
**5. Write the Nature of Chemical Carcinogens, Chemistry and Metabolism.**

Although a wide variety of chemicals and chemical classes can cause cancer in animals and humans the process is very specific. Most chemicals are not known to be carcinogenic. Within chemical classes, stereoisomers may vary widely in carcinogenicity. Genotoxic carcinogens have high chemical reactivity (such as alkylating agents) or can be metabolized to reactive intermediates by the host. They form covalent adducts with macromolecules and target DNA in the nucleus and mitochondria.[9] Because there is a good correlation between the ability to form DNA adducts and the potency to induce tumors in laboratory animals, DNA is considered the ultimate target for most carcinogens. Genotoxic carcinogens may transfer simple alkyl or complexed (aryl) alkyl groups to specific sites on DNA bases. These alkylating and arylalkylating agents include, but are not limited to, N-nitrosocompounds, aliphatic epoxides, aflatoxins, mustards, polycyclic aromatic hydrocarbons (PAHs), and other combustion products of fossil fuels and vegetable matter. Others transfer arylamine residues to DNA as exemplified by aryl aromatic amines, aminoazodyes, and heterocyclic aromatic amines; the latter is produced by overcooking meat, poultry, or fish at high temperatures. For genotoxic carcinogens, the interaction with DNA is not random, and each class of agents reacts selectively with purine and pyrimidine targets.[9] Furthermore, targeting of carcinogens to particular sites in DNA is determined by nucleotide sequence, by host cell, and by selective DNA repair processes (see DNA Repair Protects the Most from Chemical Carcinogens, later in this chapter), making some genetic material at risk over others. As expected from this chemistry, genotoxic carcinogens are potent mutagens, particularly adept at causing base mispairing or small deletions, leading to missense or nonsense mutations. Others may cause macrogenetic damage such as chromosome breaks and

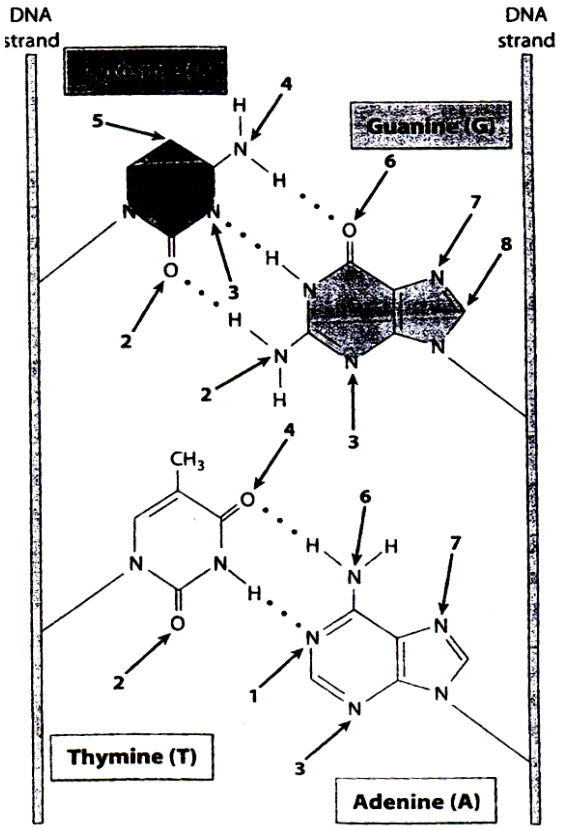
large deletions.[10] In all cases, mutations detected in tumors represent a combination of the effect of the mutagenic change on the function of the protein product and the effect of the functional alteration on the behavior of the specific host cell type.

A number of chemicals that cause cancers in laboratory rodents are not demonstrably genotoxic. Synthetic pesticides and herbicides fall within this group, as do a number of natural products that are ingested. In general, these agents are carcinogenic in laboratory animals at high doses and require prolonged exposure. The mechanism of action by nongenotoxic carcinogens is controversial and may be related in some cases to toxic cell death and regenerative hyperplasia. Induction of endogenous mutagenic mechanisms such as DNA oxyradical damage,[3] depurination, and deamination of 5-methylcytosine by exposure to nongenotoxic carcinogens may contribute to carcinogenicity of these agents. In other cases, nongenotoxic carcinogens may have hormonal effects, influencing hormone-dependent tissues directly. Although the contribution of nongenotoxic carcinogens to human cancer causation is not certain, they may also serve as modifiers in concert with genotoxic agents, altering tissue homeostasis to provide an environment conducive to the selective expansion of a neoplastic clone. A number of endogenous metabolic enzymes activate or detoxify carcinogens and procarcinogens (chemicals that can be transformed into active carcinogens).[11] These pathways are complex and interactive, and genetic polymorphisms in animal models and humans are thought to be major determinants of cancer susceptibility and indications of risk for particular exposures.[11] Furthermore, a number of metabolic pathways are inducible and modified by diet, hormones, and additional exposures, adding further complexity to the process of environmental carcinogenesis.

**Table 5-3** Examples of Several Carcinogens Activated by Epoxide Formation

| Carcinogen | Major Active Metabolite* | Site of DNA Modification**† |
|---|---|---|
| Benzo[*a*]pyrene (BP)  | BP 7,8-diol 9,10-epoxide  | N2 of guanine<br>N6 of adenine |
| Aflatoxin $B_1$  | Aflatoxin $B_1$ 8,9-epoxide  | N7 of guanine |
| Vinyl chloride  | Chloroethylene oxide  | N3 and N4 of cytosine***<br>N1 and N6 of adenine<br>N2 and N3 of guanine |

*Green shading is used to highlight the epoxide group.
**The numbers in the third column refer to the numbered positions of nitrogen atoms illustrated in Figure 5-11.
***Vinyl chloride simultaneously attacks two positions on the same base, forming a cyclic adduct.




**6. How is DNA Repair Protects the Host from Chemical Carcinogens?**

DNA repair defects have been identified in a number of cancer-prone individuals, and repair-deficient mammalian cells are susceptible to transformation by chemical and physical carcinogens.[13] Nucleotide excision repair commonly removes carcinogen-DNA adducts or ultraviolet photoproducts by a complex process involving at least ten gene products, each potentially associated with mutations that lead to human DNA repair defect syndromes and increased cancer rates. Nucleotide excision repair commonly favors adduct removal on the transcribed strand to protect protein synthesis. Genetically engineered mice deficient in genes involved in nucleotide excision repair are particularly sensitive to chemical and ultraviolet carcinogenesis at particular organ sites. The highly mutagenic $O^6$-methylguanine, a consequence of exposure to certain methylating agents, is repaired by $O^6$-alkyl-deoxyguanine-DNA-alkyltransferase, an enzyme that protects the host from thymic lymphomas, colonic preneoplastic lesions, and colonic K-*ras* mutations after exposure of mice to methylating agents. Mutations in genes involved in nucleotide mismatch repair increase risk for colon cancer in humans, and engineered mice that are null for a gene in this pathway are predisposed to development of tumors.[14] The human cancer susceptibility genes BRCA1 and -2 and ATM participate in repair of carcinogen-induced DNA double-strand breaks in pathways linked to homologous recombination.[13]

**7. Write the Genetic Susceptibility to Chemical Carcinogenesis.**

The identification and characterization of genes that modify risks for cancer development have been facilitated by substantial variation in susceptibility to chemically induced carcinogenesis at specific tissue sites among inbred strains of rodents and spontaneous or genetically modified mutant strains. For a variety of tissue sites, including lung, liver, breast, and skin, pairs of inbred mice that differ by 100-fold in risk for tumor development after carcinogen exposure have been characterized. Genetically determined difference in the affinity for the aryl hydrocarbon hydroxylase (Ah) receptor or other differences in metabolic processing of carcinogens is one modifier that has a major impact on experimental cancer risk. Other loci regulate the growth of premalignant foci, the response to tumor promoters, the immune response to metastatic cells, and the basal proliferation rate of target cells. In mice susceptible to colon cancer due to a carcinogen-induced constitutive mutation in the *apc* gene, a locus on mouse chromosome 4 confers resistance to colon cancer.[16] The identification of the phospholipase $A_2$ gene at this locus and subsequent functional testing in transgenic mice revealed an interesting paracrine protective influence on tumor development.[16] This gene and several other genes mapped for susceptibility to chemically induced mouse tumors (*ptprj*—a receptor-type tyrosine phosphatase and *STK6*/*STK15*—an aurora kinase) have now been shown to influence susceptibility to organ-specific cancer induction in humans.

**8. Write briefly about Physical Carcinogenesis.**

Among the best-known and well-characterized carcinogens that are known from direct evidence to increase the risk of cancer in humans are physical agents, including ionizing radiation, ultraviolet (UV) light, and asbestos. For all these agents the evidence that exposure will increase the risk for cancer development is clear and unequivocal. Because their carcinogenic potential is well known, questions about these agents tend to focus on the degree of risk to humans as a function of exposure level or dose and on underlying mechanisms. The issue of risk as a function of exposure level or dose is not an academic exercise; rather it is an extremely important consideration in medicine for which the techniques using ionizing radiation are powerful tools in the diagnosis and treatment of a wide range of diseases. Risks from radiation exposures that result from the application of these medical procedures must be weighed against the potential or real benefits of the procedure. Although the doses to individuals are generally low, without knowledge of the relationship between radiation dose and subsequent cancer risk such decisions cannot be made with confidence. This risk-versus-benefit issue is exacerbated when procedures result in relatively large numbers of individuals being exposed. In this instance, although the risk to any particular individual might be low, because the numbers of individuals exposed is very large, the potential for significantly increasing the cancer risk in the population as a whole may be substantial. Mechanistic studies provide insight into potential risks at low levels of exposure, for which effects cannot be directly measured by epidemiologic studies and provide

information on approaches to reducing or preventing the carcinogenic effects of these agents. Such studies are also helpful in identifying potential sensitive subpopulations. As will be seen, focusing on sensitive subpopulations is helpful for reducing risks but has also been important in dissecting underlying mechanisms.

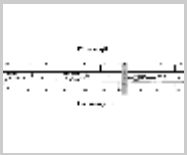
Exposures to ionizing radiation can come from natural and from human-made sources. We are continuously exposed to naturally occurring radioisotopes contained in soil, rocks, and plants and, as a result, in building materials. One of the greatest sources of naturally occurring radiation is radon. In addition, we are exposed to cosmic rays. The amount of exposure is related to where we live. At higher altitudes the amount of exposure from cosmic rays is higher than at sea level. The levels of this naturally occurring radiation, often referred to as *background radiation*, varies with altitude, geology, and the predominant type of materials used to construct homes and other materials. It is often not appreciated that the most significant human-made source of radiation exposure comes from medical procedures, including diagnostic imaging, nuclear medicine, and therapeutic procedures. On average, the dose to the general population from medical procedures is similar to that received from background radiation. However, the medical contribution to radiation exposure is rapidly increasing. This is a result of the wider application of more powerful imaging tools, such as helical computed tomography (CT) scans and the movement to the use of digital images rather than film and from the application of techniques such as intensity-modulated radiation therapy (IMRT).

UV light from the sun is responsible for an increasing number of skin cancers throughout the world. Risks for skin cancer vary with altitude, latitude, and pigmentation, all of which modify the dose of UV light delivered to target cells in the skin. The understanding of the underlying mechanisms of UV-induced skin cancer has benefited greatly from the identification and study of individuals who are extremely sensitive to the effects of ionizing radiation. More recently, the development of genetically engineered mice with specific gene defects has also proved to be invaluable in the dissection of underlying mechanisms.

Although asbestos fibers have a chemical composition, they have generally been classified as a physical carcinogen because it is believed that their physical interactions with cells rather than specific chemical interactions are responsible for their carcinogenic effects. Asbestos is a naturally occurring mineral silicone that results from fibrous crystallization. Health effects, including lung cancer, are well documented from high occupational exposures from its commercial uses. More controversial and uncertain are the health effects at low levels to which the general public might be exposed.

**9. Write the Interactions of Radiation with Cells and Tissues.**

Gamma rays, x-rays, and UV light are all part of the electromagnetic spectrum shown in **Figure 10-1**.[1] Their interactions with biologic material depend on the frequency or wavelength of the radiation. UV light and electromagnetic forms of ionizing radiation have the highest frequencies and energies. At the short wavelengths of x-rays and gamma rays, electromagnetic radiation has sufficient energy to produce ionizations as a result of removal of electrons from atoms. At the longer wavelengths including low-level electric and magnetic fields up to and including UV light, the energy deposition is insufficient to produce ionizations, and these forms of energy are generally referred to as *nonionizing radiations*.

| | |
|---|---|
|  View Figure | Figure 10-1<br>Electromagnetic spectrum. |

***Ionizing Radiation***

In addition to the electromagnetic forms of ionizing radiation (such as gamma rays and x-rays), there are particulate forms of ionizing radiation, including electrons, protons, α-particles, and neutrons. A full discussion of how these different radiations interact with matter is beyond the scope of this chapter, and the reader is referred to other books on this subject.[1,2] The spatial distribution of the ionizations produced by these different forms of ionizing radiation provides an additional means of classification based on their interactions in matter including biologic material. This classification is of particular relevance to the biologic effects of the different forms of ionizing radiation. This spatial distribution of ionizations is measured as the energy transferred per unit track length [linear energy transfer (LET)] in units of kiloelectronvolt per millimeter. On this basis, x-rays, gamma rays, and electrons are classified as sparsely ionizing radiation, whereas α-particles (such as those associated with radon) and neutrons are densely ionizing. The density of the ionizing radiation can have a substantial impact on the biologic effects of the radiation exposure. These differences can be qualitative as well as quantitative. The quantitative differences are measured by comparing the dose of a test radiation (e.g., α-particles) to produce the same level of effect as a specific dose of x-rays (or sometimes gamma rays). The ratio of these doses is called the *relative biologic effectiveness* (RBE).[3] For cell-killing effects, RBE values for α-particles and neutrons have been found to be in the range of 3 to 5. An RBE for cell killing after irradiation with α-particles on the order of 5 would mean that an alpha dose of 1 Gy would result in the same level of cell killing as that produced by an x-ray dose of 5 Gy. For cancer induction, estimates for RBE values from experimental studies can be 20 or higher for α-particles and neutrons. RBE tends to

increase as a function of LET to a maximum of approximately 100 keV/mm. At this LET, the average separation of ionizing events is approximately the diameter of the double helix, which would tend to maximize the probability that a single track of radiation can produce a double-strand break (DSB). Qualitative differences between high and low LET radiations are suggested by the observation that, for all effects examined (including cell killing, chromosome aberrations, mutation induction, and cancer induction), the radiation damage responsible for these effects appears to be less easily repaired by the cell or organism after exposure to high LET radiation.[3]

Energy deposited in biologic material can produce ionizations in target molecules, such as DNA, directly through production of ionizations in those molecules or indirectly through interactions with water molecules that result in the formation of free radicals. These free radicals then produce the damage to the DNA. Because ionizing events from low LET radiations are more sparsely distributed, damage to DNA and other targets is less likely to be a result of direct ionizations but rather is principally a result of indirect mechanisms mediated by free radicals. Because of the density of the ionizations, high LET effects are more generally mediated via direct effects on target molecules.

Whether these effects are directly or indirectly produced, ionizing radiation results in base damage and single-strand breaks and DSBs in DNA. As discussed earlier, for low LET radiation, these effects are mediated via reactive oxygen species much like those produced by normal cellular processes. The reason that ionizing radiations are able to cause the degree of damage that they do is because of the differences in spatial distribution of energy that result in a markedly different distribution of these reactive oxygen species than occurs during normal cellular processes. Exposure to ionizing radiation results in highly clustered ionization events. As such the damage produced by ionizing radiation either directly or indirectly is more complex, with localized areas of DNA molecules with multiple and complex lesions consisting of a combination of base damage and single-strand breaks and DSBs.[4–7] These complex lesions are less easily repaired with fidelity than are more simple forms of DNA damage.[8] For high LET radiations, because of the density of the ionizations, the molecular damage can be particularly complex and difficult to repair.

***Ultraviolet Light***

UV radiation does not have sufficient energy to produce ionizations. Rather, its effects are the result of molecular excitation after absorption of energy by the target molecule. UV light can be categorized into three types, based on wavelength: UVC with wavelengths ranging from 240 to 290 nm, UVB ranging from 290 to 320 nm, and UVA ranging from 320 to 400 nm. UVC is not in sunlight that reaches the earth because it is readily absorbed by the earth's atmosphere. It has proven to be useful, however, for a number of applications. It is produced by low-pressure mercury lamps commonly used for sterilization. Because the peak wavelength for these lamps

(254 nm) is very close to the peak for absorption in DNA molecules (260 nm), it has been an important experimental tool in photobiology for studies of the effects of UV light on DNA. UVB, as is discussed later in Sunlight and Skin Cancer, appears to be primarily responsible for skin cancer induction after sunlight exposure. This appears to be a result of direct damage to DNA mediated by UVB.

The amount of UV light to which an individual or a population is exposed depends on many factors, including the ozone layer. Effects of UVB and UVC appear to be mediated via effects on DNA interactions that result in a number of molecular changes, the most prevalent of which are dimers between adjacent pyrimidines.[9] The most biologically important of these are the cyclobutane dimer and the 6-4 photoproduct. Although other products are produced, these two, especially the cyclobutane dimer, appear to play the major role in the mutagenic and carcinogenic effects of UVB.[10] UVA is not absorbed by the atmosphere and penetrates deeper into the skin than UVB. Because of its wavelength, DNA and proteins only weakly absorb UVA, but, interestingly, it has been shown to be carcinogenic. It is speculated that this carcinogenic effect is due to the production of reactive oxygen species through its interactions with target chromophores. These reactive oxygen species are then able to produce DNA damage indirectly.[11]

For UVC and UVB, the distribution of specific changes in genomic DNA depends on base sequence and secondary and tertiary genomic structure. For example, cytosine absorbs higher wavelengths of UV radiation than thymine. As a result, dimers containing cytosine are more readily formed after UVB radiation. Data have shown that methylation at specific sequences in the p53 molecule enhance formation of dimers in specific regions as well.[12] This results in mutations in p53 that are relatively specific for UV damage and that have been found to be relatively early events in certain forms of skin cancer.[10,13]

### *Ionizing Radiation and Cancer*

#### Studies of Cancer in Exposed Human Populations

The benefits of ionizing radiation in the diagnosis and treatment of disease were recognized by the medical community very soon after the discovery of x-rays and radioactivity. Almost as quickly, the risks of exposure began to be recognized as well. The first cancers that were related to radiation exposure were skin cancers detected only a few years after the discovery of x-rays.[14,15] These cancers were the result of high skin doses received by early workers, who often used their hands to test the output of x-ray tubes. These cases were followed by cases of radiation-induced leukemias among radiologists and radioisotope workers. These early studies provided clear evidence that radiation exposure could result in the development of cancer in humans, but the extent of the risk as a function of dose was not known. Because these early cancers were a result of relatively high levels of exposures, it was thought that tissue injury was

probably required to increase cancer risk. Potential risks at low doses were not appreciated. This began to change with the study of the Japanese survivors of the atomic bombs, the study of patient populations exposed to radiation for therapeutic and diagnostic procedures, and occupationally exposed populations such as radiologists, uranium miners, and nuclear industry workers.[16] A partial list of principal sources of information on cancer risks in humans after radiation exposure is shown in **Table 10-1**. Studies of such populations began in the 1950s and 1960s and continue today. Such studies have provided and continue to provide information on risks as a function of dose, organ and tissue sensitivity, and risk-modifying factors, such as age and genetic background.

The largest population studied and the one that continues to serve as the primary source for understanding risks for cancer development in humans after exposure to radiation are the populations in Hiroshima and Nagasaki, Japan, who survived the atomic bombings of these two cities.[16–18] The doses received were single acute exposures of a mixture of gamma rays and a small amount of neutrons to the entire body. Although the doses received to those very close to the bomb were quite large and often acutely lethal, the survivors of the bombings received a range of doses that has provided substantial information on the relationship between cancer risk and radiation dose. What is generally not appreciated is that the majority of the survivors were not exposed to very large radiation doses. The average dose received by survivors was less than 0.3 Sv. As a result, this population represents the major source of information for determining potential risks at low doses. It is also not appreciated that approximately half of the individuals, those who were children, adolescents, and young adults at the time of the bombing, are still alive today. As is discussed later in Tissue Sensitivity and Latent Period, because the latent period for the development of solid tumors is quite long, important information on solid cancer risks at low doses is just beginning to emerge from these studies. Although the study is not complete, ongoing analyses of this population have provided the majority of the information available on the risk of cancer in humans as a function of dose as well as insight into variations in tissue and organ sensitivity. Because the age distribution of the population was wide, including the old and very young (as well as children exposed *in utero*), this study is also an important source of information about the effects of age on risks and on the tumor latent period, that is, the time between exposure and the appearance of radiation-induced tumors.

Information about radiation cancer risks in humans has also come from the study of patient populations exposed to ionizing radiation as a result of therapeutic or diagnostic procedures.[16,19] The numbers of patients in each individual study are smaller, but the number of such studies is relatively large. Nevertheless, only a few have been useful for the quantification of risks as a function of dose. Because such populations generally receive localized exposures, these groups generally can provide information on risks in specific organs and tissues. Such populations have also provided insight into modifying factors such as age and genetic background. In the past, radiation was used to treat a variety of diseases and medical disorders,

including enlarged thymus glands and tonsils, tinea capitis, ankylosing spondylitis, and peptic ulcers. Epidemiologic studies of these populations have provided information on radiation-induced leukemia, as well as thyroid, breast, and stomach cancers. In general, diagnostic procedures result in very low radiation doses; however, a few studies have provided evidence for increased cancer risks. In one of the most extensive studies, tuberculosis patients were subjected to multiple diagnostic fluoroscopies during the course of being treated for their tuberculosis. Although the individual doses were low, the large numbers of procedures resulted in the accumulation of relatively large total doses. In these studies, females have been shown to be at a significantly increased risk for breast cancer. No increase in lung cancer risk was observed, although from the doses received it might have been expected. An increased risk of childhood cancer has also been attributed to diagnostic radiation exposures *in utero*.

As is the case for other carcinogenic agents, occupational exposures are a valuable source of information.[20,21] Studies of uranium miners and other underground miners have been a particularly important source of information on cancer risks associated with exposure to radon. The analysis of nuclear workers has also provided and will continue to provide an important source of information of risks after chronic prolonged exposure to ionizing radiation. Likewise, because a major source of exposure is from medical procedures, medical workers, including radiologists, technicians, and nurses, are being studied intensively.[22]

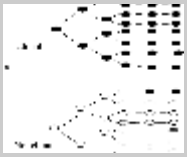
**10. Write the Mechanisms of Radiation-Induced Cancer.**

Although radiation-induced tumorigenesis in experimental animals and in humans has been the subject of intense study for many years, until recently direct evidence with respect to underlying mechanisms of radiation carcinogenesis has been lacking and models have relied heavily on indirect inferential data. For example, it has been suggested for many years that low LET ionizing radiation acts principally on early events; that is, radiation's primary effect is as a tumor-initiating agent. This is based on several observations. First, the generally increased sensitivity of animals and humans to the tumorigenic effects of ionizing radiation at young ages is more consistent with effects on tumor initiation than with promotional effects that accelerate the development of preexisting neoplasms.[16,56–59] Second, experimental animal data on skin cancer development specifically designed to examine the influence of radiation on different stages of tumorigenesis show radiation to only weakly promote the development and progression of chemically initiated tumors while having significant initiating activity.[60] Finally, the observation in humans and animals that single acute doses of low LET radiation are sufficient to produce a dose-dependent increase in cancer risk and that in quantitative animal studies dose protraction decreases that risk also supports the view that the major effect of radiation is on early events in the carcinogenic process.[16,31] Although this inference appears to be logically based, until recently there has been no direct evidence.

Advances in cell biology, cytogenetics, molecular biology, and mouse genetics over the past several years have made it possible to more directly investigate events in the tumorigenic process after radiation exposure. Such studies are providing valuable insights into mechanisms as well as a better understanding of potential risks by linking cell and molecular effects directly to the tumorigenic process. Of particular importance in this regard have been animal studies using newly developed models in inbred mice and rats and in genetically engineered rodents. Quantitative studies using mouse and rat models for radiation-induced mammary cancer and for thyroid cancer in rats have provided direct evidence to indicate that the principal effects of ionizing radiation are on early events.[60–69] Cellular, cytogenetic, and molecular data for acute myeloid leukemia, intestinal tumors, and mammary tumors also provide evidence for early monoclonal development of radiation-induced preneoplasms, implying an initial, single cell target.[65,70,71] Cytogenetic and molecular studies on the induction of acute myeloid leukemia and mammary tumors in inbred mouse strains and a variety of tumors in transgenic mouse models have provided more specific information on the potential nature of these early events.[40,41,71–74] These studies provide direct support for the view that the critical radiation-associated events in the tumorigenic process are predominantly early events involving DNA losses targeting specific genomic regions harboring critical genes. Because many of the radiation-associated DNA loss events in these tumorigenesis models involve large chromosomal regions within the genome, it can be concluded that mechanisms for radiation-induced chromosome aberration induction involving DNA DSB induction and postirradiation error-prone nonhomologous end-joining repair appear to play a critical role in the pathogenesis of cancer. More recently, experimental studies have questioned whether the initiating events produced by radiation are direct chromosomal or mutational effects or whether the mutations and chromosomal rearrangements result indirectly as a consequence of genomic instability induced by the radiation exposure.[75–79]

It is well known that the development of tumors is frequently accompanied by the acquisition of genomic instability phenotypes that serve to promote the mutational evolution involved in neoplastic progression. This form of genomic instability is increasingly well understood, and many of the responsible tumor gene mutations have been identified.[80] This instability, however, differs from radiation-induced genomic instability described during the last decade.[78] Over this time, evidence has accumulated that, under certain experimental conditions, the progeny of cells surviving radiation appear to express new chromosomal and gene mutations over many postirradiation cell generations. The observation of genomic instability induced by radiation is relatively recent. It has been generally believed that all mutagenic and cytogenetic effects of ionizing radiation occurred in the first few cell divisions. It has now been shown that increased mutation rates and new cytogenetic damage can occur in a large proportion of the progeny of irradiated cells many generations later (**Fig. 10-2**). What may be unique about radiation-induced instability with respect to its potential role in tumorigenesis is that, because of the high frequencies of instability observed after radiation exposure (10% to 50% of irradiated cells), such instability would not appear to be a result of radiation-induced mutations in a specific

gene or family of genes.[78,81,82] On the basis of data discussed earlier in Mechanisms of Radiation-Induced Cancer on radiation-induced genomic instability and the previously reported high frequency of neoplastic cell transformation,[78,83] it has been suggested that such can serve to destabilize the genomes of a substantial fraction of the progeny of irradiated cells and that it is the elevated postirradiation mutation rates in cell progeny rather than gene-specific initial mutations that act to drive radiation tumorigenesis[78] (**Fig. 10-3**).



View Figure

Figure 10-2
Comparison of mutations and cytogenetic damage as a result of direct radiation damage or radiation-induced genomic instability. **A:** Directly induced mutations or chromosome aberrations are passed to all progeny (i.e., the alterations are clonal). **B:** In contrast, mutations or aberrations arising as a result of radiation-induced instability arise in the progeny of irradiated cells that have not been directly irradiated. This leads to a nonclonal, or mosaic, pattern. Because the alterations arise in the progeny of the cells, another characteristic of instability is that the mutational or clastogenic effects are delayed with respect to the radiation exposure.

View Figure

Figure 10-3
Proposed role of radiation-induced cytogenetic instability in radiation-induced cancer. Radiation exposure induces instability in a high percentage of the progeny of the irradiated cells (*striped* cells represent unstable progeny). As a result of this instability, the rate of chromosome aberrations and mutations is increased. Some mutations result in cell death (black) or slow-growing cells (gray), whereas some occur in critical genes involved in the regulation of cell growth and differentiation or in the maintenance of the stability of the genome. These mutations result in the persistence and amplification of genomic instability or in cells with a growth advantage. As these cells continue to develop into a clonal outgrowth, further mutations result in additional cellular changes, which lead to death or progression toward neoplasia. Cells with other patterns represent cells with specific mutations or sets of mutations that arise subsequent to radiation exposure.

One form of instability that appears to be of particular relevance to tumorigenesis is that associated with telomere dysfunction. Such dysfunction can be manifest in several forms. Telomeric repeat sequences $(TTAGGG)_n$ cap the ends of mammalian chromosomes and serve to protect against replicative erosion and chromosomal fusion; in normal human cells in culture, telomere shortening and instability are natural features of replicative cell senescence. In often degenerate forms, telomeric repeats are also found in subtelomeric and interstitial chromosomal locations, and there is some evidence that these loci may act as sites at which radiation-induced

and other forms of genomic damage are preferentially resolved. Good evidence has also been shown that telomeric instability is a recurrent feature of tumorigenic development. Of particular relevance to the question of unstable translocation junctions are the so-called segmental jumping translocations, which have been well characterized in spontaneously arising human leukemias. With respect to radiation-induced leukemia, detailed cytogenetic analyses suggest an excess of complex aberrations and segmental jumping translocations in leukemias arising at old ages in high-dose–exposed A-bomb survivors. Telomeric instability at radiation-associated deletion/translocation breakpoints in mouse myeloid leukemia has also been reported, but this is not a general characteristic of these tumor-associated events. Interestingly, excess spontaneous telomeric instability is often found to be associated with DNA repair or damage response deficiency.[84]

Evidence for the involvement of telomeric sequences in the pathogenesis of at least some forms of radiation-induced instability comes from several laboratories. Early studies on the postirradiation development of chromosomal instability in *in vitro* passaged human diploid fibroblasts were among the first to suggest a link between telomeres and instability. Initial studies using this *in vitro* model were suggestive of instability effects in a high proportion of irradiated cells. Subsequent studies by the same research group have served to address issues related to the pathogenesis of instability as well as to its frequency. Detailed cytogenetic analyses suggested that passage-dependent instability in cultured human fibroblasts primarily represented telomeric events expressed in cell clones naturally selected by growth rate during passage. Overall, the data obtained can be interpreted as initial radiation exposure bringing forward in time the natural process of clonal telomeric instability associated with cell senescence and telomere shortening. Equally important is the suggestion that selection processes lead to an overestimate with respect to the frequency of induction of instability by radiation. Whether selection processes impact estimates of the frequency of instability in other systems remains to be addressed.

A different form of postirradiation telomere-associated instability is expressed in a hamster-human hybrid cell system in which, in some clones, chromosomal instability is persistently expressed at translocations that have telomeric sequences at their junction. Similar unstable structures have been observed in nonirradiated hamster cells undergoing gene amplification. Such data suggest that radiation is inducing genomic structures that enhance the natural expression of instability. A number of other reports have also suggested that radiation-associated chromosomal exchange can lead to the formation of unstable junctions that undergo secondary change leading to the formation of complex chromosomal aberrations.

Although the role of radiation-induced genomic instability in radiation-induced cancer is still a matter of investigation, several observations provide a framework for its potential role in cancer development after radiation exposure. In the case of radiation-associated persistent telomeric rearrangement and unstable chromosome translocation junctions, a strong case can be

made that a certain fraction of misrepaired genomic damage after radiation may be prone to ongoing secondary change in clonal progeny. Because there is evidence that such secondary genomic rearrangement can be a normal component of tumor development, it is reasonable to assume that instability of this type would be involved in the pathogenesis of some radiation-associated tumors. The question of whether it plays a major role and for which tumor types is unclear. The genetic evidence from mouse mammary studies that postirradiation instability can associate with mammary tumor development supports a role for genomic instability in this system. Thus, in certain genetic settings, such as individuals harboring specific types of DNA repair deficiencies, a role for postirradiation instability in tumorigenesis appears reasonable.

Of interest, recent data in the SCID and in the BALB/c mouse, both of which have defects in DNA-PKcs (catalytic subunit of DNA-dependent protein kinase), suggest that telomeric instability may be the underlying mechanism for the induction of instability, with the resulting cytogenetic instability playing an important role in early carcinogenic events in the mouse mammary carcinogenesis model discussed above. In particular, it appears that dysfunctional telomeres have a propensity to interact with sites of radiation-induced DSBs, increasing the probability of the misrepair.[84,95,96] It would be predicted that mechanisms involving DNA DSB and telomeric sequence interactions would be particularly important at low doses at which DNA DSBs are in relatively low abundance. This appears to be consistent with observations that instability is induced in a dose-dependent manner at radiation below 50 cGy but no dose dependence is observed at higher doses, at which the response appears to plateau. Importantly, the emerging evidence suggests a role for radiation-induced DSBs in the induction of instability and provides a mechanistic link between DSBs, chromosome aberrations, and cancer not unlike that for more directly induced effects.

It is well known that the probability that individual initiated cells will progress to become tumors can be modulated by interactions with surrounding cell and tissue components as well as systemic host factors.[97] Studies have also provided evidence that radiation can influence these cell–cell, cell–tissue, and host factor interactions. Renewed interest has been shown in these effects as a result of studies that have begun to identify potential underlying mechanisms involved in modulation of tumorigenic progression and expression. Research in this area will be extremely important in understanding the overall processes involved in neoplastic development, but a clear understanding of their potential impact on radiation-induced cancer remains to be determined.

***11. Write the exposure of Sunlight leads to Skin Cancer.***

The evidence that UV light is responsible for a large proportion of skin cancer is considerable. Skin cancer is more frequent in populations in regions with high ambient solar radiation and in individuals exposed to sunlight as a result of their occupations (e.g., farmers). Nonmelanoma skin cancer is most frequent in sites that are the most exposed to sunlight, such as

the head, neck, and arms. Pigmented skin is less susceptible to nonmelanoma skin cancer, and lack of pigment increases risk. In the United States, the incidence of basal cell carcinoma and squamous cell carcinoma increase by 2% to 3% for every 1% increase in ambient UV light, and malignant melanoma increases by 0.5% to 1.0%.[107] On a worldwide level, the incidence of skin cancer is extremely dependent on latitude, which directly equates with level of UV light. Specifically, it is seen that the closer to the equator one lives, the greater is the risk. This is exacerbated in countries, such as Australia, in which a large proportion of the population is lightly pigmented.[108] In addition to family history, the known risk factors for skin cancer are all related to increased propensity for damage from UV light from the sun: light pigmentation, inability to tan, propensity to burn, history of sunburns, and/or cumulative exposure to UV radiation.

***12. Write about Asbestos Exposure in Cancer Risk.***

The most common form of cancer associated with asbestos exposure is malignant mesothelioma, but the risk of bronchogenic cancer is also significantly elevated. Although it is lung cancer that is generally associated with asbestos exposure, other cancers that have been reported to occur at an increased frequency include cancers of the larynx, oropharynx, kidney, esophagus, and gallbladder/bile duct.

Because it is very rare, it has been relatively easy to link the risk of mesothelioma with asbestos exposure. Occupational exposure can be linked to 50% to 80% of all patients with malignant mesotheliomas.[125] In a study of tile workers exposed to asbestos over a 50-year period, it was found that the incidence of mesothelioma was as high as 2%. A study of a large group of asbestos insulation workers found that mesothelioma was responsible for approximately 8% of all deaths. The latent period between exposure and development of malignant mesothelioma is usually quite long, typically 30 to 40 years.[125] Approximately half of all malignant mesotheliomas are epithelioid, and the other half are sarcomatoid, mesenchymal, or mixed.

A link between asbestos and bronchogenic carcinomas was first reported in the 1930s and has been subsequently confirmed in several investigations.[125,127] Although the vast majority of bronchogenic carcinomas are related to smoking, it has been estimated that from 3% to 17% of such cancers are from occupational exposure, including asbestos. Asbestos and smoking appear to interact in a multiplicative manner, and the risk is decreased when exposure to either agent is stopped. Whereas the majority of smoking-related tumors are squamous cell carcinomas originating in the upper lobes of the lung, those associated with asbestos are more often adenocarcinomas located in the lower lobes. The asbestos-related tumors are also often associated with areas of fibrosis.

*Mechanisms*

Asbestos fibers are cytotoxic and genotoxic.[128] They have been shown to induce DNA damage, including DSBs, mutations, and chromosomal damage. Evidence also indicates that asbestos fibers can impair mitosis and chromosomal segregation, which can result in aneuploidy. The majority of these effects are believed to be due to oxidoreductive processes that result in the formation of reactive oxygen species. Support for this view comes from studies showing that the amount of damage induced is increased if iron is present in the chemical structure of the fibers. Besides the direct induction of reactive oxygen species, these effects may also be indirectly induced as a result of phagocytosis of the asbestos fibers. Fibers also tend to induce inflammatory response, resulting in the release of cytokines. Such inflammatory responses may facilitate the growth, clonal selection, and expansion of initiated cells.

Loss of one copy of chromosome 22 is one of the most common chromosomal alterations in malignant melanoma. A wide range of other changes have also been reported, including deletions in chromosomes 1p, 3p, 6q, 9q, 13q, 15, and 22q. Analyses of tumors have found some common features. First, deletions of CDKN2A, located on chromosome 9p, have been observed. Second, mutations in NF2 (the neurofibromatosis type 2 gene, located on chromosome 22q) have been found; such mutations are often coupled with the loss of the normal NF2 allele as a result of one copy of chromosome 22.

## UNIT – III

## PART – A

**1. Give example of DNA Virus.**

Adenoviruses - Human Adenoviruses
Hepadnaviruses - Hepatitis B virus
Herpes Viruses - Epstein-Barr virus
Papillomaviruses - Human papillmavirus

**2. Give example of RNA Virus.**

Retroviruses - Rous sarcoma Virus
Flaviviruses - Hepatitis C Virus
Reoviruses - Wound tumor virus

**3. What is oncogenesis.?**

Oncogene abnormality appears to be the universal feature of cancer. Both viral and cellular oncogenes can transform cells, causing malignant tumours under certain conditions. Harmless cellular proto oncogenes can convert to true oncogenes, resulting in the formation of tumours is Called oncogenesis.

**4. Write the multi step development of colorectal cancer.**

Changes in the tumor parallel three genetic alterations in tumor suppressor genes and the mutation of one oncogene. In each of four cases, the mutations are in genes that normally regulate cell growth or division, causing a breakdown in regulation.

1 Normal colon epithelial cells - Loss of TSG from chromosome 5
2 Small polyp - cell division continous
3 Class I adenoma (benign) - Activation of ras oncogene on chromosome 12
4 Class II adenoma (still benign) - loss of DCC TSG from chromosome 18
5 Class III adenoma (still benign) - loss of p53 TSG from chromosome 17
6 Carcinoma (now malignant) - more genetic alterations
7 Metastasis

**5. What are the general schemes for accumulating cancerous mutations?**

Cells can accumulate cancerous mutations by at least two different means.
Viruses carrying oncogenes can infect cells, after which the viral oncogenes may Integrated ino the cells chromosomes. If these integrated oncogenes are expressed, they will transform the cell alternatively. Mutations in proto-oncogenes that can result in oncogenes that can cause transformation. Such mutations may arise spontaneously, through exposure to radiation or chemical carcinogens, or as a result of chromosomal rearrangements.

**6. Give any two mechanisms by which proto oncogene are converted to oncogenes.**

Harmless cellular proto oncogenes can convert to true oncogenes, resulting in the formation of tumours (oncogenesis). The mechanism included 1. Point mutation 2. Proviral insertion 3. Deletion 4. Translocation & 5. Amplification

**7. Define Oncogene.**

A oncogene is a gene that

- when mutated or
- expressed at abnormally – high levels

Contributes to converting a normal cell into a cancer cell. Cancer cells are cells that are engaged in uncontrolled mitosis.

**8. Which proteins of Adeno virus are responsible for cellular transformation?**

The W1A proteins & E1B.

E1A proteins are transactivates proteins that greatly stimulate transcription from nearly all adeno virus promoters. They stimulate transcription from genes transcribed by both RNA Polymerase III (VAI and VAII) and RNA Polymerase II (all other genes). The E1B region has transforming function, causing morphological changes in cells. The Adenovirus early proteins are onco proteins that bring about transformation of host cells by adenoviruses.

**9. Write any two RNA viruses and its associated cancer.**

All RNA tumour causing viruses belong to the retrovirus family example of viruses are the Rous sarcoma virus, Avian Leukemia virus mouse mammary tumour virus.

Tumour causing retroviruses are also grouped into two general classes, non-defective viruses and acute transforming viruses, on the basis of how they cause tumours.

(i) Non defective virus do not carry oncogene in their genome, but induce tumours by their ability to activate a cellular proto-oncogene. Eg. FLV, MMTV. Acute transforming viruses have acquired an oncogene, which is not present in the ancestral non transforming virus.

**10. Define : Telomere**

Special tandemly arrange, guanine-rich, repeated sequences that prevent loss of DNA at te end of a DNA strand during chromosome replication. So are required for faithful replication of the DNA in a chromosome

**11. What is the role of Telomeric sequence?**

Special sequence on the ends of DNA strands that are required for synthesis of the terminal segments of the lagging strand. Telomeric sequence are present on the end of chromosomes are used in the construction of yeast Artificial Chromosome (YAC)

## PART – B

**1. Write the comparison of DNA and RNA oncogenic viruses.**

| DNA tumor viruses | RNA tumor viruses |
|---|---|
| 1. Examples of DNA viruses are the hepatitis B viruses, SV 40 & polyoma viruses, papilloma viruses, Adeno viruses and herpes virus. | RNA tumor viruses are all retroviruses. |
| 2. Some cause protective infection in permissible cells of one species producing progeny viruses and tumors in another species. (non permissive cells) | Usually cause tumors in most species in which they cause productive infection. |
| 3. Infection of most non permissive cells is abortive, with very few cells becoming cancerous. | Most permissive cells on infection become tumor cells. |
| 4. There is no production of progeny virions, since viral genes are lost when the prophase integrates into the host cell genome. | For production of virions, integration of viral DNA is essential. |
| 5. Although DNA tumour viruses are oncogenic, they do not carry oncogenes. | RNA tumour viruses transform cells through oncogenes in the viral genome. |
| 6. DNA Tumour viruses transform cells through the action of genes that are essential parts of the viral genome oncogenes of some of the viruses encode essential early proteins for viral replication. | RNA tumor viruses transform cells through oncogenes in the viral genome that are not essential for the production of progeny virions. |
| 7. Virus – Induced protein kinases are not found in DNA tumour viruses. | Some RNA tumours viruses produce virus – induced protein kinases. |

**2. What is Retrovirus Infectious Cycle?**

The retrovirus infectious cycle consist of two stages, the early and late stages. The essential feature of early stage of retrovirus replication is the conversion of the SS RNA genome into the ds DNA copy. During the early stage, the virus containing the ss RNA genome within its core attaches to a host cell. Binding of the virus to the host cell receptor takes place through the surface protein (su) or the spike. The core of the virus passes through the host cell membrane into the cytoplasm (penetration and uncoating). The enzyme reverse transcriptase (RT) synthesizes DNA using the RNA genome as a template. This is converted into double-stranded viral DNA (ds DNA) which is then transported to the nucleus. Here it is integrated into the host genome by the help of the integration protein (IN). Thus the early stage is dependent on the Pol gene products RT and IN brought with the viral core.

During the late stage, the integrated DNA expresses itself by transcription and translation of the viral genetic information, providing components of the progeny virus particles. The late stage depends primarily on host cell functions, by using host ribosomes, initiation factors, t RNAs and RNA polymerase II.

The replication cycle of a retrovirus can be divided into three stages the pre-integrative stage (a-e), the integrative stage (f) and the post integrative stage (g-j). The major event in the pre-integrative stage is reverse transcription, during which the SSRNA viral genome is copied into ds DNA. During the integrative stage the ds DNA copy is inserted into the host cell genome. In the post integrative stage, transcription of the viral genome to form mRNA, translation of the MRNA to form viral proteins and assembly of progeny viral particles takes place.

a. Binding of the virus particle to the host cell and membrane fusion.
b. Uncoating of the viral capsid.
c. Synthesis of complementary (-) DNA strand on the RNA Template.
d. Degradation of RNA strand by RNase activity of reverse transcriptase.
e. Synthesis of the second (+) DNA strand by reverse transcriptase.
f. Integration of ds DNA into the host – cell chromosomal DNA.
g. Transcription of viral genes into RNA by host cell RNAP II.
h. Translation of mRNAs into viral polyproteins.
i. Assembly and budding of retrovirus particles.

**3. What is a viral transactivator protein?**

TransActivation is the process of which are or more proteins encoded by the viral genome greatly stimulate transcription from all promoters. The mechanism of transactivation is of interest both from point of view of transcription stimulation and oncogenic transformation. Transactivation proteins have been described for many different viruses. Viral proteins that enhance the rate of transcription or interact with DNA sequences or proteins with promoter domains, and function at the level of transcription can be divided inhto two groups on the basis of whether or not they exhibit sequence – specific DNA binding.

Transactivation by transactivator proteins is not restricted to the genes of the virus encoding the proteins. Genes of other viruses can also be transactivated. (i) The adenovirus E1A protein transactivates promoters of all adenovirus genes. (ii) The pseudorabies immediate early (IE) protein, a 180 KDa nuclear phosphoprotein, is analogus to the adenovirus E1A protein. It is required for transactivation of early genes of the pseudorabies virus. It can also induce transcription from adenovirus early genes at even higher rates than E1A. (iii) The HTLV type II X protein transactivates promoters from HTLV genes as well as adenovirus promoters. It can also transactivate adenovirus genes transcribed by RNAP III. (iv) The SC4o.

**4. Explain about Nuclear onco proteins.**

Several proto-oncogenes encode transcription factors which regulate the transcription of genes activated in growing cells. Progression through the cell cycle requires several genes to be activated (or repressed). The proteins encoded by these genes regulate cell growth and division.

Therefore, any alterations in the proteins that control the expression of these genes would disturb the normal growth patterns of the cell. The proto-oncogenes may have corresponding oncogenes that encode oncoproteins that may result in unregulated cell division. Several proto-oncogenes coding for transcription factors have corresponding viral oncogene (V-onc). These include myc, fos, jun, erbA, rel and myb.

The myc gene encodes the myc protein which has a role in the promotion of cell cycle progression. Cells that are not actively growing and dividing enter a shunt stage of the cell cycle, referred to as $G_0$. When the cell is stimulated to re-enter the cell cycle, the myc protein is one of the first to appear. The protein plays a role in the continuous proliferation of the cells, as is shown by the fact that it is synthesized as long as the cells continue to divide. Experimental blocking of myc gene expression by antisense oligonucleotides results in the arrest of progression of the cell cycle through $G_1$. The myc proteins function as transcriptional factors that are regulated by mitogenic stimuli. Abnormal expression of myc oncogenes can lead to the development of a variety of human tumors. In human cancers, myc oncogenes often undergo amplification and rearrangement by chronosomal inversion or translocation. Over expression of myc may result in continuous cell proliferation by overriding the action of tumour suppressor gene products such at the Rb and P53 proteins.

The products of the C-fos and C-Jun genes are the Fos and the Jun proteins.

The products of the C-fos and C-jun genes are the Fos and the Jun proteins, respectively. Transcription factor AP-1 is heterodimes of one subunit of Fos, and one of Jun. If activates the transcription of a number of target genes in cells stimulated by growth factors. The oncogene V-fos and V-jun encode the oncoproteins V-Fos and V-Jun, respectively.

**5. Write the mechanism of converting proto-oncogenes to oncogenes.**

**Mechanisms converting proto oncogenes to oncogenes**

Oncogene abnormality appears to be the universal feature of cancer. Both viral and cellular oncogenes can transform cells, causing malignant tumours under certain conditions. Harmless cellular proto oncogenes can convert to true oncogenes, resulting in the formation of tumours (oncogenesis). The basic question about the induction of cancer is: how do cellular genes fulfilling normal basic regulatory functions become transformed into oncogenes? There are several different mechanisms for this phenomenon. All involve the overexpression of a normal cellular proto-oncogene or the mutational alteration of proto oncogene into an oncogene, thus affecting the structure and function of the encoded protein, or both mechanisms. The mechanisms include (i) point mutations, (ii) Proviral insertion, (iii) deletion, (iv) translocation and amplification

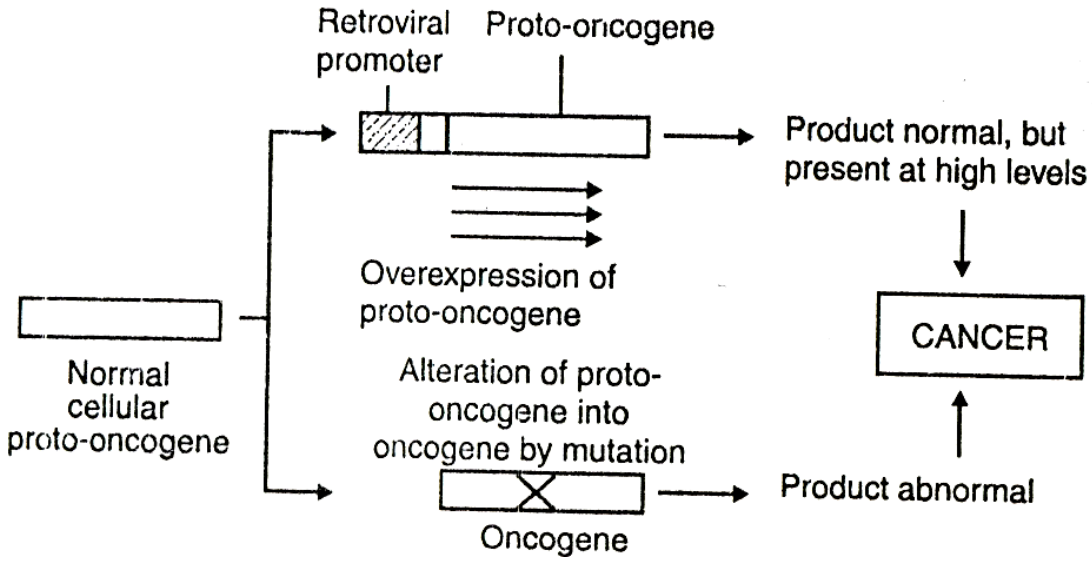
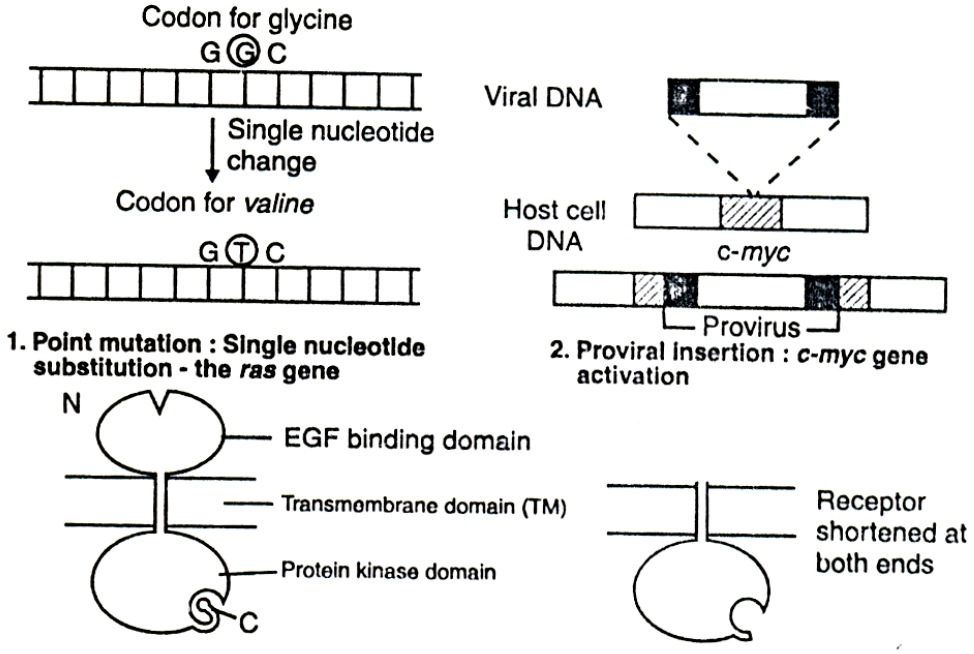



**Fig. Cancer caused by overexpression of cellular proto-oncogene or by alteration of proto-oncogene into oncogene by mutation**

(ii) proviral insertion, (iii) deletion, (iv) translocation, and (v) amplification (Fig. 5.2)



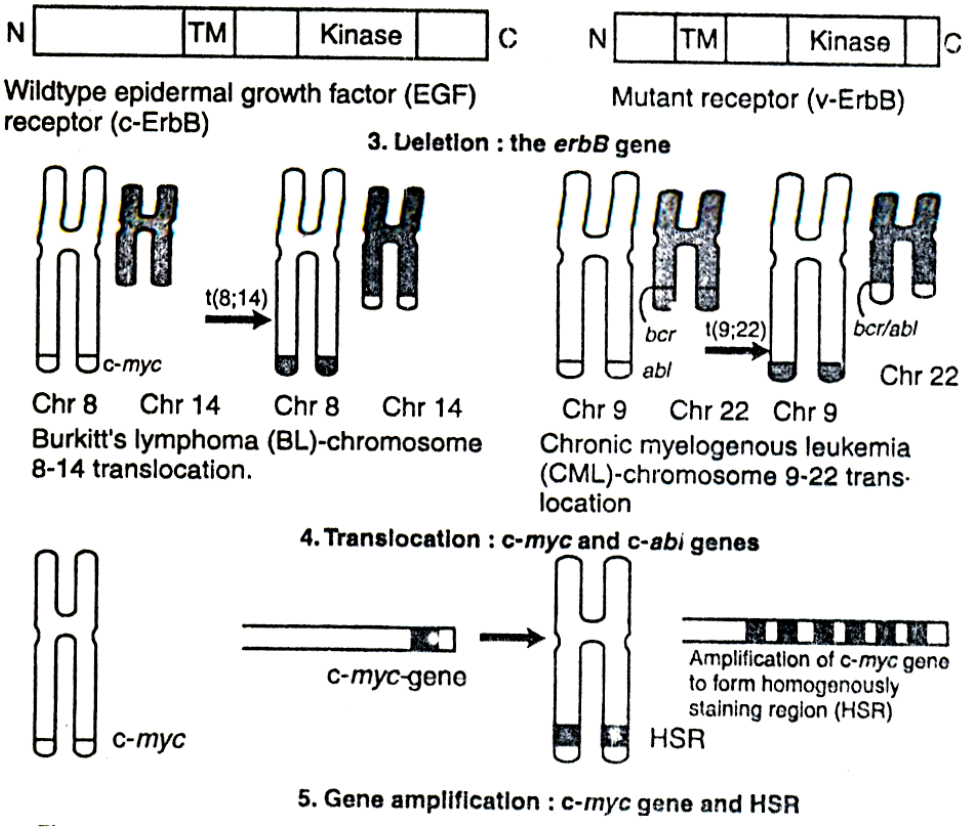



**Fig. Molecular mechanisms of oncogenesis**

**1. Point mutations : single nucleotide substitution-the ras oncogene**

A point mutation is any gene mutation arising from the substitution, addition or deletion of one or a few bases initially. Since cellular proto oncogenes resemble viral oncogenes, a few mutations can cause their conversion into oncogenes. The ras oncogene family consists of three active c-ras genes, H-ras, K-ras and N-ras in both humans and rat. The H-ras and K-ras cellular oncogenes have v-ras counterparts carried by the Harvey and Kirsten strains of murine sarcoma viruses, respectively. The ras family of genes encodes closely related proteins about 21 kDa in size, and hence called p21. These proteins are membrane-associated proteins that bind GTP, hence their name G proteins. They plan an important role in cellular signal transduction.

Normal bladder and lung cells contain the harmless c-H-ras proto oncogenes gene. A point mutation resulting in a single amino acid substitution can convert the cellular proto-oncogene into an oncogene. The c-H-ras proto-oncogene codes for the c-Ras normal protein with glycine at position 12 and glutamine in position 61 (wildtype positions). A point mutation in the codon for amino acid 12 transforms the c-H ras proto-oncogene into the v-H-ras oncogene of human bladder carcinoma. This mutation (G→T transversion) involves a substitution of the amino acid glycine at position 12 with valine. Similarly, a glutamine to leucine substitution at position 61 results in lung carcinoma.

**Table : Mutational changes in c-H-ras genes in human tumour cells**

| Gene | Amino acid at position of p21 | | Type of tumour |
|---|---|---|---|
| | 12 | 61 | |
| Proto-oncogene (c-H-ras) | Glycine | Glytamine | Wildtype positions |
| Oncogene (V-H-ras) | Valine | Glutamine | Bladder carcinoma |
| Oncogene (v-H-ras) | Glycine | Leucine | Lung carcinoma |

**2. Proviral insertions : C-myc gene activation**

Tumour viruses have genomes of either DNA (e.g. adenovirus and Simian Virus 40) or RNA (all retroviruses). Tumour viruses can transform cells because they contain genes that direct the transformation process. These viral oncogenes (v-onc) in many cases are homologous to cellular proto-oncogenes (c-onc) found in normal uninfected human cells. Oncogenes do not appear to have originated in viruses, but were picked up by the viruses from cells.

The majority of retroviruses, are non-tumorigenic, i.e. they do not cause cancers. They simply infect a cell and produce a persistent infection in which the infected cell continually produce viruses. The genome of such viruses contains only three genes, gag, pol and env (for envelope surface proteins). On each side of the genome is a direct repeat called long terminal repeat (LTR). An example of a non-tumorigenic virus is the avian leucosis virus (ALV). Retroviruses also include tumorigenic viruses, whose infection results in the conversion of the cell into a cancer cell. An example of a tumorigenic retrovirus is the Rous sarcoma virus (RSV), also called the avian sarcoma virus (ASV). This was shown to cause connective tissue cancer in chickens by Peyton Rous in 1911. The genome of RSV contains an additional gene, src, for a transforming protein that causes cancer.

The capture of a cellular gene by a retrovirus generates a transducing virus. Figure illustrates a hypothesis for the conversion of a cellular oncogene (c-onc) to a viral oncogene (v-onc). The provirus DNA consists of gag, pol and env genes flanked by LTRs, but lacks an oncogene. The cellular oncogene is shown consisting of three exons (E1, E2 and E3) and two introns (11 and 12). When the retrovirus infects the cell, recombination may take place between the retroviral DNA (provirus) and the cellular proto-oncogene. The recombinant provirus transcribes a combined RNA molecule consisting of viral RNA with proto-oncogene RNA. During the processing of the combined RNA, splicing takes place, during which the introns are excised and the exons ligated to form the viral oncogene (v-onc). The resulting RNA is packaged into a retrovirus particle, which can infect a new cell.

In the example given above, the entire gag-pol-env region is retained in the infective retrovirus particle. The particle is therefore capable of carrying out replication, since it has all the viral functions needed for reproduction. Usually, however, one or more viral genes may be lost during recombination with cellular sequences. The resulting viruses are unable to replicate, and are called defective interfering (DI) virus particles. They can replicate only in the presence of non-deleted helper viruses which may coinfect the cell and provide the functions that were lost with the deletion of genes during recombination.

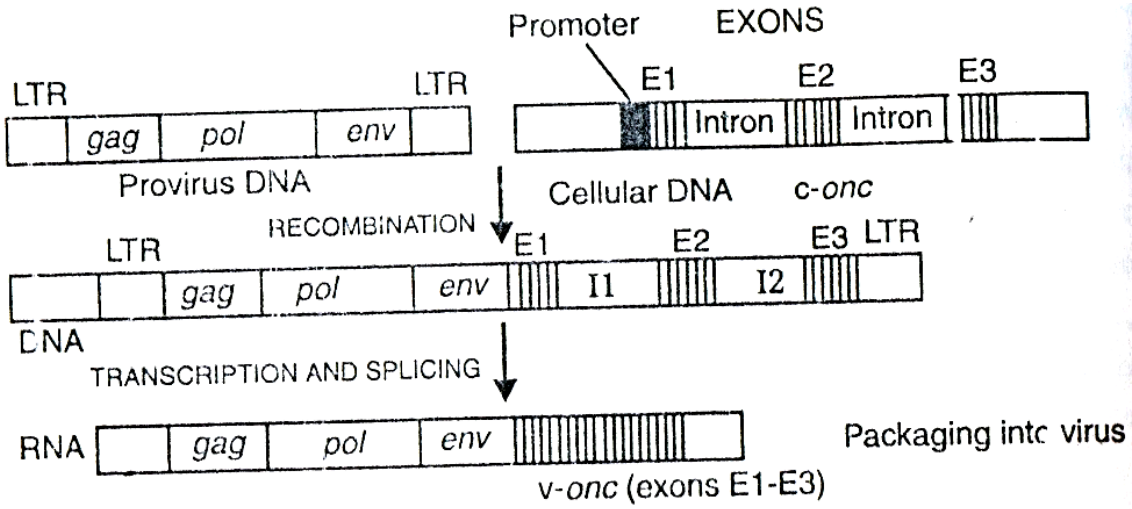



**Fig. Conversion of a cellular oncogene (c-onc) with introns to a viral oncogene (v-onc) without introns**

Though AVL completely lacks an oncogene, it can cause tumours by activating cellular proto-oncogenes. The ALV provirus inserts next to a cellular oncogene known as c-myc, which is localized at band 124 on human chromosome-8. The c-myc gene consists of three exons separated bytwo introns. The first exon does not take part in the coding of a functional c-myc protein, and is untranslated. It appears to have a regulatory role. The c-myc gene has a unique promoter with two different startsites, P1 and P2. The c-myc gene can change its expression without changing its coding sequence. Mechanisms of gene activation include proviral insertion, chromosomal translocation and gene amplification. The c-myc gene promotes growth of tumour cells by quantitative increase in the level of protein synthesized rather than by qualitative changes in the protein.

The activation of the c-myc gene byproviral insertion will now be considered. ALV proviral DNA has been found to integrate close to or within the c-myc gene. Insertions of ALV DNA at the c-myc locus activate the gene and generate tumour cells. Three types of insertions have been found.

**(i) Insertion in intron 1**, between exons E1 and E2, has a promoter effect. The right hand LTR, which normally functions as a terminator, now acts as an efficient promoter. The retroviral transcript continues into the read-through transcript of exons E2 and E3. Because of the efficient LTR promoter, 20-50 fold increased level of protein as synthesized, resulting in tumour formation.

**(ii) Insertion in intron 1,** but in the reverse direction, has an enhancer effect. In this orientation, the LTR does not function as a promoter but provides an enhancer that acts on an upstream sequence that resembles a promoter.

**(iii) Insertion 3′ (downstream)** of exon 3 has an enhancer effect through the LTR. Transcription is initiated at the usual c-myc promoter and enhanced by the LTR, which also serves to terminate he transcript.

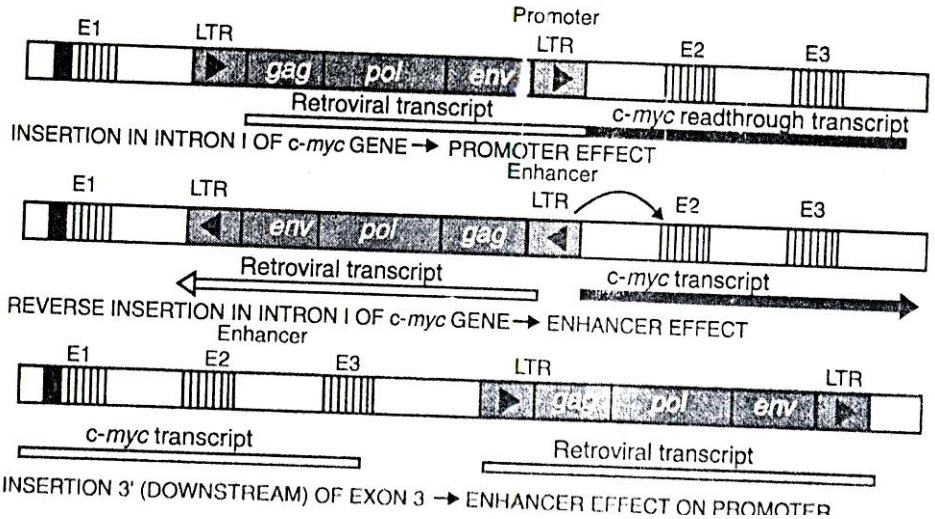



**Fig. Activation of c-myc gene by a proviral insertion of ALV genome**

In all the three cases mentioned above, the c-myc gene is transcribes only weakly, if at all, before insertion of viral DNA. After ALV provirus insertion, however, c-myc transcription is greatly stimulated by the promoter or enhancer effects of the LTR, contributing to the transformation process. Many tumours arising after retrovirus infection contain mutant cellular oncogenes activated by proviral insertions. Such insertions are believed to be the primary events in the formation of tumours. Their effect is to stimulate the expression of the target gene by providing strong promoters or enhancers located within the viral DNA/RNA. They increase the expression of the gene, interfering with the normal growth control. Transformation in retroviruses is usually caused by increased levels of normal cellular proteins, rather than by introducing new viral proteins into the cell. About 30 types of acute transforming viruses are known.

**3. Deletion : the erbBgene**

Tumours may arise by the removal (deletion) of a part of the coding sequence. The erbB gene encodes the epidermal growth factor (EGF) receptor, which is a large glycoprotein with intrinsic kinase activity. Binding of EGF to its receptor makes the kinase more active. This results in the receptor as well as other cellular proteins becoming rapidly phosphorylated. In the erbB gene, deletion of the extracellular ligand-binding domain and loss of the C-terminus is the oncogenic sequence change producing oncogenecity. The truncated mutant EGF receptor loses its EGF-binding capacity and also its normal controls. It therefore constantly signals the cell to divide, even in the absence of EGF, leading to transformation.

**4. Translocation: c-myc and c-abl genes**

Translocation is a chromosomal structural change in which there is a change in the position of a chromosome segment within the chromosome complement. Translocations result in fused elements from different chromosomes, and are generally a common feature of cancer. Cellular proto-oncogenes can be translocated from a non-expressed silent region into one that is actively transcribed. There are two different mechanisms of oncogene activation, one exemplified by Burkitt's lymphoma (BL) and the other by chronic meylogenous leukemia (CML) which results in the Philadelphia chromosome.

**(i) Burkitt lymphoma results from 8;14 translocation.** A t(8;14) translocation converts the c-myc proto-oncogene to an oncogene, resulting in Burkitt's lymphoma, a human cancer. In this translocation, a segment of chromosome 8 bearing the c-myc proto-oncogene is translocated to the part of chromosome 14 carrying a cluster of immunoglobin (antibody) heavy chain locus (IgH). Antibody genes normally undergo a translocation in which a variable region (V) is juxtransposed with a constant region (C). In the t(8;12) translocation, instead of the V region, the myc gene is translocated to the C region of the heavy chain. During this translocation, the myc gene loses the first of its three exons. Though this does not affect the protein product of the gene (exon 1 does not code any amino acids), it affects the regulation of the gene. Translocation of the myc gene from its normal location in a silent region close to the very actively transcribed IgH gene has an activating effect on the myc gene. This results in increased protein synthesis, which in turn results in Burkitt's lymphoma.

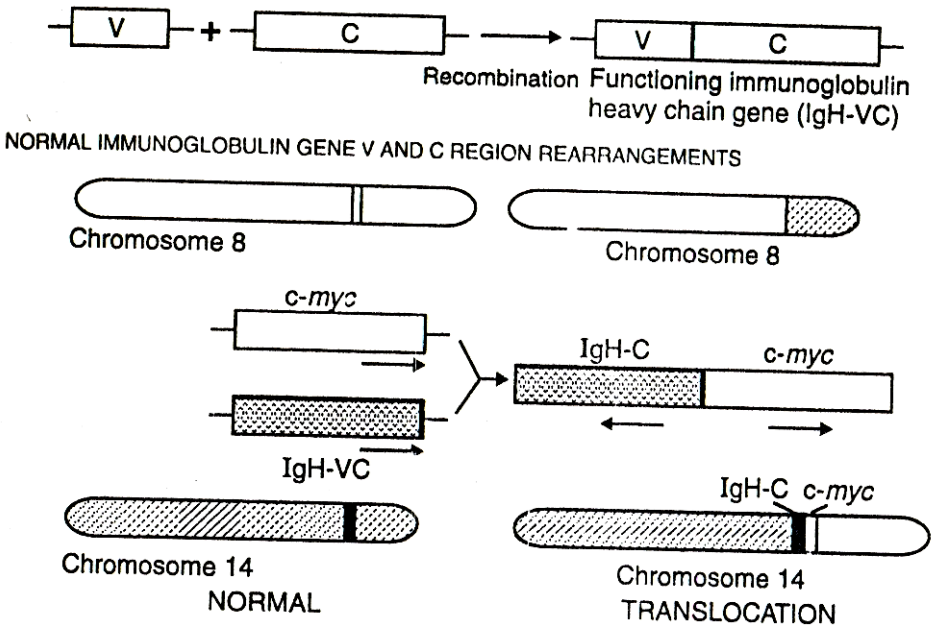



**Fig. Translocation t(8;14) in Burkitt lymphoma**

**(ii) Chronic myelogenous leukemia (CML) results from 9;22 translocation.** The **Philadelphia chromosome** ($Ph^1$), which is present in the CML cells, results is an example of a balanced reciprocal translocation t(9;22) in which a piece of chromosome 9 is transferred chromosome 22. Chromosome 9 carries the c-abl proto-oncogene and chromosome 22 the breakpoint cluster region (bcr) within which the translocation breakpoints occur. The c-abl gene consists of at least 10 exons (only four shown in the figure). The bcr region lies within the bcr gene. In CML, the breakpoints usually occur within one of two introns in the middle of the gene. In a related disease, acute lymphoblastic leukemia (ALL), the breakpoint occurs in the first introns of the bcr gene. The t (9;22) translocation results in the Philadelphia chromosome with a bcr/c-abl transcription unit. This transcribes a bcr/c-abl RNA molecule which undergoes splicing to produce mRNA. The latter translates an unusually large 210 kDa fusion protein consisting of a 70 kDa Bcr protein linked to a 140 kDa c-Abl protein. The usual c-Abl protein is 145 kDa. Thus translocation results in the loss of a few N-terminal amino acids of the c-Abl sequence.

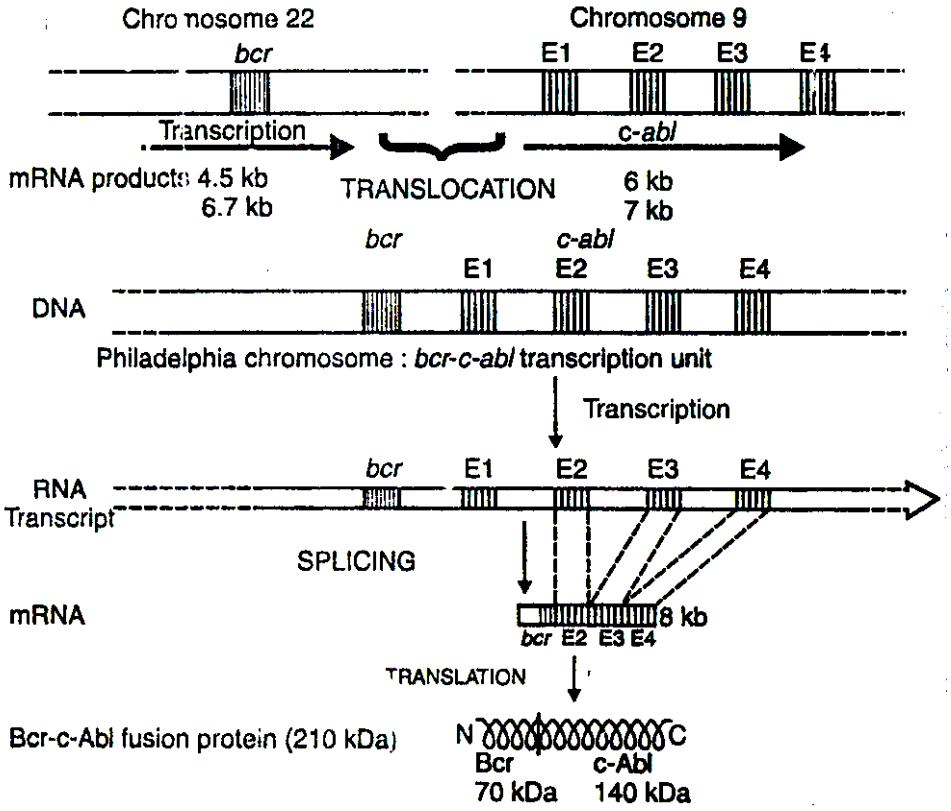



**Fig. A t(9;22) translocation resulting in the Philadelphia chromosome that synthesizes Bcr/c-Abl fusion protein**

The oncogenecity of the fusion protein depends on an interaction between the N-terminal region provided by Bcr with the c-Abl protein. The bcr gene codes for a serine/threonine kinase activity encoded within the first exon. This activity autophosphorylates amino acid residues in this part of the protein. The phosphorylation enables the Bcr protein to interact with the c-Abl protein sequences, possibly resulting in a change in their conformation and activating latent oncogenic potential. The N-terminus region of the c-Abl protein regulates a tyrosine kinase activity. Deletion or replacement of this region activates the kinase activity and brings about transformation.

**5. Amplification : DMs and HSRs**

One commonly found chromosomal anomaly in tumour cells is localized reduplication of DNA. This gene amplification is indicated by the presence of double minute (DM) chromosomes and homogeneously staining regions (HSRs). A proto-oncogene may be copied over and over again in these structures so that many more than the usual two copies are present. Consequently additional gene products are produced which may lead to cell transformation.

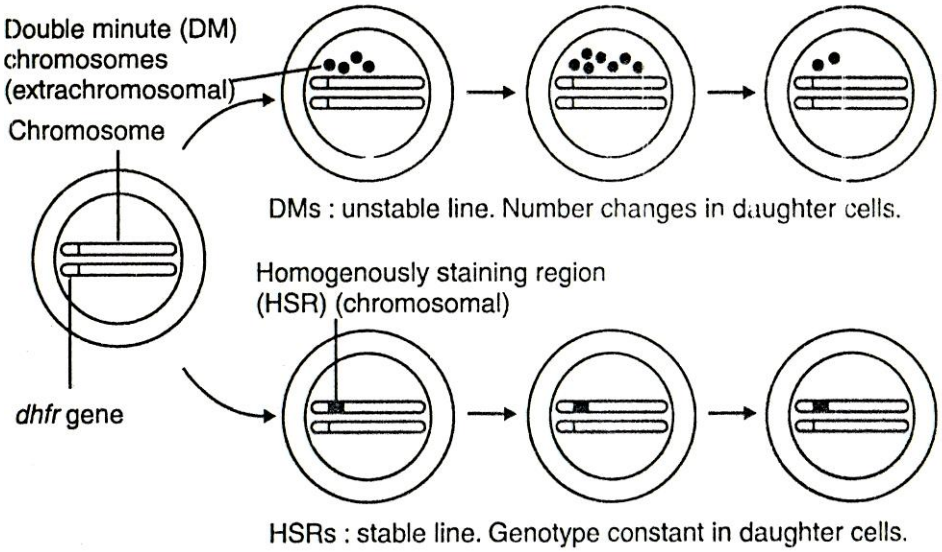



**Fig. Amplification of the dhfr gene to form extrachromosomal double minute (DM) chromosomes and chromosal homogeneously staining regions (HSRs)**

**(i) Double minute (DM) chromosomes.** During metaphase, DMs appear as small, usually paired and spherical, chromosome-like structures. They can be distinguished from small chromosomes by their lack of functional centromeres. Typically DMs are dot-like structures 0.3-0.5 μm in diameter. Larger DMs may be rod-shaped or ring-shaped. Some cell lines may have only one or two DMs per cell, while others may have more than 1,000. DMs replicate early and only once during the cell cycle. DMs have been demonstrated in a wide variety of tumours in man and animals, especially neuroblastomas. In the Y1-DM cell line from tumours of the mouse adrenal gland, the K-ras gene has been found to be amplified 30-fold.

**(ii) Homogeneously staining regions (HSRs)** are located within the chromosomes. They are additional, amorphous, ill-stained regions that lack bands after treatment such as G-staining. HSRs have been found in a variety of tumour cells from humans and animals, especially (as with DMs) in neuroblastomas. Both DMs and HSRs are found only in cancer cells. HSRs represent localized reduplication of DNA, resulting in as many as 100 copies. The duplicated DNA may be found at a single site on the chromosome in a tandem organization. HSRs containing oncogenes may be located in chromosomes other than those in which the oncogene is normally situated. For example, the c-myc gene which is normally located in human chromosome 8 has been found amplified within HSRs on a different chromosome in several neuroblastoma cell lines. Oncogenes amplified in various tumours include c-myc, c-myb, c-abl, c-erbB and c-K-ras. HSR replication is completed before the middle of the S-phase, when other chromosome regions are still actively synthesizing DNA. Unlike the DMs, whose number changes in the daughter cells, the HSR genotype is constant in daughter cells.

Gene amplification was first demonstrated in cell lines from tumours of the mouse adrenal glands. The cell line used with double minute chromosomes was Y1-DM, and that with homogeneously staining regions Y1-HSR. DNA from the two tumour cell lines was cut with restriction enzyme EcoR1, the fragments electrophoresed and Southern blotted, the blots hybridized to a K-ras probe and autoradiographed. DNA from the Y1-DM and Y1-HSR tumour cell lines gave × 60 and × 30, respectively, stronger hybridization to the probe han did the same amount of DNA from the untransformed mouse cell line 3T3.

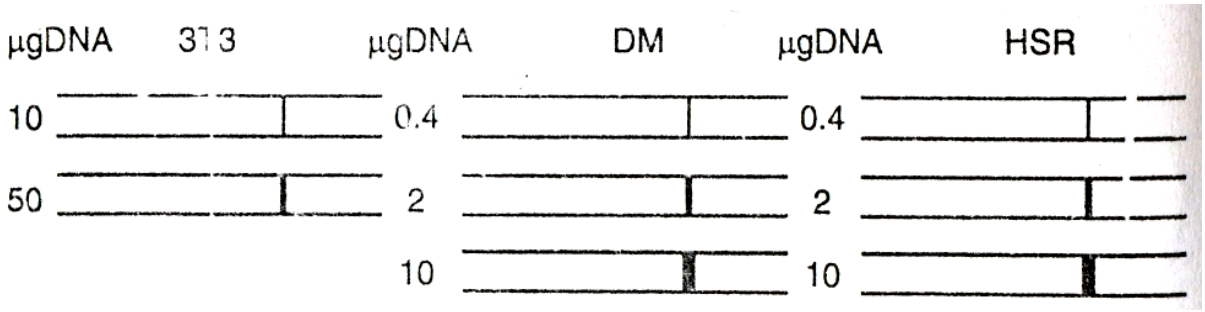



**Fig. Amplification of the c-myc gene in 3T3, DM and HSR cells**

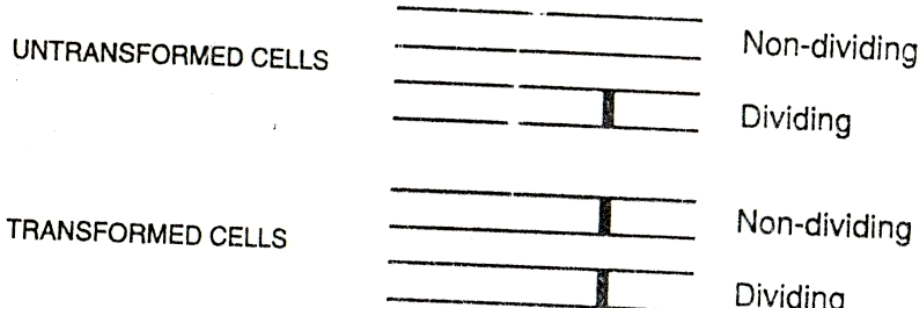



**Fig. Loss of response to normal regulation in c-myc in transformed cells**

**6. Write about Virus.**

Viruses have long been hypothesized to cause some cancers. Although several DNA viruses are associated with the development of malignancy, members of only two RNA virus families—*Retroviridae* and *Flaviviridae*—have thus far been associated with development of neoplastic disease.[1–3] In humans, these viruses include the retroviruses human T-lymphotropic virus (HTLV) and human immunodeficiency virus (HIV) and the flavivirus hepatitis C virus (HCV). HTLV type 1 (HTLV-1) appears to contribute directly to the development of adult T-cell leukemia (ATL); HIV and HCV are associated with human malignancy but likely contribute to its development in an indirect manner.

However, a number of animal retroviruses cause cancer in their natural hosts and have been important tools for understanding oncogenesis in humans and animals.[4] The discovery and

characterization of oncogenes and the subsequent elucidation of protooncogene functions have been closely intertwined and made possible by the study of retroviruses. In this section, the discussion focuses on the molecular genetics and the characteristics of retroviruses relevant to oncogenesis and explores the roles of the retroviruses HTLV-1, HTLV-2, and HIV and the flavivirus HCV in human cancers. In many examples, although these viruses may play key initiating or contributing roles to carcinogenesis, additional events are needed for infection to yield the full malignant phenotype.

**7. Describe briefly about Retroviruses.**

**INTRODUCTION:**

In the past, retroviruses had been classified on the basis of pathogenesis into the oncoretrovirus (the retroviruses associated with tumor formation), lentivirus, and spumavirus groups or on the basis of virus particle morphology (virus types A through D). Now, however, the retroviruses are organized into seven genera based on molecular genetic analysis: *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, and *Epsilon-retroviruses*; *Lentiviruses*; and *Spumaviruses*. This taxonomic system divides the oncoretroviruses into five genera. Few human retroviruses are known: the *Deltaretroviruses* HTLV-1 and HTLV-2 and the *Lentiviruses* HIV-1 and HIV-2. The spumavirus formerly termed *human foamy virus* is, in fact, a simian retrovirus isolated from contaminated human cell cultures (it is now termed *chimpanzee foamy virus human isolate*). Human endogenous retroviruses (HERVs) are endogenous retroviral elements contained within the human genome but are not classified as retroviruses per se.

Retroviruses are unique among animal viruses in having an RNA genome that replicates through a DNA intermediate.[5,6] Retroviral virions contain two identical plus-sense RNA molecules. The RNA genome (**Fig. 7.1-1**) contains a 5' untranslated region; the three genes common to all retroviruses—*gag*, *pol*, and *env*; and a 3' untranslated region and polyadenylated tail. In general, the *gag* gene encodes viral structural proteins, *pol* encodes viral enzymatic proteins, and *env* encodes viral envelope glycoproteins.

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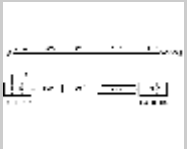
|  View Figure | Figure 7.1-1<br>Retrovirus genetic organization. General genome structure of a typical replication-competent oncoretrovirus. The structure of the double-stranded DNA copy (provirus) is shown below that of the genomic RNA. In the provirus, *gag* (encoding viral structural proteins), *pol* (viral replication enzymes), and *env* (viral envelope glycoproteins) genes are flanked on each end by the long terminal repeats (LTRs). The viral promoter/enhancer is located in the 5' U3, with transcription termination and polyadenylation signals located in the 3' LTR. The arrow indicates point and direction of transcription initiation. R, repeat; U3, unique 3' region; U5, unique 5' region. |

After entry into a cell, the single-stranded viral genome is converted to a double-stranded DNA copy by reverse transcriptase,[7] an RNA-dependent DNA polymerase (see **Fig. 7.1-1**). Then, the retroviral integrase protein inserts the double-stranded DNA viral genome into a host cell chromosome, where it permanently resides as a provirus.[8] An integrated retroviral provirus resembles cellular genes in that it is duplicated along with the cell's genome, passed on to daughter cells during mitosis, and subsequently transcribed and processed into messenger RNA (mRNA).

Reverse transcription is a complicated process that can use both viral RNA molecules as templates for DNA synthesis and involves RNA and DNA template strand-switching events during nucleotide polymerization. This process results in duplication of the 5' and 3' ends of the genome, thus forming the long terminal repeats (LTRs), which are composed of the unique 3'(U3), repeat (R), and unique 5' (U5) regions. The viral promoter and enhancer functions reside within the LTR. Transcription initiates in the 5' LTR and proceeds to the polyadenylation signal, usually located in the 3' LTR.

In summary, a number of features unique to the retroviral replication cycle demonstrate how retroviruses may be involved in the development of or be used for the study of the molecular basis of cell transformation.[9,10] Integration of the provirus into the cellular genome can permanently introduce genes into a cell or can result in mutation or altered regulation of genes. In addition, the molecular mechanism by which reverse transcription occurs is a fertile environment in which alterations of genes or the creation of cancer-causing genes might take place.[11,12]

**Mechanisms of Retroviral Oncogenesis**

Infection by members of the *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, and *Epsilon-retrovirus* genera may lead to the development of neoplastic disease in different ways (the genera are classified based on variations in genetic structure, not by shared mechanisms of pathogenesis), but, in general, individual members of each genus can be thought of as either acutely or slowly transforming viruses. In the case of the acutely transforming retroviruses, virtually all infected cells are swiftly transformed, whereas for other retroviruses, transformation is an unusual and much delayed outcome that often depends on the cell's accrual of additional alteration of its DNA. This latter group includes the classic slowly transforming retroviruses and the *trans*-acting retroviruses. Reverse transcription and integration of the viral genome into the cell favor the three major mechanisms by which oncogenic retroviruses may participate in the malignant transformation process (**Fig. 7.1-2**) :

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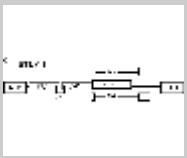

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Figure 7.1-2
Three types of oncogenic retroviruses. **A:** Slowly transforming retroviruses. In the upper example, a provirus has integrated upstream of a cellular protooncogene. The viral promoters may alter protooncogene expression directly [via read-through transcription originating from the 5' long terminal repeat (LTR) or from transcripts aberrantly originating in the 3' LTR] or indirectly via the effect of viral enhancers increasing transcription from cellular promoters (the viral enhancer can also affect expression of cellular genes 5' to and in the opposite orientation of the site of provirus integration). In the second example, insertional mutagenesis and consequent gene disruption are illustrated. This process may lead to inactivation of a tumor suppressor or to production of a mutant cellular protein that could lead to oncogenesis. In both cases, aberrant splicing could result in production of chimeric viral-cellular proteins. Arrows indicate points of initiation and direction of transcription. **B:** Acutely transforming retroviruses. Rous sarcoma virus (RSV) and simian sarcoma virus (SSV), retroviruses that contain oncogenes, are shown. RSV is unique in that the oncogene, *v-src*, has been added to the viral genome without concomitant loss of replicative genes. SSV, an example of the more common oncogene-containing retroviruses, is not replication competent; the recombination that led to *v-sis* insertion resulted in deletion of some viral sequences. **C:** *Trans*-acting retroviruses. In addition to *gag*, *pol*, and *env*, the human T-lymphotropic virus type 1 (HTLV-1) genome encodes the proteins Tax, Rev, $p12^{I}$, $p13^{I}$, and $p30^{II}$, derived from open reading frames I to IV located in the pX region. Tax (and possibly $p12^{I}$) is implicated in the genesis of adult T-cell leukemia through interactions with cellular transcription factors, resulting in alteration of expression of many T-cell growth regulation genes, including cytokines and cell-cycle control elements.

1. Slowly transforming viruses [e.g., avian leukosis virus (ALV), an *Alpharetrovirus*] alter cellular gene expression by random integration of a provirus within or adjacent to cellular protooncogenes (insertional mutagenesis). Direct physical disruption of a gene or effects of viral promoters and enhancers on cellular gene expression can lead to a malignant phenotype in infected cells.
2. Acutely transforming retroviruses [e.g., Rous sarcoma virus (RSV), an *Alpharetrovirus*] have incorporated into their genomes viral oncogenes derived from cellular protooncogenes (protooncogene capture) and subsequently transfer these altered or deregulated oncogenes into newly infected cells, thus leading to development of a malignant phenotype.
3. *Trans*-acting retroviruses (e.g., HTLV-1, a *Deltaretrovirus*) alter cellular gene expression and function and, consequently, the control of cell growth via viral protein(s) that act in *trans*.

### *Slowly Transforming Retroviruses: Insertional Mutagenesis*

Simple integration of a provirus into the genome of a cell can, rarely, be tumorigenic by leading to aberrant activity of cellular genes. Retroviruses that act in this manner have been termed *chronic, or slow-acting, tumor viruses*: They do not transform cells in tissue culture and, *in vivo*, a long latency period between infection and tumorigenesis is typical. In general, in addition to the mutagenesis caused by provirus integration, tumors produced by slowly transforming retroviruses require additional mutagenic events to take place for their transforming properties to become apparent.

Most proviral integrations have no effect on the phenotype of the infected cell but rarely can lead to phenotypically evident insertional mutagenesis in one of a number of ways.[13,14] Proviral integration might disrupt a gene (typically a tumor suppressor gene) by integrating within the gene (in an exon or intron) or by disrupting the promoter of the gene, thereby preventing production of a functional protein. Alternatively, insertion upstream or within a gene can also result in aberrant production of a cellular protein if the retroviral promoters in either the 5' or 3' LTRs are active and, because of read-through transcription, result in production of chimeric viral/cellular mRNAs. The protein derived from this mRNA may be wild type but produced in increased amounts, or may be a truncated or viral/cellular chimeric protein due to read-through transcription and aberrant RNA splicing. The enhancers present in the retroviral LTRs also can affect expression of cellular genes either upstream or downstream of the provirus integration site and genes in either the sense or antisense orientation relative to the provirus.

With the exception of rare cases of T-cell malignancy that appear to involve a monoclonal expansion of an HIV-1–infected cell, naturally occurring retroviruses have not been shown to cause malignancy by insertional mutagenesis in humans. However, humans are clearly susceptible to malignancy associated with retroviral-mediated insertional mutagenesis. In a gene transfer clinical trial using a murine leukemia virus (MLV) (a *Gammaretrovirus*) vector, two of the treated pediatric patients developed T-cell acute lymphoblastic leukemia (T-ALL) that appears to have been caused, at least in part, by insertion of the MLV vector provirus within or near the *LMO-2* gene.

### *Acutely Transforming Retroviruses: Oncogene Transduction*

Acutely transforming retroviruses have taken the oncogenic potential of slow-acting retroviruses one step further and are capable of rapidly transforming infected cells, which is followed by subsequent tumor formation. Although there are no known acutely transforming human retroviruses, the animal viruses are important because of the roles they played in the discovery of oncogenes and in the continued study of the molecular mechanisms of tumor formation.

Acutely transforming retroviruses are mutant retroviruses that encode oncogenes that, when integrated into the genome of an infected cell and subsequently expressed, result in cell transformation. These viral oncogenes are derivatives of cellular protooncogenes that were "captured" from the genome of a previously infected cell and incorporated into the retroviral genome, thereby creating a recombinant retrovirus containing a cancer-causing gene.

Many acutely transforming retroviruses contain different oncogenes. RSV is the prototype of such viruses. In fact, it was the study of RSV (and then other transforming retroviruses) that eventually led to the discovery of cellular protooncogenes and their potential roles in cancer development.

RSV arose when a retrovirus incorporated an oncogene into its genome; it is unique among acutely transforming retroviruses in that it encodes an oncogene (*v-src*) and functional retroviral *gag*, *pol*, and *env* genes. Typically, the recombination events that incorporate the oncogene into the retroviral genome also result in deletion of some or all of the retroviral genes. Therefore, the recombinant viruses are only capable of being propagated and infecting new cells when cells are coinfected with the parental, wild-type virus, which can produce all necessary retroviral proteins.

Acutely transforming retroviruses are believed to be formed after a number of complex events due to recombination during reverse transcription. The first such event is the integration of a wild-type retroviral provirus within or near a cellular protooncogene, as has been described for slowly transforming retroviruses. The next step involves formation of chimeric retroviral/cellular protooncogene mRNA, resulting from transcription from the 5' LTR, with subsequent read-through transcription into the cellular protooncogene. This chimeric viral/cellular mRNA can then be copackaged into a virion (which normally contains two identical copies of the viral genome) capable of infecting a new cell. Then, because reverse transcription of the retroviral genome involves template strand-switching events and also may lead to recombination events, nonhomologous recombination between wild-type and chimeric molecules can occur, thereby resulting in a novel provirus with an oncogene incorporated into its genome.

A protooncogene generally is not transduced intact from the cell to the retroviral genome: Deletion, frameshift, and point mutations are likely to occur during the process. In addition, the chimeric molecule can result in production of a viral/cellular fusion protein. Any or all of these alterations to the cellular protooncogene might contribute to altering the activity, level of expression, stability, function, or localization of the resulting protein, therefore resulting in the conversion of a cellular protooncogene with the potential to cause cancer into a viral oncogene fully capable of doing so.

*Trans-Activating Retroviruses*

A third manner of transformation involves *trans*-acting viral proteins that affect the expression or function of cellular growth and differentiation genes. This mechanism of oncogenesis is illustrated by HTLV-1, the only human retrovirus known to directly cause cancer.

**8. Discuss briefly about Telomeres.**

Our knowledge of the basic biology of telomeres is now beginning to yield fundamental insights into the pathophysiology of complex diseases, as studies in humans and model organisms have demonstrated that telomere maintenance and the cellular response to telomere dysfunction play crucial roles in processes of genomic instability, organ homeostasis, chronic diseases, aging, and tumorigenesis. With respect to the latter, the study of telomeres has begun to provide mechanistic insights into how advancing age fuels the development of epithelial cancers as well as how chronic inflammation and degeneration may engender increased cancer risk in affected organs. These advances in the basic understanding of telomere maintenance are now being translated into clinically relevant applications that may have an impact on the diagnosis and management of a broad spectrum of cancers.

**Telomeres**

Telomeres are specialized nucleoprotein complexes at the ends of linear chromosomes consisting of long arrays of double-stranded TTAGGG repeats, a G-rich 3' single-strand overhang, and associated telomeric repeat binding proteins.[1,2] The work of Muller and McClintock in the 1930s led to the concept that telomeres function to "cap" chromosomal termini and prevent end-to-end recombination, thereby maintaining chromosomal integrity. Subsequent work has substantiated this model across the animal and plant kingdom, underscoring the critical roles served by the telomere complex.

Telomere structure and function have been studied extensively in mammals. Although the overall structural features of telomeres are preserved among different mammalian organisms, lengths can vary considerably from species to species: for example, 5 to 15 kilobases (kb) for humans and 20 to 80 kb for the laboratory mouse. On the structural level, electron microscopy and other studies show that telomeres form complex secondary and tertiary structures via DNA-DNA interactions between the telomeric repeats, DNA-protein interactions between the telomeric DNA and the telomeric repeat binding proteins, and protein–protein interactions between the telomeric repeat binding proteins themselves and other associated proteins (**Fig. 3.4-1**). The formation of this well-documented higher-order DNA-protein complex has provided a working model of how the telomere functions as a capping structure, preventing the ends of linear

chromosomal DNA from being recognized and repaired as a double-strand DNA break (DSB), thereby avoiding the formation of chromosomal end-to-end fusions.

View Figure

Figure 3.4-1
Human telomere structure. Human telomeres form telomere loop (T loop) and displacement loop (D loop) secondary structures. Long stretches of telomeric repeats create a loop-back structure (T loop), completed by the invasion of the single GT-rich 3' overhang into the double-stranded DNA molecule (D loop), thus protecting the chromosome terminus. In human cells, double-stranded telomeric repeats are bound directly by two proteins, TRF1 (TTAGG repeat binding factor 1) and TRF2. Cell culture studies have suggested that TRF1's main function is to regulate telomere length, whereas TRF2 functions to protect telomeres from activating nonhomologous end joining (NHEJ) and other DNA repair or DNA damage response pathways. Biochemical studies also suggest that the formation of the T loop is dependent on TRF2. Another protein, Pot1 (protection of telomere 1), has been shown to bind to the single-stranded human telomeric 3' overhang. Pot1 has been proposed to interact with TRF1 complexes to regulate telomere length. Thus, there is significant interplay between telomeric binding proteins and the formation of the secondary/tertiary structures that protect the ends of chromosomes. Several other proteins have been shown to localize to the telomeres via protein–protein interactions with TRF1 and TRF2. Three TRF1-interacting proteins have been identified: PinX1, tankyrase1/2, and TRF1-interacting nuclear protein 2 (TIN2). TRF2 also interacts with the human Rap1 protein (hRap), the DNA-damage response Mre11 complex composed of Mre11, Rad50, and the Nbs1 protein. Chromatin precipitation experiments have shown that Ku70, Ku86, and DNA-PKcs proteins, involved in NHEJ repair of double-stranded DNA break, are also localized to the telomeres.

Paradoxically, many DSB repair proteins, involved in nonhomologous end joining and homologous recombination processes, have been found to be physically associated with the telomeres.[3,4] These findings have fueled speculation that DSB repair proteins are somehow reprogrammed to assume a protective role at the telomere, for example, by sequestering the telomere end from the DNA damage surveillance/repair machinery. Experimental support for this hypothesis has emerged from the mouse, in which germline inactivation of various repair proteins (e.g., Ku and DNA-PK) results in reduced telomere length or loss of capping function, or both, leading to increased end-to-end fusions.[5] Correspondingly, in cultured human cells, experimental disruption of telomere-binding proteins results in the unraveling of higher-order nucleoprotein structure and telomere localization of DNA DSB surveillance/repair proteins (e.g., 53BP1, gamma-H2AX, Rad17, ATM, and Mre11), establishing that dysfunctional telomeres can indeed serve as substrates for the classic DNA repair machinery.[6] A further understanding of the

molecular mechanisms governing the repression versus activation of the DNA DSB surveillance/repair apparatus at the telomere could lead to the development of novel cancer therapeutic options. For example, the design of agents that can uncap telomeres while preserving the DNA damage checkpoint response yet neutralize the actual DNA damage repair process would be ideal, as they would produce unrepaired DSBs and elicit cell-cycle arrest or apoptosis responses. Lastly, in the near future, agents designed to uncap the telomeres will likely be used in combination with conventional chemotherapeutic agents that create DSB for cancer treatment, thereby simultaneously targeting these intertwined pathways.

**9. Write Briefly about Telomerase.**

**Telomerase Structure, Regulation, and Function**

Conventional DNA polymerases operating in the S phase of the cell cycle require an RNA primer for reverse strand synthesis, resulting in incomplete DNA replication of telomeres during each cell division. The solution to this "end-replication problem" is the telomere-synthesizing telomerase enzyme, a specialized ribonucleoprotein complex with reverse transcriptase activity. The functional telomerase holoenzyme is a large multisubunit complex that includes an essential telomerase RNA (hTERC) component serving as a template for the addition of telomere repeats and a telomerase reverse transcriptase (hTERT) catalytic subunit.[7] In normal human cells, telomerase levels are insufficient to maintain telomere length, resulting in progressive attrition with each cell division. This forms the basis for the theory that the metered loss of telomeres can serve as a cellular mitotic clock that ultimately limits the number of cell divisions and cellular life span.

Many normal somatic human cells and differentiated tissues express readily detectable levels of the hTERC component. In contrast, hTERT expression and activity are more restricted due to stringent regulation on the levels of transcriptional initiation, alternative RNA processing, posttranslational modification, and subcellular localization. With the identification of an increasing number of TERT-associated proteins, it is likely that additional regulatory mechanisms will surface, such as those governing the accessibility of the telomerase holoenzyme onto the telomere end.[8] Here again, a more complete elucidation of these regulatory mechanisms may provide additional therapeutic strategies that can preferentially target telomerase activity in cancer cells. Indeed, the development of such selective strategies may become paramount because studies have revealed low telomerase levels in cycling somatic human cells that were previously thought to have no telomerase activity.[9] Eradication of residual telomerase function in these primary cells alters the maintenance of the 3' single-strand telomeric overhang without changing the rate of overall telomere shortening, resulting in diminished proliferation rates and overall reduction in proliferative capacity. These studies support an additional protective function of telomerase at the telomeres[10] and raise concerns that generalized antitelomerase therapy could

lead to the immediate uncapping of telomeres in normal cells, thus limiting the use of antitelomerase therapy in cancer patients.

**Crisis, Telomerase Reactivation, and Alternative Lengthening of Telomeres**

In human cell culture, the first cell division barrier triggered by critically shortened telomeres manifests as a cellular senescence response, termed the *Hayflick limit* (or mortality stage 1, M1). Because the loss of RB or p53 pathway function, or both, in primary human cells permits additional cell divisions beyond the Hayflick limit, these pathways appear to be involved in the activation of this senescence program brought about by the "shortened telomere" signal. Under circumstances of extended cell divisions, progressive telomere erosion ultimately leads to loss of capping function, resulting in increasing chromosomal instability. This leads to progressive loss of cell viability and proliferative capacity across the cell population, ultimately resulting in "cellular crisis" (or mortality stage 2, M2). The cellular phenotypes of massive cell death and growth arrest are a likely by-product of rampant chromosomal instability and associated loss of essential genetic material. Emergence from crisis is a rare event in human cell culture and requires restoration of telomere function to a level compatible with cell viability, achieved either by up-regulation of telomerase activity or activation of the alternative lengthening of telomeres (ALT) mechanism.[11] Finally, the extent to which normal tissues experience Hayflick and crisis transitions is a subject of ongoing study. Although clear evidence of the presence of telomere-activated Hayflick limit is still lacking, support is mounting for telomere-based crisis, particularly during early stages of neoplastic development.

Transcriptional up-regulation of the TERT gene seems to be a key rate-limiting step in telomerase reactivation, whereas the telomerase-independent ALT pathway appears to be executed via a homologous recombination pathway. The analysis of pathways regulating TERT gene transcription has forged links to well-known oncoproteins and tumor suppressors including Myc, Mad, and Menin, among others, demonstrating the capacity of these proteins to engage the TERT gene promoter directly.[12–14] The regulation of ALT remains a mystery. It appears to be most often activated in the setting of p53 deficiency and in tumors of mesenchymal origin.[15] Studies in yeast have also shown that ALT is enhanced in mismatch repair-deficient cells, owing to increased homologous recombination between chromosomes.[16] The rare use of ALT by epithelial-derived tumors, coupled with functional comparisons of telomerase- versus ALT-mediated telomere maintenance, has shown that ALT may not be as biologically robust in advancing malignancy, a finding that diminishes the theoretic concern that ALT may provide a robust resistance mechanism to antitelomerase therapy in advanced malignancy. The idea that ALT may be a less effective telomere maintenance mechanism derives additional support from studies in human cell culture and the mouse revealing that telomerase per se is needed for full malignant transformation, including metastatic potential. The fundamental mechanistic differences between ALT and telomerase reactivation in telomere maintenance may provide an explanation for the

report of more favorable clinical outcomes for ALT-positive compared to telomerase-positive glioblastomas,[17] although analysis of 71 human osteosarcoma cases failed to show a more favorable clinical outcome for the ALT-positive subset.[18] However, it should be noted that in the latter, the absence of any telomere maintenance mechanism was more associated with improved survival than stage or response to chemotherapy, further emphasizing the general importance of telomere maintenance in cancer.

**Telomere Maintenance and Cancer**

Robust telomerase activity is observed in greater than 80% of all human cancers,[19] a profile consistent with its role in promoting malignant progression. However, another side to the telomerase-cancer connection has emerged from mouse models and correlative data in staged human tumors. These data have indicated that a lack of telomerase and associated telomere attrition during the early stages of neoplastic growth may provide a mutator mechanism that enables would-be cancer cells to achieve a threshold of cancer-promoting changes required to traverse the benign to malignant transition. Indeed, telomeres of human cancer cells are often significantly shorter than their normal tissue counterparts, suggesting that telomere attrition has occurred at some time during the life history of these cancers, presumably during early phases when telomerase activity is low. The subsequent reactivation of telomerase appears to restore telomere function, albeit at a shorter set length. Thus, although reactivation of telomerase is critical to the emergence of immortal human cells, a preceding and transient period of telomere shortening and dysfunction appears to contribute to carcinogenesis by leading to the formation of chromosomal rearrangements through breakage-fusion-bridge (BFB) cycles. Serial BFB in turn begets rapid and wholesale genetic changes in the population, with rare cells incurring a threshold number of relevant procarcinogenic changes needed to initiate the transformation process. Although, at first glance, the cancer-promoting effects of telomere-based crisis seem contradictory to the hackneyed role of telomerase activation in cancer progression, this mechanism is less paradoxic if one considers that many early-stage cancers deactivate pathways essential for telomere checkpoint responses, thus increasing the survival and proliferation of cells with increasing genomic instability[20,21] (**Fig. 3.4-2**). This hypothesis of "episodic instability"–derived support from genetic studies in the mouse shows that telomere-based crisis coupled with loss of the p53-dependent DNA damage response can act cooperatively to effect malignant transformation. In humans, the accumulation of oncogenic lesions during normal aging or accelerated accumulation of DNA damage (e.g., environmental carcinogen exposure or oxidative damage) may deactivate the telomere checkpoint response, accelerate telomere attrition, and drive the affected premalignant cells into crisis. It is the rare transformed cell that may emerge from this process with reactivated telomerase. Thus, telomeric shortening can be viewed as a barrier to cancer development in the presence of intact checkpoint response and as a facilitator for numerous genetic changes necessary for the emergence of nascent cancer cells in the absence of the checkpoint response pathways.

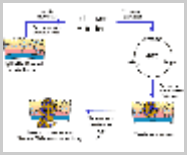

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Figure 3.4-2
Dysfunctional telomere-induced genomic instability model of epithelial carcinogenesis. Continuous epithelial turnover during aging coupled with somatic mutations inactivating checkpoint responses is thought to lead to critical telomere erosion, resulting in telomere uncapping and the initiation of breakage-fusion-bridge (BFB) cycles. The double-strand breaks created by the BFB cycles are nidi for amplifications and deletions for the resulting daughter cells. The broken chromosome may become fused to another chromosome, generating a second dicentric chromosome and perpetuating the BFB cycle. This facilitation of the accumulation of genetic changes (via aneuploidy, nonreciprocal translocations, amplifications, and deletions) by the BFB cycles coupled with the reactivation of telomerase enables cells to emerge from crisis and proceed to malignancy. ALT, alternative lengthening of telomeres.

**Aging and Cancer**

The study of telomeres has also provided some insights into the link between advancing age and increased cancer risk. In humans, there is a dramatic escalation in cancer risk between the ages of 40 and 80, resulting primarily from a marked increase in epithelial malignancies such as carcinomas of the breast, lung, colon, and prostate. A conventional view is that the cancer-prone phenotype of older humans reflects the combined effects of cumulative mutational load, decreased DNA repair capabilities, increased epigenetic gene silencing, and altered hormonal and stromal milieus. Although these factors are almost certain to contribute to increasing cancer incidence in aged humans, it is less evident why such processes would spur the preferential development of epithelial cancers. Moreover, these mechanisms do not readily explain one of the cardinal features of adult epithelial carcinomas—namely, a radically altered genome typified by marked aneuploidy and complex nonreciprocal chromosomal translocations.

The study of telomere dynamics in normal and neoplastic cells of the mouse has provided a potential explanation for the observed tumor spectrum and associated cytogenetic profiles in aged humans. In *Terc p53* compound mutant mice, the presence of telomere dysfunction results in a dramatic shift in the tumor spectrum toward epithelial cancers, including those of the lung, colon, and skin. Moreover, in contrast to the somewhat normal cytogenetic profiles of cancers arising in mice with intact telomeres, the cancers generated in the *Terc p53* compound mutant mice had cytogenetic profiles with a striking resemblance to human epithelial cancer genomes.[22]

In attempting to assign relevance of these murine studies to humans, it is worth considering that the typical adult cancer, an epithelial carcinoma, derives from a compartment that has undergone continued renewal throughout the human life span. Against this backdrop of physiologic cell turnover, combined with the occasional proproliferative oncogenic mutation,

telomere lengths would shorten in self-renewing progenitor cells of these epithelial tissues. If somatic mutations also neutralize RB/INK4a/p53-dependent senescence checkpoints, continued growth beyond the Hayflick limit further drives telomere erosion and loss of the capping function, culminating in cellular crisis with attendant genomic instability. In this manner, telomere-based crisis provides the means to generate many additional mutations required to reach the early stages of malignant transformation. The subsequent reactivation of telomerase in transformed clones would serve to stabilize the genome to a level compatible with cell viability, allowing these initiated neoplasms to mature further.[23] It is unclear whether additional somatic mutations, beyond telomerase activation, would be needed to produce a fully malignant phenotype that includes invasive and metastatic potential. Thus, a transient period of explosive chromosomal instability before telomerase reactivation appears to be required for the stochastic acquisition of the relatively high number of mutations thought to be required for adult epithelial carcinogenesis.

The episodic instability model of epithelial carcinogenesis fits well with current knowledge regarding the timing of telomerase activation and evolving genomic changes during various stages of human carcinoma development, particularly those of the breast, esophagus, and colon. Comparative genome hybridization has demonstrated that dysplastic human breast, esophageal, and colon lesions sustain widespread gains and losses of regions of chromosomes early in their development, often well before these tissues exhibit carcinoma *in situ* or invasive growth.[24–26] The ploidy changes detected by comparative genome hybridization appear to correlate tightly with the presence of complex chromosomal rearrangements, and these markers of genomic instability are evident in the stages of advanced dysplasia of these tissues (e.g., ductal carcinoma *in situ*, Barrett's esophagus, etc.). As these cancers progress through invasive and metastatic stages, genomic instability continues, apparently at a moderate rate, but further mutations would be predicted to derive from nontelomere-based mechanisms. Correspondingly, the measurement of telomerase activity in adenomatous polyps and colorectal cancers has established that telomerase activity is low or undetectable in small- and intermediate-sized polyps, reflecting less intact telomere function. In contrast, telomerase increases markedly in large adenomas and colorectal carcinomas, reflecting stabilization of telomere function.[27] Therefore, it appears that widespread and severe chromosomal instability is present early on during human tumorigenesis at a time when telomerase activity is low.

Additional support for this episodic instability model derives from the documentation of anaphase bridging (a reasonable correlate of telomere-based crisis) in evolving human colorectal cancers and in genomically unstable pancreatic cancers.[28,29] This suggests that the DSB-induced conditions (including but not limited to telomere dysfunction), coupled with mutations that allow survival in the face of a DSB, could provide amplification/deletion mechanisms across the genome. Biologic forces would in turn lead to the selection of clones with the amplifications and deletions that target cancer-relevant loci. Studies in the telomerase mutant mouse have begun to

provide mechanistic insight into how BFB leads to cancer-relevant changes. In particular, telomerase p53 compound mutant mice with telomere dysfunction have increased end-to-end fusions, and the ensuing BFB process is associated with chromosomal regional gains and losses that appear linked to nonreciprocal translocations.

In future human studies, it will be important to document telomere attrition in renewing epithelial stem cells and to perform a simultaneous comparison of telomere status, telomerase activity, and chromosomal instability in the same tumor samples, particularly during the earliest stages of human epithelial cell transformation. Defining the temporal point at which telomerase is reactivated in the genesis and progression of the different cancers may also lead to the development of biomarkers for diagnosis, prognosis, and outcomes prediction. Such studies are needed to more firmly establish a causal link between telomere dysfunction and early chromosomal instability in human neoplasms.

The importance of telomere dysfunction in human epithelial carcinogenesis is also gaining experimental support from research of other human diseases. The discovery that germline mutations of the telomerase complex cause dyskeratosis congenita, a progeroid syndrome characterized by increased susceptibility to a variety of malignancies, lends additional support to the hypothesis that telomere dynamics are intricately linked to aging and cancer.[32]

**10. Write briefly about Telomere Dynamics, Inflammatory Diseases, and Cancer.**

The telomere dysfunction-induced genomic instability model also suggests some unanticipated opportunities for the therapies of other human diseases. For example, this model provides a potential explanation for the high cancer incidence associated with diseases characterized by chronic cell destruction and renewal. One of the most notable examples of this tight link is the high incidence of hepatocellular carcinoma in late-stage cirrhotic livers. Cirrhosis is the phenotypic end point of prolonged cycles of hepatocyte destruction and regeneration, and cirrhotic livers show a documented reduction in telomere length over time. Mouse models involving the telomerase null mouse have shown that critical reductions in telomere length and function can accelerate the development of cirrhosis and hepatocellular carcinoma in chronic liver injury experiments.[33,34] Another example of a telomere-based pathogenetic relationship between chronic tissue turnover, telomere-based crisis, and increased cancer risk is ulcerative colitis, a condition characterized by rapid cell turnover and oxidative injury to the intestines, and a high incidence of intestinal dysplasia or cancer.[28] In addition to the progressive telomere attrition resulting from the cell turnover, accelerated telomere attrition might occur via increased oxidative stress and from the altered inflammatory microenvironment milieu. Together, such observations suggest the intriguing possibility that early somatic reconstitution of telomerase could attenuate telomere attrition and paradoxically reduce the occurrence of cancers in these high-turnover disease states, a theory that requires additional preclinical studies. In addition, serial analyses of

telomere length from these tissues may provide prognostic information regarding the rising risk of cancer development. Thus, progress in our understanding of telomere biology has mechanistically connected diverse fields in medicine involving chronic inflammatory diseases, degenerative diseases, geriatrics, and oncology (**Fig. 3.4-3**).

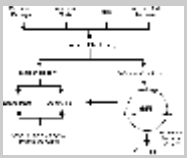

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Figure 3.4-3
Schematic of the roles of telomere maintenance in cancer, chronic diseases, and aging. ALT, alternative lengthening of telomeres; BFB, breakage-fusion-bridge.

**Telomere Status As a Biomarker for Cancer**

Given the important roles of telomerase and telomere maintenance in the development of various cancers, intense efforts have been made to develop assays based on telomerase activity or telomere length to ascertain malignant progression, to detect malignant cells, and to predict clinical outcome. Using the polymerase chain reaction–based telomerase assay, telomeric repeat amplification protocol, telomerase activity is detected in approximately 80% to 90% of the most common cancers, such as breast, prostate, lung, liver, pancreatic, and colon.[35] Therefore, it is a near universal marker of human cancer, with low or absent expression in normal somatic cells. Accordingly, the telomeric repeat amplification protocol assay for hTERT expression is being used for the diagnosis of various cancers from cells obtained from biopsy specimens or from cytologic specimens obtained from secretion samples, washing or brushing samples, and fine-needle aspirates. In a group of cancers, such as non–small cell lung cancer, gastric cancers, and neuroblastoma, in which telomerase activity is up-regulated during cancer progression, telomerase expression is not only useful to evaluate malignant grade of the tumors but also correlates with the prognosis of the patients.[36] However, these advances must be tempered with the fact that some normal differentiated cell types as well as stem cells in various organ compartments also express telomerase. Thus, the specificity of these tests must be evaluated closely before they are widely applied in the clinical diagnostic field.

Efforts have also been directed toward assessing whether telomere length itself in the tissue of interest could serve as a biomarker for the risk of developing cancer and other diseases or serve as a prognostic marker in the setting of established disease, or both. In addition, several studies have begun to explore the use of telomere length determination in accessible peripheral blood lymphocytes (PBL) rather than the diseased tissue per se. Such studies in nonneoplastic diseases have suggested that PBL telomere lengths can provide predictive information on the risk of developing atherosclerosis, premature myocardial infarctions, coronary artery disease, Alzheimer's disease status, and overall mortality. It has also been shown that the presence of short

telomeres in PBL is associated with increased risk for the development of carcinomas of the head and neck, kidney, bladder, and lung.[37] Further substantiation of the use of PBL telomere length determination is necessary before it can serve as a reliable and noninvasive surrogate marker that predicts the malignant propensity of epithelial lineages.

In the years ahead, basic telomere science discoveries will continue to yield mechanistic insights into cancer pathogenesis that can then translate into new tools for the screening and diagnosis of cancer. Parallel advances in technology may make possible the determination of telomere length *in vivo* at the level of a single cell, improving its sensitivity and specificity for diagnosis. Such advances in telomere-based biomarkers may greatly enhance general screening for cancer. For example, periodic screening for the rate of telomere attrition in specific organs may eventually be used to assign risk of cancer development in those organs. This approach would be particularly appealing for the assessment of cancer risk for patients with chronic hepatitis or ulcerative colitis, as an accelerated rate of telomere attrition has been documented for these conditions. It seems likely that serial telomere length measurements in such premalignant conditions will be useful as a biomarker to stratify more accurately where these patients lie on the risk curve.

**11. Write briefly about Telomerase and Telomere Maintenance As Therapeutic Targets.**

A significant body of evidence supports the view that telomerase-mediated telomere maintenance represents an ideal and near universal therapeutic target for cancer. Indeed, cell culture–based studies of human cancer cells have established that inhibition of telomerase culminates in cell death after extended cell divisions. The past few years have witnessed intense efforts to design therapeutic strategies capable of targeting telomere structure and the telomerase holoenzyme[38,39] (**Fig. 3.4-4**).


View Figure

Figure 3.4-4
Schematic of the different therapeutic strategies for cancer treatment that are targeting telomere and telomerase. hTERC, essential telomerase RNA; hTERT, telomerase reverse transcriptase.

Many natural compounds and small molecules that have inhibitory activity against the reverse transcriptase enzymatic function of telomerase have been identified. However, all these compounds have a significant lag time (measured in months) between the inhibition of telomerase and the time telomeres in the cancer cell are shortened sufficiently to produce an effect on tumor growth. Such a lag time is not necessarily a hurdle to telomerase therapy, as the median survival of many metastatic epithelial cancers such as breast and colon now significantly exceeds 1 year. However, this lag time could pose significant economic and logistic challenges for the clinical development of these compounds.

Several different classes of agents have been developed to target the two components of the core telomerase enzyme, namely the RNA (hTERC) or the catalytic subunit (hTERT). Antisense oligonucleotides or hammerhead ribozymes that target hTERC RNA have had limited success in preclinical studies because of pharmacokinetic and long lag time issues. Second-generation antisense oligonucleotides against hTERC, chemically modified (bonded to 5'-phosphorylated 2'-5'–linked oligoadenylate) to activate the cellular RNase L system to cleave single-stranded RNA, have been shown to induce apoptosis in telomerase-positive cancer cells within 3 to 6 days after the treatment. This effect was not seen in cells that lack telomerase and occurred before telomere length shortening. One explanation for this rapid response independent of telomere shortening is that this treatment may cause degradation of the core telomerase enzyme, which, as described above, appears to have other cellular activities independent of its enzymatic activity (including capping of the telomeres). The safety profile in humans of this agent, particularly the impact on tissue stem cell reserves, will only become evident with completion of human phase I studies.

Similar approaches have been used to target the hTERT component of telomerase. One strategy uses a ribozyme against the hTERT RNA that, on application of cultured cancer cells, results in the immediate induction of apoptosis independent of initial telomere length in these cancer cells. Interestingly, several studies have also shown that expression of mutant hTERT in human cancer cells exerts a strong transdominant negative effect that rapidly causes increased apoptosis and decreased cell viability independent of telomere shortening. The presumed mechanism for this effect is altered telomere structure stemming from altered DNA-protein or DNA-DNA interactions that uncap the telomeres. Again, the effects of these approaches on normal human cells that possess telomerase activity await entry into clinical trials.

The physical structure of the telomere itself has provided another potential therapeutic target. The G-rich 3' overhang at the ends of telomeres can form a core stack of guanines arranged in an almost planar hydrogen-bonded tetrad, called a G-quadruplex structure. This structure may hinder telomerase from elongating the 3' overhang of the telomere. Chemical compounds such as telomestatin, TMPyP4, and specific oligonucleotides that can facilitate and stabilize the formation of the G-quadruplex structure have been shown to be effective telomerase inhibitors. One potential advantage of some members of this class of compounds, such as TMPyP4, is that they appear to suppress ALT-positive cells in addition to telomerase-positive cells. However, many studies have demonstrated that these compounds also have a significant lag time in achieving antiproliferation effects. The effects of these compounds on normal human cells and organs are also not known.

Other approaches to devise tumor-specific gene delivery have exploited the restricted expression pattern of hTERT in normal cells and its nearly universal expression in most advanced cancers. Studies using adenoviral vectors or plasmids to deliver toxic genes such as FADD, Bax, and caspases driven by the hTERT promoter have shown that expression of these toxic gene products is limited to telomerase-expressing cells. The clinical application of this delivery system

is limited, as only a small subset of cancer cells can feasibly be infected with adenoviral vectors or transfected with plasmids using current delivery technologies. A more promising alternative strategy is the construction of adenoviral vectors under the control of the hTERT promoter that could infect normal and cancer cells, but the virus would only replicate in those cells that are expressing telomerase activity. The hTERT-driven adenovirus would replicate and eventually lyse the infected cells and release additional viral particles to continue the next round of infection of the neighboring cells. However, given the discoveries that most cycling human somatic cells express a low level of hTERT, it is not clear whether this strategy will have detrimental effects *in vivo*. Furthermore, this is one area in telomere biology in which clinical research is ahead of the basic science, as little is known about the regulation of hTERT in different tissues.

Lastly, another telomerase-related cancer treatment strategy is immunotherapy, targeting immune recognition and the destruction of cells that express telomerase. Immune responses, specifically cytotoxic T-cell responses, have been generated against peptide sequences of the hTERT protein, and it has been demonstrated that these cytotoxic T cells are capable of selectively lysing target cells that express TERT peptides presented on the cell surface in the context of major histocompatibility complex class I molecules. Preclinical studies suggest that telomerase is a poor autoantigen either due to the low level of hTERT protein or, alternatively, the inefficient processing of the hTERT peptide in these normal cells. Several phase I trials using peptides from telomerase as a vaccine are ongoing. Although there are no reports of severe toxicity including autoimmunity, the clinical efficacy of this approach is not yet known.

Overall, in contrast to the diagnostic areas, the cancer therapeutic applications are not firmly anchored on the basics of telomere biology. Many unresolved questions still exist regarding the exact targets and mechanisms of action for all these approaches. An important caveat is that analyses of the telomerase-deficient (*mTerc*–/–) mouse with ample telomere reserve showed that the cells from the various organ compartments are phenotypically normal and viable in the absence of telomerase activity. (It remains to be seen whether the deletion of the mTert catalytic protein subunit will yield a similarly benign phenotypic outcome.) The proposed alternative capping or other functions of telomerase that are independent of its enzymatic function have not been fully elucidated. (This would also suggest that there might be use in the disruption of the telomerase protein even in ALT cancer cells.) It is also not totally clear whether the induction of telomere dysfunction in the absence of intact p53 checkpoint response would cause reentry into a phase of rampant genomic instability from which more aggressive and resistant tumors would emerge. Last, inhibition of telomerase activity might cause the cancer cells to activate the ALT.

**12. How is oncogene detected?**

Some oncogenes can be detected by using a direct assay for transformation in which 'normal' recipient cells are transfected with DNA obtained from animal tumours. The procedure is illustrated in figure. The established mouse NIH 3T3 fibroblast line usually is used as a recipient. Historically these experiments started by using DNA extracted en masse, but now they are usually performed with a purified oncogene. The ability of any individual gene to convert wild-type cells into the transformed state constitutes one form of proof that it is an oncogene. Another Assay that can be used is to inject cells into 'nude' mice (which lacks the ability to reject such transplants immunologically). The ability to form tumours can then be measured directly in the animal.

When a cell is transformed in a 3T3 culture (or some other 'Normal" culture), its descendants pile upto a focus. The appearance of faci is used as a measure of the transforming ability of a DNA prepn. starting c̄ a prepn. Of DNA isolated from tumour cells, the efficiency of focus formation is low. However, once the transforming gene has been isolated and cloned, greater efficiencies can be obtained. In fact, the transforming 'strength' of a gene can be characterized by the efficiency of focus formation by the cloned sequence. DNA with transforming activity can be isolated only from tumorigenic cells: It is not present in normal DNA. The transforming genes isolated by this assay have two revealing properties.

DNA from tumour cells can transform normal cells.

They have closely related sequences in the DNA of normal cells. This argues that transformation was caused by mutation of a normal cellular gene (a proto-oncogene) to generate an oncogene. The change may take the form of point mutation or more extensive recognition of DNA around the c-onc gene.

They may have counterparts in the oncogenes carried by known transforming viruses. This suggests that the repertoire of proto-oncogene is limited, and probably the same genes are targets for mutations to generate oncogenes in the cellular genome or to become viral oncogenes.

Oncogenes derived from the c-ras family are often detected in the transfection assay. The family consist of several active genes is both man and rat, dispersed in the genome. (They are also some pseudo genes). The individual g4enes, N-ras, H-ras, and K-ras, are closely related, and code for protein products N21 KD and known collectively as $P21^{ras}$.

The H-ras and K-ras genes have V-ras counterparts, carried bythe Harvey and Kirsten strains of murine Sarcome virus, respectively, each V-ras gene is closely related to the corresponding C-ras gene, c̄ only a few individual A. Acids substitutions. The Harvey and Kirsten virus strains must have originated in independent recombination events in which a progenitor virus gained the correspondency c-ras sequence.

## UNIT – IV

## PART – A

**1. Write some natural stimulators of Angiogenesis.**

**Proteins:**

1. Angiogenin
2. Epidermal Growth Factor
3. Fibroblast Growth Factor
4. Granulocyte colony – stimulating factor
5. Hepatocyte Growth Factor
6. Vascular Epithelial Growth Factor (VEGF) etc.

**Small molecules:**

**Proteins**

1. Angiostatin
2. Canstatin
3. Endostatin
4. Thrombospondin
5. TIMP – 1 (tissue inhibitor of metalb proteinase -1) etc.

**2. Which drug used as a Angiogenesis Inhibitor in colon cancer?**

Avastin.

**3. What is Invasion? and Metastasis?**

Cancer spread through the body via two distinct mechanisms: Invasion & metastasis.

Invasion refers to the direct migration and penetration of cancer cells into neighboring tissues, whereas metastasis involves the ability of cancer cells to enter the blood stream (or other body fluids) and travel to distant sites, where they form new tumours that are physically contiguous with the primary tumor.

**4. What are the steps involved in metastasis?**

Metastasis involves a complex cascade of events, beginning with the process of angiogenesis. There are three steps:

1. First, cancer cells invade surrounding tissues and penetrate through the walls of lymphatic and blood vessels, thereby gaining access to the blood stream.
2. 2nd, these cancer cells are then transported by the circulatory system through out the body.

The cancer cells leave the blood stream and enter particular organs, where they establish new metastasis tumors.

**5. Three stepTheory for Invasion.**

Degradation, Adhesion and migrations of cancer cells. In general this model led to a large body scientist studying the role of tumour cell interactions with the extra cellular matrix, characterizing the extra cellular molecules and discovering the enzyme inhibitors and adhesion molecules. The role of ECM and the tumour microenvironment continues to be a fertile area.

**6. What is Pagets "Seed and Soil hypothesis?**

Pagets "Seed and Soil" hypothesis emphasizes the importance of the interaction between the tumour cell and its environment in order for metastasis to occur.

Stephen Pagets 1889 proposal that metastasis depends on cross-talk between selected cancer cells (the 'seeds') and specific organ micro environment (the 'soil'). It is known that the potential of a tumour cell to metastasize depends on its interactions with the homeostatic factors that promote tumour-cell growth, survival, angiogenesis, invasion and metastasis.

## PART – B

**1. What are the mechanism involved in the Invasion process?**

1. Cell Adhesion
2. Motility
3. Protease Production

1. The first involves changes in the adhesive forces between cells. In most tissues, adjoining cells are held together by binding interactions between cell – cell adhesion proteins found on the outer surface of each cell. These adhesion proteins found on the outer surface of each cell. Ex. E-Cadherin, a cell – cell adhesion proteins that normally binds epithelial cells to one another.

   These adhesion molecules, which normally function to keep cells is place, are often missing or deficient in cancer cells, thereby allowing cells to separate from the main tumor mass more readily.

2. A second property involved in tumour invasion is cell motility, which is activated after the loss of cell – cell adhesion permits cancer cells to detach from one another. Cancer cells possess all the normal cytoplasmic machinery required for cell locomotion but their actual movement needs to be stimulated by signaling molecules produced either by surrounding host tissues or by cancer cells themselves. Besides activating cell motility, some of these signaling molecules act as chemo attractants that guide cell movement by serving as attracting signals towards which the cancer cells will migrate.

3. Production of proteases (Protein degrading enzymes)

   The purpose of these enzymes is to break down structures that would otherwise represent barriers to cancer cells movement. For example, epithelial cells, the source of about 90% of all human cancers, are separated from underlying tissues by a thin, dense layer of protein – containing material called the basal lamina. Before epithelial cancers can invade adjacent tissues, the basal lamina must first be breached. Cancer cells break through this barrier by producing proteases that facilitate degradation of the proteins that form the backbone of the basal lamina.

   One such protease is plasminogen activator, an enzyme that converts the inactive precursor plasminogen into the active protease plasmin. The plasmin in turn perform two tasks: 1. it degrades components of the basal lamina and the extra cellular matrix, thereby facilitating tumor invasion; and 2. It cleaves inactive precursors of matrix metalloprotinases (MMPs)

produced mainly by surrounding host cells, into active enzymes that also degrade components of the basal lamina and extracellular matrix. After proteases allow the basal lamina to be penetrated, they degrade the matrix of the underlying tissues to open up path through which the cancer cells can move. The cancer cells migrate until they reach tiny blood or lymphatic vessels, which are also surrounded by a basal lamina, allowing cancer cells to pass through it and through the layer of endothelial cells that form the vessel's inner lining, at which point cancer cells have finally gained entry into the circulation.

**2. Knudson's two-hit model for retinoblastoma.?**

In 1971, Knudson proposed the Two-hit model to explain the relationship between mutations and cancer. He proposed that the occurrence of the familial (heritable) and sporadic (non heritable) forms of retinoblastoma (rare childhood cancer of the retina of the eye occurring from birth to about four years) could be explained on the basis of two mutational events. In normal cells there are two wild type copies of the retinoblastoma gene ($RB^{+}/RB^{+}$) located on chromosome 1.

In sporadic (heritable) retinoblastoma, a rare mutational event in a cell inactivates one allele ($RB^{+}$/ RB). If a second mutation in a descendent cell inactivates the other allele (RB/RB), a tumour will develop. Thus both mutations leading to cancer occur in somatic cells. In familial (non-heritable) retinoblastoma, the offspring inherits one mutated defective RB gene from the parent through the germ line. All the cells are therefore heterozygotes ($RB^{+}$/RB), with one functional wild type gene ($RB^{+}$) and one nonfunctional mutant gene (RB). A single somatic mutation will result in the loss of function of both the genes (RB/RB), (RB/RB) leading to tumour development. Thus in both sporadic and familial retinoblastoma two mutational events (hits) are required for the cancer to develop. In sporadic retinoblastoma both mutations occur in the retinal cell of the patient i.e. they are somatic mutations. In familial retinoblastoma, one mutation is inherited from the parent through the germ line while other is a somatic mutation. In familial retinoblastoma, one mutation is inherited from the parent through the germ line while the other is a somatic mutation. Knudson's two-hit model for cancer development applies to only a few types of cancer. Other cancers are the result of mutations in several critical genes (multi step nature of cancer).

**3. What is Metastasis?**

Metastasis formation is the spread of cancer cells from a primary tumor to vital organs and distant sites in the cancer patient's body. This process is the end result of a complex series of genetic alterations, epigenetic events, and host responses. The tendency of a primary tumor to form a metastasis is the hallmark of malignant cancer. Metastasis formation associated with malignant transformation has significant diagnostic, prognostic, and therapeutic implications. After a diagnosis of cancer, the primary tumor is resected and histologic examination of this

tissue is performed for evidence of metastatic potential (local invasion). The patient also undergoes clinical staging for evidence of metastatic involvement of other organs. Major factors dictating the course of cancer treatment are the malignant potential of the primary tumor (i.e., presence of local invasion and regional lymph node involvement) and the presence of distant metastases. One of the major obstacles to effective cancer diagnosis is the detection of clinically occult metastatic disease. Once the primary tumor is resected, cancer therapy is directed at the elimination of metastases. The variation in size, age, dispersed anatomic location, and heterogeneous composition of metastases makes complete eradication of metastatic disease by currently available therapeutic strategies extremely difficult. Patients with metastases succumb to organ failure secondary to anatomic compromise of organ function by nonfunctional tumor tissue or to complications associated with systemic therapy directed against the metastatic disease.

The principal objectives of current research in cancer invasion and metastasis are improvement of diagnostic markers for detection of malignant potential and clinically silent (occult) metastasis formation, as well as the design of more effective therapies to treat metastatic disease. These goals require a better understanding of the molecular events, cellular processes, and host responses involved in metastasis formation. The focus of current research efforts is directed at understanding the origins of cancer metastasis, definition of malignant potential (i.e., delineating the metastatic propensity of a primary tumor from any given patient), and identification of genes specifically associated with metastasis formation as potential therapeutic targets.

**4. What is the Origins of Metastatic Tumor Cells?**

The central question with regard to metastasis formation, and with significant clinical implications for the diagnosis and treatment of cancer metastasis, is how metastasis arise from the primary tumor. Two dominant hypotheses have been formulated regarding the evolution of cancer metastasis. The following section briefly reviews the origins of these hypotheses.

Metastasis formation is the final step in tumor progression. This suggests that, like tumorigenesis, neoplastic cells with metastatic capacity arise through a process analogous to Darwinian natural selection aided by genetic instability. Initial genetic changes, mutations in specific oncogenes, and inactivation of tumor suppressor genes result in acquisition of a selective growth advantage that produces a dominant descendent population within the primary tumor mass. Subsequent genetic changes confer additional advantageous phenotypes that include self-sufficiency in growth signals (growth autonomy), insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, and the capacity for tissue invasion and metastasis. These changes and subsequent genetic or epigenetic alterations would dominate the primary tumor cell population through clonal expansion This clonal progression model is consistent with numerous studies demonstrating similar patterns of gene expression in

the primary tumor and metastases derived from the same patient. This model of metastatic progression suggests that evolution of metastatic competence is a generic predisposition that can be expressed at any time during the process of tumor development and that this phenotype will predominate in the primary tumor through clonal expansion.

In contrast, a second model suggests that metastases arise from rare, highly metastatic variants within the primary tumor. In this model, metastatic competence does not confer a selective growth advantage within the primary so that these rare tumor cells with metastatic capacity remain a minor cell population in the primary tumor, This model is principally based on classic animal model experiments of Fidler et al. showing that multiple variants of metastatic potential can be isolated from the primary tumor population by subcloning *in vitro* or by *in vivo* selection. This is an important concept in that it suggests that metastatic variants "preexist" in the primary tumor, that not all cells of the primary tumor population share the same propensity for metastasis formation, and that metastasis results from selection of an aggressive, rare subpopulation of tumor cells from the primary tumor population. Furthermore, this model of tumor progression suggests that the metastatic potential of the primary tumor would be determined by the size or behavior, or both, of this highly aggressive subpopulation. Therefore, detection of such rare variant cells within the primary tumor population would be of significant prognostic value. This would suggest that determination of the average metastatic potential of the entire primary tumor cell population, by assessment of molecular markers associated with metastatic potential, may not reflect the presence of these highly metastatic variants.

Are the clonal expansion and rare variant models for the origin of metastatic tumor cells mutually exclusive, or are they two conceptual frameworks in a continuous spectrum of mechanisms for metastasis formation? To study this experimentally, Kerbel exploited the random integration of foreign DNA into the tumor cell genome to tag metastatic cells isolated from a primary tumor population. Tumor cell clones tagged with different integration sites were mixed with a single tagged metastatic clone and inoculated subcutaneously into syngeneic experimental animals. The clonal evolution of primary tumors and metastatic foci were then followed over time by restriction fragment length polymorphism analysis of the foreign DNA integration sites. The results of these experiments indicate that a single clone, initially present in the mixture in as low as 1% to 2% of the tumor cell inoculum, grows to dominate the primary tumor and that this clone is metastatically competent. This suggested that if a rare metastatic variant preexists within the tumor cell population, it can, over time, overgrow the primary tumor mass. This mechanism is referred to as the *clonal dominance theory of cancer progression*.

The underlying implication of these studies is that metastatic competence and growth dominance in the primary tumor are somehow linked. If, instead of a single metastatic clone mixed with tagged primary tumor cells, the experiment was conducted by pooling a large number ($10^4$ or $10^5$) of metastatically competent tumor cell clones, the results were similar, in that

the tumor cell populations in primary and metastatic tumors were dominated by a few (less than 10) clones. These findings suggest that cells within the primary tumor that obtain metastatic potential also have a selective growth advantage. It must be noted, however, that the seemingly "genetically" convergent dominant clones, although homogeneous with respect to the tag used to identify them, are still heterogeneous in other characteristics due to the ongoing process of genetic instability. These other characteristics may be secondary, unrelated to growth or metastatic potential (referred to as *carrier mutations*), or primary in that they enhance the metastatic phenotype. Finally, the clonal dominance model of Kerbel suggests that the clonal progression and metastatic variant models for the evolution of metastatic tumor cells may contribute to the development of metastatic tumor cells and that these models are not mutually exclusive.

The consideration of these models of tumor progression and origin of metastatic tumor cells is increasingly important for the design of experiments to isolate and identify genes involved in tumor metastasis. With the advent of complementary DNA (cDNA) microarray technologies, researchers can simultaneously screen for the differential expression of thousands of genes in a single experiment. However, to obtain useful information from these experiments, we must understand the potential relationships between primary tumors and metastasis. For example, cDNA microarray experiments comparing the differential gene expression between a primary tumor and metastatic lesion from the same patient yield different information than an experiment that compares a series of nonmetastatic and metastatic tumors from different patients.

**5. Write briefly about the Metastatic Cascade.**

Investigators refer to the process of metastasis formation as the *metastatic cascade*. This conceptual framework divides the process into a series of discrete steps that can then be investigated for identification of cellular and molecular events requisite for metastasis to occur. It is well established, from clinical observations and from mechanistic studies, that metastasis formation is an inefficient process. What is the source of this inefficiency?

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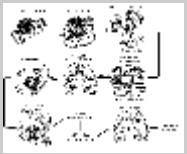
|  View Figure | Figure 4-2<br>The pathogenesis of cancer metastasis. To produce metastases, tumor cells must detach from the primary tumor, invade the extracellular basement membrane and enter the circulation, survive in the circulation to arrest in the capillary bed, adhere to subendothelial basement membrane, gain entrance into the organ parenchyma, respond to paracrine growth factors, proliferate and induce angiogenesis, and evade host defenses. The pathogenesis of metastasis is therefore complex and consists of multiple sequential, selective, and interdependent steps whose outcome depends on the interaction of tumor cells with homeostatic factors. |

Large numbers of tumor cells and tumor cell clumps are shed into the vascular drainage of a primary tumor. It has been demonstrated experimentally that, after intravenous injection of highly metastatic tumor cells, only approximately 0.01% of these cells will form tumor foci. The number of circulating tumor cells and tumor emboli correlates with the size and age of the primary tumor; that is, larger tumors shed more tumor cells and emboli. However, the number of circulating tumor cells does not correlate with the clinical outcome of metastases.

The inefficiency of tumor cells in completing the metastatic cascade is, in part, the result of the fact that successful formation of metastatic foci consists of several highly complex and interdependent steps. Each step is rate limiting in that failure to complete any of these events completely disrupts metastasis formation. Thus, the steps involved in metastasis formation also represent a Darwinian selection process. Only those tumor cells that have acquired sufficient genetic changes and accompanying alterations in gene expression can successfully complete the requisite events to allow metastasis formation.

Another source of inefficiency in the metastatic cascade is revealed by studies on tumor cells shedding into the circulation. In these experiments, highly metastatic tumor cells were grown subcutaneously in athymic nude mice and perfused *in vivo* to collect shed tumor cells. The shed tumor cells were collected and analyzed for clonogenicity in soft agar, resistance to apoptosis, and ability to form tumors *in vivo* after *in vitro* expansion and reinjection subcutaneously. These properties of the tumor cells shed into the circulation were compared with those of tumor cells isolated directly from the primary tumors by excision and dissociation into single-cell suspensions. Somewhat surprisingly, the authors found that shed tumor cells have a low metastatic potential compared to cells isolated directly from the primary tumor. Specifically, cells in shed tumor cell populations showed an increase in apoptosis (48%), compared with an average apoptotic fraction of 20% in the native tumors. This suggests that an additional source of inefficiency in the metastatic cascade is due to the fact that most of the tumor cells shed into the circulation are in the process of dying as they exit the tumor.[21] Thus, not all circulating tumor cells represent metastatically competent tumor cells capable of colonizing distant tissue sites.

As might be expected from the highly complex nature of metastasis formation, no single gene product is exclusively responsible for metastasis formation. Successful completion of many of the steps of the metastatic cascade is the result of acquisition of both positive effectors, as well as the loss of negative regulators. Unrestrained growth is not sufficient to result in tumor metastasis. Tumor metastasis is not a passive process secondary to tumor growth and requires additional genetic changes other than those associated with the tumorigenicity. Tumorigenicity and metastatic competence have some overlapping features but are clearly under separate genetic control. Ongoing research into the steps of the metastatic cascade has identified gene products that can facilitate completion of each of the steps outlined above. These are the molecular effectors of tumor metastasis. In many cases, research has also identified gene products that function to

block successful completion of each of the steps in the metastatic cascade—that is, metastasis suppressor genes. The idea that there is loss of negative effectors, as well as positive phenotypic changes associated with malignant progression and metastasis formation, is now well established.

**6. What is the Origins of Metastatic Tumor Cells ?**

The central question with regard to metastasis formation, and with significant clinical implications for the diagnosis and treatment of cancer metastasis, is how metastases arise from the primary tumor. Two dominant hypotheses have been formulated regarding the evolution of cancer metastases. The following section briefly reviews the origins of these hypotheses.

Metastasis formation is the final step in tumor progression. This suggests that, like tumorigenesis, neoplastic cells with metastatic capacity arise through a process analogous to Darwinian natural selection aided by genetic instability. Initial genetic changes, mutations in specific oncogenes, and inactivation of tumor suppressor genes result in acquisition of a selective growth advantage that produces a dominant descendent population within the primary tumor mass. Subsequent genetic changes confer additional advantageous phenotypes that include self-sufficiency in growth signals (growth autonomy), insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, and the capacity for tissue invasion and metastasis.[8] These changes and subsequent genetic or epigenetic alterations would dominate the primary tumor cell population through clonal expansion (see **Fig. 4-1**). This clonal progression model is consistent with numerous studies demonstrating similar patterns of gene expression in the primary tumor and metastases derived from the same patient. This model of metastatic progression suggests that evolution of metastatic competence is a generic predisposition that can be expressed at any time during the process of tumor development and that this phenotype will predominate in the primary tumor through clonal expansion.

In contrast, a second model suggests that metastases arise from rare, highly metastatic variants within the primary tumor. In this model, metastatic competence does not confer a selective growth advantage within the primary so that these rare tumor cells with metastatic capacity remain a minor cell population in the primary tumor (see **Fig. 4-1**). This model is principally based on classic animal model experiments of Fidler et al.[9,10] showing that multiple variants of metastatic potential can be isolated from the primary tumor population by subcloning *in vitro* or by *in vivo* selection. This is an important concept in that it suggests that metastatic variants "preexist" in the primary tumor, that not all cells of the primary tumor population share the same propensity for metastasis formation, and that metastasis results from selection of an aggressive, rare subpopulation of tumor cells from the primary tumor population. Furthermore, this model of tumor progression suggests that the metastatic potential of the primary tumor would be determined by the size or behavior, or both, of this highly aggressive subpopulation. Therefore, detection of such rare variant cells within the primary tumor population would be of

significant prognostic value. This would suggest that determination of the average metastatic potential of the entire primary tumor cell population, by assessment of molecular markers associated with metastatic potential, may not reflect the presence of these highly metastatic variants.

Are the clonal expansion and rare variant models for the origin of metastatic tumor cells mutually exclusive, or are they two conceptual frameworks in a continuous spectrum of mechanisms for metastasis formation? To study this experimentally, Kerbel[11] exploited the random integration of foreign DNA into the tumor cell genome to tag metastatic cells isolated from a primary tumor population. Tumor cell clones tagged with different integration sites were mixed with a single tagged metastatic clone and inoculated subcutaneously into syngeneic experimental animals. The clonal evolution of primary tumors and metastatic foci were then followed over time by restriction fragment length polymorphism analysis of the foreign DNA integration sites. The results of these experiments indicate that a single clone, initially present in the mixture in as low as 1% to 2% of the tumor cell inoculum, grows to dominate the primary tumor and that this clone is metastatically competent. This suggested that if a rare metastatic variant preexists within the tumor cell population, it can, over time, overgrow the primary tumor mass. This mechanism is referred to as the *clonal dominance theory of cancer progression*.[11]

The underlying implication of these studies is that metastatic competence and growth dominance in the primary tumor are somehow linked. If, instead of a single metastatic clone mixed with tagged primary tumor cells, the experiment was conducted by pooling a large number ($10^4$ or $10^5$) of metastatically competent tumor cell clones, the results were similar, in that the tumor cell populations in primary and metastatic tumors were dominated by a few (less than 10) clones. These findings suggest that cells within the primary tumor that obtain metastatic potential also have a selective growth advantage. It must be noted, however, that the seemingly "genetically" convergent dominant clones, although homogeneous with respect to the tag used to identify them, are still heterogeneous in other characteristics due to the ongoing process of genetic instability. These other characteristics may be secondary, unrelated to growth or metastatic potential (referred to as *carrier mutations*), or primary in that they enhance the metastatic phenotype. Finally, the clonal dominance model of Kerbel suggests that the clonal progression and metastatic variant models for the evolution of metastatic tumor cells may contribute to the development of metastatic tumor cells and that these models are not mutually exclusive.

The consideration of these models of tumor progression and origin of metastatic tumor cells is increasingly important for the design of experiments to isolate and identify genes involved in tumor metastasis. With the advent of complementary DNA (cDNA) microarray technologies, researchers can simultaneously screen for the differential expression of thousands of genes in a single experiment. However, to obtain useful information from these experiments, we must

understand the potential relationships between primary tumors and metastasis. For example, cDNA microarray experiments comparing the differential gene expression between a primary tumor and metastatic lesion from the same patient yield different information than an experiment that compares a series of nonmetastatic and metastatic tumors from different patients.

**7. How is Microarray Analysis of Tumor Progression and Metastasis performed?**

The introduction of cDNA microarray technology affords investigators the opportunity to examine changes in expression of thousands of genes in a single experiment. Such experiments have been used to explore the process of tumor progression and identify genes that may be involved in metastasis formation. Patterns of gene expression have been analyzed for a variety of primary tumors and their metastases. These studies include microarray profiling of primary tumor cells and metastatic lesions in animal models of tumor progression, as well as analysis of human tumor samples. The results of these experiments clearly indicate that specific patterns of gene expression can be associated with specific tumor subsets and clinical outcomes. However, these experiments also present new insights into the origins of metastatic tumor cells and the relationship between primary tumors and their metastases.

cDNA microarray (transcriptome) analysis of primary breast tumors and comparison of the gene expression profiles of nonmetastatic and metastatic primary tumors result in identification of a gene expression signature that predicts the probability of metastases.[12] In these experiments the gene expression profiles across approximately 25,000 genes were conducted using primary breast cancer tissue from two groups of patients: those in whom distant metastasis developed within 5 years and those who remained disease free after a period of at least 5 years. All of these patients with sporadic breast cancer were lymph node negative and under age 55 at the time of diagnosis. The authors found that some 5000 genes were significantly regulated across this group of patient samples and that simple hierarchic cluster analysis revealed two distinct groups of patients. In one group, only 34% of patients had metastasis within 5 years, in comparison with the second group, in which 70% of patients had metastatic progression. From this study the authors identified a set of 70 genes to establish a prognosis profile that would predict clinical outcome in node-negative primary breast cancer. Application of this gene expression profile demonstrated that it was a more powerful predictor of disease outcome than standard prognostic systems based on clinical and histologic criteria.[13]

Researchers have also compared the gene expression profiles of adenocarcinomas of multiple tumor types to unmatched primary adenocarcinomas.[14] In this study, the investigators examined the expression profiles of metastatic nodules and compared these with primary adenocarcinomas of similar tissue origin. These authors found a gene expression signature that distinguished primary from metastatic adenocarcinomas and that was associated with metastasis and poor clinical outcome. Further refinement of the initial gene set identified 17 unique genes

that recapitulated the distinction of primary tumors and metastasis across the entire set of tumor types. Application of this subset of 17 genes to specific tumor types, for example, breast cancer, lung cancer, prostate adenocarcinoma, and medulloblastomas (brain tumors), revealed the general utility of this subset in identifying primary tumors that were more likely to develop distant metastases, demonstrating the prognostic value of this approach.[14] This study also found a subset of primary tumors in which the gene expression signatures were identical to the metastatic tumors, an observation consistent with the clonal evolution model of cancer metastasis.

Primary breast carcinomas and metastases from the same patient may also show very similar gene expression profiles.[15] Examination of premalignant, preinvasive, and malignant mammary lesions, obtained by laser capture microdissection, revealed extensive similarities in the gene expression patterns (transcriptome) between these distinct stages of breast cancer progression.[16] Collectively, these findings suggest that the molecular program of a primary tumor may generally be retained in its metastasis, which is again consistent with the clonal evolution model for the origin of metastatic tumor cells. However, these methods cannot detect rare, highly metastatic variants within the primary tumor population and so do not exclude the presence of these variants or their contribution to metastasis formation.

Transcriptome analysis (cDNA microarrays) has also been used to examine the changes in gene expression that are associated with *in vivo* selection of highly metastatic tumor cell variants.[17] These experiments used techniques for the isolation of highly metastatic variants, for example, *in vivo* selection, identical to those originally used by Fidler et al.[9,10] Poorly metastatic melanoma cell lines, either the murine B16F0 or human A375P, were injected intravenously into the tail vein of host mice. Pulmonary metastases were isolated and reinjected via tail vein either two (A375) or three (B16) times for selection of highly metastatic tumor cell lines. The gene expression profiles of pulmonary metastasis were compared with the parental A375P or B16F0 cell lines grown as subcutaneous tumors. The results define a pattern of gene expression that involved 15 genes, independent of tumor site, that correlate with progression to a metastatic phenotype, independent of the tumor microenvironment. However, many of the differentially expressed genes that were identified in this study encode extracellular matrix (ECM) proteins, suggesting that enhanced expression of specific ECM proteins may promote tumor cell survival or angiogenesis, or both.[17] The pattern of transcriptome alterations observed in this animal model of progression are distinct and more restricted than those observed in studies of human clinical material. Although this is due in part to the use of tumor cell lines as a starting point, it also suggests that many of the cells within the heterogeneous tumorigenic populations of the parental tumor cell lines (A375P or B15F0) are genetically primed for acquisition of metastatic ability. This was demonstrated by additional experiments in which introduction of a single gene (RhoC), identified in the original screen, back into the parental cell population was sufficient to confer a high level of metastatic capacity.

These findings are supported by a study on the formation of osteolytic bone metastases in breast cancer. Kang et al.[18] intravenously injected the human breast cancer cell line MDA-MB-231 into athymic nude mice, which resulted in formation of metastasis in bone and the adrenal medulla. The authors isolated the cells from osteolytic bone metastasis and reinjected them to obtain cells with a stable elevated capacity to form bone metastasis. Transcriptome analysis (cDNA microarray) revealed that the parental MDA-MB-231 cell line, derived from a patient with metastatic breast cancer, demonstrated the poor prognosis gene expression signature previously defined using human clinical samples.[12] No enhanced expression of this poor prognosis pattern occurred in the osteolytic metastasis, but this comparison did yield a bone metastasis signature composed of a set of four genes. These four genes included the chemokine receptor CXCR4, interleukin-11, connective tissue growth factor, and matrix metalloproteinase 1 (MMP-1). Tumor cell populations expressing only one of these four genes were not more aggressive than the parental MDA-MB-231 cells, but expression of any three of the four genes resulted in metastatic activity that was intermediate between the parental cell and fully metastatic population (expressing all four genes). These findings suggest that the parental MDA-MB-231 cell population contains evidence of clonal evolution for the entire primary tumor cell population to a "poor prognosis" pattern of gene expression. However, superimposed on this poor prognosis signature are variant cells of high metastatic potential with gene expression profiles for metastasis at a specific tissue or organ site. These findings are consistent with our hypothesis that the "clonal evolution" and "metastatic variant" models of metastatic progression represent nonexclusive mechanisms for the development of site-specific metastatic tumor cells and that these models should not be considered mutually exclusive but rather opposite ends in the spectrum for the origin of tumor metastases.

Collectively, cDNA microarray data support the concept that clonal evolution and metastatic variants occur within the same tumor (see **Fig. 4-1**). The clonal evolution of this primary tumor results in a poor prognosis profile in tumors with enhanced metastatic potential. Superimposition of additional genetic changes (metastatic variants) on the poor prognosis profile of gene expression increases the potential for metastasis to a specific site or organ. These findings suggest that tumor formation and metastasis may be under independent genetic control.

**8. How is Determining Metastatic Potential and Site of Metastasis ?**

During investigation of the molecular events associated with specific steps in the metastatic cascade, investigators frequently use animal models of metastasis. In these assays, tumor cells are either injected into experimental animals to form a primary tumor site that subsequently metastasizes or directly into the circulation to model the later phases of metastasis formation. Animal models in which there is formation of a primary tumor and subsequent metastasis formation are known as *spontaneous metastasis* models. Intravenous injection of tumor cells, bypassing the molecular and cellular events associated with tumor invasion, focuses on

colonization of the metastatic site, and assays that use this technique are referred to as *tumor colonization* models. The end point in both types of assay systems is the formation of visible metastases at a secondary site, and both have led to the identification of specific molecular changes in tumor cells that contribute to metastatic competence. However, these end point assays are unable to examine the role of specific molecules at each individual step in the metastatic cascade.

Subcutaneous xenografts of human metastatic tumor cells in nude or severe combined immunodeficiency disease mice often fail to recapitulate the behavior of the parent tumor or demonstrate a spontaneously metastatic phenotype. However, when injected intravenously, many of these same human tumor cells are capable of forming metastatic colonies in the lung (colonization assays). In pioneering experiments, Fidler and colleagues demonstrated that the primary tumor site can directly influence metastatic potential through tumor-host interactions.[22] Using orthotopic (defined as in the normal or usual position, breast tumors in breast tissue) implantation of tumor xenografts, these investigators have demonstrated that the host microenvironment has a profound influence on a number of tumor cell parameters.[22] These include tumor growth, invasive behavior, response to chemotherapeutic agents, and growth factor and cytokine production, as well as protease profiles for urokinase and metalloproteinases. An important host contribution to tumor progression is the frequent association of MMP production by stimulated stromal fibroblasts adjacent to invading tumor cells.[23] In more recent studies, Fidler[24] has extended these findings to demonstrate that tumor-host interactions also influence gene expression patterns in metastatic foci and may contribute to tumor progression at these sites. These findings are supported by transcriptome analysis of metastatic human tumors in murine models, which show that successful formation of metastatic foci is associated with alterations in gene expression of host tissues.

These findings suggest that favorable tumor-host interactions may facilitate the outgrowth of metastatic tumor cells, whereas unfavorable interactions would suppress metastasis formation. This concept is embodied in the "seed-soil" hypothesis of metastasis originally put forth by Paget.[25] Paget noted the propensity for some types of cancer to produce metastasis in specific organs and that the metastatic site was not simply a matter of chance. This idea was later challenged by the proposal of James Ewing,[26] who suggested that the pattern of blood circulation leaving the site of the primary tumor was a principal determinant for the site of metastases.

However, these theories are not mutually exclusive, and evidence from more recent studies supports contributions from both mechanisms. For example, the direct drainage of the portal circulation through the liver can account for this organ as a principal site of metastatic involvement in patients with advanced colorectal carcinomas. However, in a study in which colorectal cancer cells of differing metastatic ability were implanted into the liver, it was found that growth regulation in the liver microenvironment influenced tumor cell growth, although the

molecular basis for these differences remains to be elucidated. As described above, the organ microenvironment can influence gene expression and response to chemotherapy. Investigators have identified soluble cytokines and cell-adhesion molecules in the tissue microenvironment that modulate tumor cell responses to chemotherapy and therapy-induced cell death. Another example is the influence of chemokine receptors on organ-specific metastasis formation. Chemokines are well characterized in their ability to modulate the "homing" of hematopoietic cells (lymphocytes) to specific organs. Studies have demonstrated that in some tumor types the malignant cells have a cytokine receptor pattern that allows homing to specific end organs. This occurs by "matching" the cytokine receptor expression on the tumor cells to the pattern of cytokines expressed in specific tissues. In breast cancer, for example, metastatic tumor cells express high levels of the chemokine receptors CXCR4 and CXCR7. These receptors have specific ligands, CXCL12 and CXCL21, which are expressed at high levels in bone marrow, liver, and lymph nodes, frequent sites of breast cancer metastases.[27] Neutralizing the CXCR4 receptor *in vivo* inhibited metastasis formation. Cytokine receptor expression has also been observed in cDNA microarray analysis of metastatic versus nonmetastatic tumors.[18]

The mechanisms of tumor cell intravasation have not been as systematically investigated as other events in the metastatic cascade. This is due in part to the lack of suitable model systems. A clear role for protease activity in tumor cell intravasation has been shown using the chick chorioallantoic membrane system.[28] In this model, human tumor cells are placed directly onto a chorioallantoic membrane in which the epithelium and basement membrane have been disrupted, allowing tumor cells direct access to the underlying connective tissue that is highly vascularized. Tumor cell intravasation is then quantified by using polymerase chain reaction amplification of human-specific Alu genomic DNA sequences of tumor cells present in the chorioallantoic membrane on the other side of the chick embryo from the initial tumor cell inoculation. These experiments demonstrate that MMPs, as well as urokinase-type plasminogen activator and the urokinase-type plasminogen activator-receptor, are involved in the escape of cells from the primary tumor.

Chambers et al.[29] have used intravital videomicroscopy to study the events and mechanisms involved in tumor cell exit from the circulation (extravasation). The results of these studies have profoundly changed current thinking about the metastatic process. It appears that circulating tumor cells may remain viable in the circulation and extravasate up to 3 days after their introduction into the circulation. Surprisingly, metastatic and nonmetastatic cells extravasate, and this process is not protease dependent. However, only a small subset (1 in 40) of cells grow and expand to form micrometastases, and even fewer (1 in 100) continue to grow, forming macroscopic tumors. Almost 40% of injected tumor cells remained as dormant solitary cancer cells. These findings suggest that the control of postextravasation growth of individual cancer cells is a dominant effect in metastatic inefficiency. Collectively, these data, as well as the studies on orthotropic effects, demonstrate that the local environment of the target organ and

primary tumor may profoundly influence the growth potential and metastatic competence of both the primary tumor and its metastases.

For the remainder of this chapter, the molecular events associated with tumor cell invasion and migration are examined, with the aim of identifying the molecular mechanisms, as well as effector and suppressor genes, that may become targets for new and effective cancer therapies. Immune modulation of cancer is discussed elsewhere, as is the process of tumor-associated angiogenesis.

**9. Explain about the Tumor Cell Motility.**

Cell motility is a critical component of the invasive phenotype. Understanding the molecular mechanisms that confer tumor cell motility should allow identification of novel targets for disrupting this process and preventing tumor dissemination. Tumor cell motility can be correlated with metastatic behavior. When parameters such as pseudopod extension, membrane ruffling, or vectorial translation are measured, there is a quantitative increase in metastatic tumor cells when compared with their nonmetastatic counterparts. A variety of stimuli have been shown to stimulate tumor cell motility *in vitro*, including host-derived factors, growth factors, and tumor-secreted factors that function in an autocrine fashion to stimulate tumor cell motility. Autocrine motility factor (AMF) is a 60-kD glycoprotein produced by human melanoma cells that stimulates tumor cell migration. AMF has been identified as neuroleukin/phosphohexose isomerase. Autotaxin (ATX), another autocrine motility agent, is a 125-kD glycoprotein that elicits chemotactic and chemokinetic responses at picomolar to nanomolar concentrations in human melanoma cells. ATX possesses 5'-nucleotide PDE (EC 3.1.4.1) activity,[30] binds adenosine triphosphate (ATP), and is phosphorylated only on threonine (Thr210), which is required for motility-stimulating activities. ATX possesses no detectable protein kinase activity toward histone, myelin basic protein, or casein. These results have led to the proposal that ATX is capable of at least two alternative reaction mechanisms, threonine (T-type) ATPase and 5'-nucleotide PDE/ATP pyrophosphatase, with a common site (Thr210) for the formation of covalently bound reaction intermediates, threonine phosphate and threonine adenylate, respectively.[30] The identification of AMF and ATX suggests that stimulation of tumor cell movement occurs in response to autocrine mechanisms that are unique to metastatic tumor cells.

**10. Describe about Tumor Invasion of the Basement Membrane.**

During the transition from benign to invasive carcinoma, extensive changes occur in the quantity, organization, and distribution of the subepithelial basement membrane. A primary histopathologic feature of malignant tumors is the disruption of the epithelial basement membrane and the presence of cancer cells in the stromal compartment.[31] Benign proliferative disorders such as fibrocystic disease, sclerosing adenosis, intraductal hyperplasia, intraductal

papilloma, and fibroadenoma are all characterized by disorganization of the normal epithelial architecture. No matter how extensive this disorganization may become, however, these benign lesions are always characterized by a continuous basement membrane that separates the neoplastic epithelium from the stroma.[31] In contrast, malignant tumors are characterized by a loss of basement membrane around the invasive tumor cells in the stromal compartment. Once the basement membrane barrier is compromised, it is impossible to determine the quantity or location of tumor cells that may have escaped from the primary tumor. Thus, local invasion is paramount to metastatic competence, which is the hallmark of malignant conversion.

The ability to invade across basement membrane barriers is not unique to malignant tumor cells, however. During an inflammatory response, nonneoplastic immune cells regularly cross the subendothelial basement membranes, as do endothelial cells during the angiogenic response. Trophoblasts invade the endometrial stroma and blood vessels to establish contact with the maternal circulation during development of the hemochorial placenta. Nonneoplastic invasive cells, such as trophoblasts, endothelial cells, and inflammatory cells, all use mechanisms for invasion that are functionally similar to those of tumor cells. The difference between these normal invasive processes and the pathologic nature of tumor metastasis is therefore one of regulation. An understanding of the factors that control cellular processes essential to cell invasion should allow identification of novel targets for therapeutic intervention to prevent and treat angiogenesis and inflammatory diseases, as well as tumor metastases.

**11. What is the Initiation of Cell Migration?**

The initial events in cellular migration are changes in cell adhesion. These changes consist of alterations in cell-cell adhesion as well as interactions of cells with the ECM. A variety of cell surface receptors that mediate these interactions have been characterized. These include the cadherins, integrins, immunoglobulin (Ig) superfamily members, and CD44. Tumor cells must decrease cell- and matrix-adhesive interactions to escape from the primary tumor. However, at later stages in the metastatic cascade, tumor cells may need to increase adhesive interactions with cells or ECM, or both, such as during arrest and extravasation at a distant site. The apparent contribution of each class of cell-adhesion molecule to invasive behavior will, in some way, be dependent on the tumor cell population and model system used to study these interactions. This chapter reviews the contribution of changes in cell-cell adhesion to tumor progression before considering alterations in tumor cell adhesion to the ECM.

**12. Write about Cell–Cell Adhesion and Metastasis Suppressor.**

The majority of human cancers arise in epithelial cells. Several types of junctional structures, such as desmosomes, tight junctions, and adherens-type junctions, tightly interconnect normal epithelial cells. The formation and maintenance of these contacts require $Ca^{2+}$-dependent

homophilic interactions mediated by the cell-adhesion molecules known as *cadherins* (**Fig. 4-3**). Cadherins are a superfamily of single-pass transmembrane glycoproteins that mediate $Ca^{2+}$-dependent cell–cell adhesion. The cadherin superfamily now consists of five subfamilies. These are the classic type I and type II cadherins, desmosomal cadherins, protocadherins, and cadherin-related proteins.[32] The classic cadherin, epithelial cadherin (E-cadherin) mediates homotypic cell adhesion in epithelial cells. E-cadherin is a transmembrane glycoprotein that has five extracellular homologous domains (ectodomains), a single membrane-spanning region, and a cytosolic domain. E-cadherin is physically anchored to the actin cytoskeleton by cytoplasmic proteins termed *catenins*. β-Catenin is also a major component of the wnt signaling pathway.

View Figure

Figure 4-3
Disruption of cell-cell adhesion concomitant with tumor progression. Epithelial cadherin (E-cadherin) is a homotypic cell-adhesion molecule containing five homologous, extracellular domains (ectodomains) that bind divalent calcium ions. Calcium binding promotes homophilic cell–cell E-cadherin complexes found in such structures as desmosomes, tight junctions, and adherens-type junctions. The cytoplasmic tail of E-cadherin involved in cell–cell adhesion interacts with β-catenin, α-catenin, and $p120^{CAS}$ (p120). Loss of E-cadherin function, by germline mutation, promoter hypermethylation, or destruction of the ectodomains by matrix metalloproteinase (MMP) activity, results in an increase in free cytosolic β-catenin levels. Increased cytoplasmic β-catenin can be directed to the proteosome complex by glycogen synthase kinase-3β (GSK3β) phosphorylation and subsequent interaction with the adenomatous polyposis coli (APC) gene product. The frizzled (FRZ)-disheveled (DSH) pathway for WNT signaling can down-regulate the activity of GSK3β. Activation of the WNT signaling pathway or loss of APC function facilitates the increase in cytosolic β-catenin levels that are associated with loss of E-cadherin function or mutations of the β-catenin gene that result in reduced association with E-cadherin cytoplasmic domain. Translocation of β-catenin to the nucleus results in association with members of the TCF/LEF-1 transcription factor family. This is associated with gene expression associated with cell transformation and tumor growth (i.e., c-Myc, cyclin D1). It is noteworthy that this cascade of cellular transformation can be initiated by expression of an extracellular protease that culminates in enhanced chromosomal instability. EGF, epidermal growth factor; P, phosphorylated amino acid residues; TCF, tissue coding factor. (From **ref. 36**, with permission.)

Any disruption of the intracellular E-cadherin–catenin complex results in loss of cell adhesion. This includes changes in E-cadherin expression or function, as well as genes other than E-cadherin required for junctional complex formation and function. Abundant evidence has been shown that E-cadherin function is frequently lost during progression of many human cancers,

including those arising in the breast, prostate, esophagus, stomach, colon, skin, kidney, lung, and liver.[33] This loss of E-cadherin function arises via several different mechanisms. In familial gastric carcinomas, germline mutations in the E-cadherin gene predispose an individual to the development of malignant cancer. Mutations in β-catenin are found in many primary tumors, including prostatic cancer and melanoma, as well as gastric and colon cancer. Another mechanism disrupting E-cadherin function during tumor progression is hypermethylation of the E-cadherin promoter, resulting in decreased gene expression. This has been found to be a major mechanism in papillary thyroid cancer in that 83% of cases demonstrated hypermethylation of the E-cadherin promoter.[34] Yet another mechanism to alter E-cadherin function is proteolytic modification. Lochter et al.[35] reported that E-cadherin function can be disrupted by degradation of E-cadherin extracellular domains by stromelysin-1, a member of the MMP family that has been closely linked with tumor progression. Constitutive expression of active stromelysin in mammary epithelial cells results in cleavage of E-cadherin and progressive phenotypic changes *in vitro*, including loss of catenins from cell-cell contacts, down-regulation of cytokeratins, up-regulation of vimentin, and MMP-9. These changes result in a stable epithelial-to-mesenchymal transition of cellular phenotype. *In vivo* stromelysin expression promotes mammary carcinogenesis that includes genomic changes that are distinct from those seen in other mouse breast cancer models.[36] It has been reported that loss of H-cadherin expression occurs during the progression of breast cancer, but little is known about the function of other cadherin family members during tumor progression. In summary, a decrease in cell–cell adhesion is associated with malignant conversion. Forced expression of E-cadherin in tumor cell lines results in reversion from an invasive to a benign tumor cell phenotype,[33] implicating E-cadherin as a metastasis suppressor.

In normal cells, β-catenin is sequestered in the intracellular adhesion complex with the cytoplasmic domain of E-cadherin, α-catenin, γ-catenin, and $p120^{CAS}$. Loss of cell–cell adhesion results in disruption of the adhesion complex and an increase in free cytosolic β-catenin. This free β-catenin is bound by the adenomatous polyposis coli (APC) gene product and is rapidly phosphorylated by glycogen synthase kinase-3β. Phosphorylated β-catenin is subsequently degraded in the ubiquitin-proteosome pathway. In many colon cancer cells, the tumor suppressor gene APC is nonfunctional. This can lead to accumulation of high levels of cytoplasmic β-catenin that are subsequently translocated to the nucleus. The wnt-1 protooncogene-initiated signaling pathway, which includes the frizzled and disheveled gene products, blocks the activity of the glycogen synthase kinase-β and results in accumulation of β-catenin. In the nucleus, free nonphosphorylated β-catenin can bind to members of the TCF/LEF-1 family of transcription factors. It has been demonstrated that, after inactivation of APC function, the increase in available cytosolic β-catenin results in translocation to the nucleus, where it complexes with transcription factor Tcf-4 and up-regulates c-Myc expression.[37] It has also been shown that β-catenin activates transcription from the cyclin D1 promoter and contributes to neoplastic transformation by causing accumulation of cyclin D1.[38] These findings link changes in cell–cell adhesion with intracellular signaling, oncogene expression, and tumor cell growth. Thus, loss of cadherin-

mediated cell–cell adhesion is an important event that has many far-reaching consequences for acquisition of the invasive phenotype and tumor progression.

Other types of cell–cell adhesive interactions can actually facilitate metastasis formation. They may be particularly important during tumor cell arrest and extravasation. These molecules include members of the Ig superfamily such as NCAM and VCAM-1. This superfamily has a wide variety of members involved in cellular immunity and signal transduction, as well as cell adhesion. Members of the Ig superfamily share the Ig homology unit that consists of 70- to 110-amino acid residues organized into seven to nine β-sheet structures. The diversity of superfamily members precludes generalization about their role in tumor cell invasion and metastasis. However, the role of one family member seems straightforward. VCAM-1 is an endothelial cell, cytokine-inducible, counter-receptor for VLA-4 integrin, also known as $\alpha_4\beta_1$-*integrin receptor*. The role of integrin receptors is discussed separately in Role of Integrins in Tumor Progression, later in this chapter. Normally, VLA-4 is expressed on leukocytes and functions in mediating leukocyte attachment to endothelial cells. VLA-4 is also found on tumor cells in malignant melanoma and metastatic sarcoma, but not in adenocarcinomas. It is thought that expression of VLA-4 may facilitate interaction of circulating tumor cells with endothelium before tumor cell extravasation. This was demonstrated by intravenous injection of human melanoma cells into nude mice pretreated with VLA-4–inducing cytokines, which results in an enhanced number of lung metastases compared with no cytokine pretreatment of the mice.[39] Cell-cell adhesive interactions can either suppress or facilitate metastasis formation. Either role is dependent on the specific context and molecular mechanisms of cell–cell interaction.

**13. Write about Cell Matrix Interactions and Tumor Cell Migration.**

As stated previously in Initiation of Cell Migration, the interaction of the tumor cell with the ECM, in particular the basement membrane, defines the invasive phenotype, and tumor invasion is paramount to metastasis. It is now recognized that the ECM exerts a profound influence on the behavior of nonneoplastic cells and that cells can direct the assembly/disassembly of the matrix. This concept is known as *dynamic reciprocity* and also applies to the interaction of malignant tumor cells with the ECM. During the process of metastasis formation, malignant tumor cells must interact with a variety of different types of ECM. These include the subepithelial basement membrane of the tissue of origin, stromal elements of the tissue of origin, subendothelial basement membranes during extravasation, and the stromal matrix and basement membranes of the organ(s) at the site of metastasis growth. Attachment of nonneoplastic cells to the ECM is prerequisite for cell survival. A fundamental difference for neoplastic cells is the loss of anchorage requirement for cell survival and growth. The anchorage-independent growth of tumor cells may result from an uncoupling of cell survival signals transduced from the ECM via ECM receptors together with autonomous growth mechanisms associated with neoplastic transformation. Tumor cell interactions with the ECM have profound implications for cell-cycle regulation and for migration.

## 14. What is the Role of CD44 in Tumor Invasion and Metastasis?

CD44 is a transmembrane glycoprotein with a large ectodomain and single cytoplasmic domain. CD44 is involved in cell adhesion to hyaluronan (HA). The gene encoding CD44 is on the short arm of human chromosome 11 and contains constant and variable exons.[40] As a result of this gene structure, a number of differentially spliced isoforms of CD44 can be generated. The isoform containing no variant exon sequences is referred to as *standard CD44* (CD44s). A total of nine variant regions can encode protein sequences v2 to v10. Alternatively, spliced messenger RNA variants of CD44 (CD44v) can contain one or more variant coding regions. More than 30 different splice variants have been detected by polymerase chain reaction analysis. In addition to these variants, there are also cell type–specific differences in glycosylation of the core protein. The pattern of glycosylation and presence of variant exons influence the ability of CD44 to function in HA binding.

Several lines of evidence suggest that CD44 expression plays a role in metastasis formation. Clinical studies demonstrate that a variety of different types of cancer express high cell surface levels of CD44, which correlate with a poorer clinical outcome compared with tumors that have low CD44 surface expression.[41,42] Forced expression of CD44 v4 to v7 confers metastatic ability to a nonmetastasizing rat pancreatic carcinoma cell line, and metastasis formation could be blocked using anti-CD44 variant–specific antibodies. However, the exact role of CD44v in metastasis formation remains elusive.

In some tumors, CD44-associated increases in tumor growth and metastatic potential correlate with CD44-mediated cell attachment to HA.[43] CD44 also functions in HA uptake and degradation correlated with invasive tumor cell behavior.[44] These studies demonstrate that CD44 aggregation on the cell surface creates a binding site for the active MMP-9. It is postulated that bound MMP-9 may liberate ECM-bound HA and facilitates tumor cell HA uptake and degradation. These findings link cell adhesion and ECM turnover. In addition, they suggest that CD44 may function at different stages of tumor cell invasion and metastasis and that the specific CD44 role may depend on the specific stage of metastasis formation that is examined.

## 15. What is the Role of Integrins in Tumor Progression?

Integrins are heterodimeric transmembrane proteins that are formed by the noncovalent association of αand βsubunits.[45,46] Considerable redundancy within cell-ECM interaction mediated by integrins exists, as most integrins bind to several individual matrix proteins, and ECM components, such as laminin, fibronectin, vitronectin, and collagens, can bind to several different integrin receptors. This suggests that integrins are capable of providing the cell with detailed information about the surrounding ECM environment, which is then integrated at the cellular level to generate a cellular response (**Fig. 4-4**). It is now well established that integrins can signal

across the cell membrane in both directions.[47] Binding of ECM ligands to integrins is known to initiate signal transduction pathways that can result in cell proliferation, differentiation, migration, or cell death (apoptosis, anoikis). This is referred to as *outside-in signaling*. It is also known that intracellular events can modulate the binding activity of integrins for their ligands in the ECM; this is referred to as *inside-out signaling*. Integrin clustering and ligand occupancy are crucial for the initiation of intracellular integrin-mediated signal pathways.

View Figure

Figure 4-4
Role of integrins in tumor cell invasion and metastasis: integration of kinase and phosphatase activities. Binding of extracellular matrix (ECM) components to integrin receptors initiates an intracellular signaling cascade that results in formation of a focal adhesion complex that consists of cytoskeletal and signal transduction molecules. Ligand binding to the integrin receptor results in integrin clustering and association of signal transduction molecules. Integrin receptor clustering induces autophosphorylation of focal adhesion kinase (FAK) on tyrosine 397. Subsequently, an Src homology 2 (SH2)-containing (Shc) adapter protein of the Src kinase family that binds to specific phosphotyrosine residues (Y397) on FAK is recruited to the integrin-FAK complex. Recruitment of additional proteins, such as α-actinin, talin, and paxillin, to this complex connects the focal adhesion complex to the filamentous actin cytoskeleton. Interaction of Shc with FAK results in additional sites of phosphorylation on the FAK molecule and subsequent recruitment of additional SH2 adapter proteins, such as Grb2 and the nucleotide exchange factor Sos. These interactions lead to activation of the mitogen-activated protein (MAP) pathway that stimulates tumor cell growth, adhesion, and migration. Similarly, receptors for growth factors can transiently associate with the focal adhesion complex to synergistically activate the MAP kinase pathway. FAK activation also acts upstream of the Akt/protein kinase B (PKB) signaling pathway that promotes cell survival. Association of the p85 subunit of phosphatidylinositol 3 kinase (PI3K) with tyrosine 397 in FAK mediates this effect. The rapid elevation of phosphatidylinositol(3,4,5)triphosphate ($PIP_3$) lipid product of PI3K activity stimulates the Akt/PKB pathway, leading to enhanced cell survival. Crk-associated substrate ($p130^{CAS}$) is another SH2- and SH3-containing signal transduction that associates with FAK on integrin binding to the ECM. Interaction of $p130^{CAS}$ with FAK is mediated by a proline-rich region on FAK (residues 712-178) that interacts with the SH3 domain of $p130^{CAS}$. Activation of $p130^{CAS}$ promotes cell migration and invasion, which are associated with enhanced metastatic behavior. The tumor suppressor gene PTEN inhibits cell adhesion, migration, and invasion. This inhibition is mediated by direct PTEN dephosphorylation of FAK and Shc. This leads to negative regulation of the $p130^{CAS}$ pathway that affects cell attachment, migration, and invasion. Down-

| | |
|---|---|
| | regulation of the MAPK pathway by PTEN dephosphorylation of FAK and Shc negatively affects cell growth in addition to attachment and migration. PTEN is also known to directly dephosphorylate $PIP_3$ and negatively regulate the downstream Akt/PKB cell survival pathway. PTEN may also disrupt this pathway indirectly by dephosphorylation of FAK, which alters PI3K activation. Thus, integrin-mediated regulation of cell growth, adhesion, migration, and invasion is a complex network of signal transduction cascades that have positive (kinase) and negative (phosphatase) regulatory elements. EGF, epidermal growth factor; Erk, extracellular signal–regulated kinase; MEK, MAP kinase or ERK kinase. |

The roles of specific integrins in tumor progression and metastasis formation are dichotomous. The decreased expression of some integrins is associated with cellular transformation and tumorigenesis. These include $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins. HT-29 colon carcinoma cells lacking $\alpha_5\beta_1$ expression were either significantly less tumorigenic or completely nontumorigenic when forced to express $\alpha_5$.[48] Loss of $\alpha_2\beta_1$ expression in breast epithelial cells correlates with the transformed phenotype, and reexpression of this integrin abrogates the malignant phenotype.[49] On the other hand, expression of some integrins directly correlates with tumorigenicity and tumor progression. For example, the $\alpha_v\beta_3$ integrin is expressed in metastatic melanoma but not in benign melanocytic lesions.[50] Antibodies against $\alpha_v$ integrins blocked the growth of human melanoma xenografts in nude mice.[51] Integrin $\alpha_6$ expression is increased in oropharyngeal and bladder cancers as well as lung tumors.[39] Tumor progression is associated with expression of $\alpha_3\beta_1$ in 82% of tumors. The molecular events associated with enhanced expression of this integrin and tumor progression are not well defined. The role of integrins in tumor invasion and metastasis may only be secondarily related to growth control. Integrins are also directly involved in cell migration.

Integrin-mediated signal transduction involves direct activation of signaling pathways and collaborative signaling (also referred to as *cooperative signaling*), in which integrins modulate signaling events initiated through receptor tyrosine kinases.[52] The *cis* association of integrins with other receptors on the same cell surface results in formation of multireceptor complexes. Little evidence has been shown that these complexes signal exclusively through integrin-specific pathways; instead they cooperate with the other receptors to influence a variety of signaling pathways. Direct integrin signaling starts after the engagement of these receptors with their cognate ECM ligands, which results in lateral clustering of the integrin receptors. Lateral aggregation leads to interaction of the integrin cytoplasmic domains to form complexes that link to the cytoskeleton. This results in organization of cell structures known as *focal adhesions*.

Autophosphorylation of the focal adhesion kinase (FAK) was among the first integrin-mediated signaling events to be identified. This autophosphorylation event on tyrosine 397 results in recruitment of Src family protein kinases that in turn leads to phosphorylation of

additional tyrosine residues on FAK. Phosphorylation of FAK can result in activation of the extracellular signal–regulated kinase/MAPK pathway. Activation of the MAPK pathway has been linked to induction of cell migration. The activation of MAPK can be mediated by Grb-2 recruitment to FAK, which then binds Sos, a guanine nucleotide exchange factor, for Ras.[53] Alternatively, activation of the MAPK pathway can result from FAK phosphorylation of paxillin and the Crk-associated substrate (p130Cas) that leads to binding of link proteins, such as Nck. In addition, phosphorylation of FAK at tyrosine 397 creates a binding site for the regulatory subunit of phosphoinositol 3 kinase and triggers activation of this signaling pathway.

The knowledge that FAK is tyrosine phosphorylated on integrin activation suggests that focal adhesion-associated protein tyrosine phosphatases (PTP) could modulate Fak function. Several PTPs that interact with components of the focal adhesion complex have been identified. These are PTP αand PTP-PEST (PTP rich in proline, glutamic acid, serine, and threonine), which negatively regulate Src and paxillin, respectively.[54] It has been shown that PTP-PEST–deficient cells have a defect in cell motility that correlates with an increase in the size and number of focal adhesions.[55] This defect appears to be due to in part to the constitutive increase in tyrosine phosphorylation of paxillin, as well as p130CAS and FAK.

A PTP that interacts directly with FAK is PTEN. PTEN was identified as a tumor suppressor gene on human chromosome 10q23, which is frequently mutated or deleted in a wide variety of human cancers, including gliomas, prostate, breast, lung, bladder, endometrial, kidney, and oropharyngeal cancers.[53,56] This gene encodes a phosphatase domain and also has extensive sequence homology to the cytoskeletal protein, tensin. PTEN functions as a dual-specificity phosphatase in that not only is it a PTP but it also dephosphorylates the lipid phosphatidylinositol(3,4,5)triphosphate (PIP3). The current view is that phosphatase activities function as tumor suppressor activities. The lipid phosphatase activity regulates levels of PIP3 that in turn regulate activation of the protein kinase B (PKB/Akt) pathway, which is protective against programmed cell death (apoptosis, anoikis). As a PTP, PTEN can also regulate the phosphorylation status of FAK and Shc, which, in turn, regulate cell adhesion, migration, cytoskeletal organization, and MAPK activation.

To summarize, loss of PTEN function results in alterations in integrin-mediated signaling via the FAK and PIP3 pathways, which results in a migratory and invasive phenotype.[53,56] The protein kinase activities function to promote cell invasion. The activities of these kinases are countered by the PTPs, which act as tumor suppressors.

**16. Write the Role of Proteases in Tumor Cell Invasion.**

Proteolytic remodeling of the ECM has been recognized as essential for tumor cell invasion. Tumor cells must be able to move through connective tissue barriers such as the basement membrane and interstitial matrix to spread from their site of origin. Although a variety

of proteases have been implicated in this process, the family of proteases that has received the most attention has been the MMPs. MMPs and their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), play important roles in physiologic remodeling of the ECM. Approximately 26 MMPs and four TIMPs have been characterized in humans and other animals.[23,65] The MMPs share a common domain structure (**Fig. 4-6**), although not all domains are represented in all family members. All of the enzymes have a signal peptide sequence; a propeptide domain (prodomain); a catalytic domain, which includes a highly conserved binding site for the catalytic zinc ion; and a hemopexin-like domain. Two family members, MMP-2 and MMP-9, have a gelatin-binding domain containing three fibronectin type II repeats inserted into the catalytic domain just on the amino side of the active site sequence. Five family members have a carboxy-terminal transmembrane domain after the hemopexin domain. This subgroup is also known as the *membrane-type MMPs*, or *MT-MMPs*. These MT-MMPs reside on the cell surface, in contrast to the other family members, which are all secreted as proenzymes into the extracellular milieu.

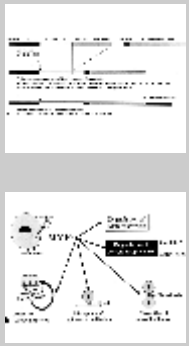


Figure 4-6
Matrix metalloproteinase (MMP) family members. **A:** Domain structure of MMPs. Simplified schematic domain structure of the MMPs. Most MMPs have the same basic domain structure, consisting of signal, pro, catalytic, hinge, and hemopexin-like domains. MMP-7 lacks the hemopexin-like domain, and the gelatinases and membrane-type MMPs have additional domains. The arrow indicates the location of the furin RXKR cleavage site of MMP-11 and the membrane-type MMPs. The length of each segment is roughly proportional to the number of amino acids that comprise the domain. **B:** Emerging roles of MMPs in tumor progression.

The TIMPs also share a high level of homology, including 12 conserved cysteine residues that are all involved in intramolecular disulfide bonds. TIMPs are divided into two domains by the disulfide-bonding pattern. An amino-terminal domain contains the inhibitory site and a carboxy-terminal domain has other binding interactions. Because most of these enzymes are secreted in their proenzyme forms, activation is a key regulatory step. Many of the MMPs are activated by an initial protease cleavage with the prodomain by another MMP or by a serine protease such as plasmin or urokinase-type plasminogen activator. This destabilizes the bond between a conserved prodomain cysteine sulfhydryl group and the catalytic zinc in the active site. The bond breaks and the prodomains are released, which frees the active site for catalysis. Unlike the other family members, which are typically activated outside the cell, MMP-13 and the MT-MMP subgroup are activated intracellularly by a furin-dependent cleavage of a conserved RXKR sequence that lies between the prodomain and the catalytic domain. The activation mechanisms have been the subject of a review.

An early indication of the importance of MMPs in tumor biology was the characterization in 1980 of an MMP secreted from a melanoma cell line that was able to degrade basement

membrane collagen type IV and was initially referred to as *type IV collagenase* (now MMP-2).[67] This was followed by numerous studies showing that secretion of MMPs enhanced tumor cell invasion in experimental model systems and that inhibition of protease activity by TIMPs or by synthetic metalloproteinase inhibitors impeded invasion. For example, the invasion of HT-1080 fibrosarcoma cells through Matrigel (a reconstituted basement membrane) is enhanced by the addition of activated MMP-2 and inhibited by the addition of TIMP-2 or by zinc chelators.[68] Such *in vitro* evidence has been supported by the results of *in vivo* experiments using transfected cell lines. For example, when MYU3L bladder carcinoma cells are transfected with *mmp-2*, they have enhanced metastatic potential, whereas transfecting the highly metastatic LMC19 cell line with *timp-2* reduces its metastatic potential. Mice that are genetically engineered to overexpress MMP-3 in breast epithelial cells develop spontaneous breast carcinomas, possibly through the effects of MMP-3 on the E-cadherin/β-catenin system.[36] These experiments and many others like them have demonstrated the key role of MMP-initiated degradation in tumor invasion and metastasis. The ability of TIMPs to inhibit tumor growth and metastasis suggests that targeted inhibition of MMPs could be an effective therapeutic strategy for cancer therapy. However, clinical trials of synthetic MMP inhibitors have been disappointing.

The role of these enzymes in this process is more complicated than a "degradation equals invasion" paradigm would suggest. Uninhibited matrix degradation may lead to complete dissolution of matrix proteins and would prevent tumor cells from being able to form attachments to each other or to matrix proteins, which is a necessary part of the tumor invasion mechanism. Thus, there is an implied balance between active proteases and inhibitors that would result in an optimal invasive phenotype. As a demonstration of this principle, when A2058 melanoma cells are transfected with either sense or antisense *timp-2*, the invasive potential is decreased. Increasing TIMP-2 expression in this cell line enhances cell attachment and decreases motility, whereas decreasing expression decreases cell attachment and motility. Thus, although protease activity in tumor cell invasion is abnormal, it cannot be totally unregulated.

Another possible explanation for the ineffectiveness of synthetic metalloproteinase inhibitors in clinical trials is that MMPs have been shown to have effects other than removal of structural barriers to invasion.[65] For example, some MMPs act on other proteins to reveal hidden biologic activities. MMPs, such as MMP-2 and MT-1-MMP, specifically degrade the γ2 chain of laminin-5, a structural protein in the basement membrane, to reveal a site on the α3 chain that has chemotactic properties.[69] Although the physiologic role of this fragment may be to act as a wound-related chemoattractant, in tumors this peptide may attract tumor cells to breaks in the basement membrane. In contrast, MMPs have also been shown to process monocyte chemoattractant protein-3 (MCP-3).[70] However, in this case the MMP processing of MCP-3, which results in removal of five amino acids from the N-terminus, results in loss of chemoattractant function and conversion of MCP-3 into an agonist that blocks inflammatory cell recruitment. Further complicating their role in tumor progression, MMPs have been shown to degrade

plasminogen into angiostatin, the angiogenesis inhibitor.[71] Studies have focused on the interaction between cell surface adhesion molecules and the MMP family. One obvious interaction is the simple degradation of cell surface adhesion molecules. For example, MMP-3 degrades E-cadherin on mammary epithelial cells, inducing an increased expression of vimentin and decreased expression of keratin.[35] The cell matrix adhesion molecule CD44 is also cleaved by MMPs, permitting detachment from the matrix.[72] Decreased cell-cell or cell matrix adhesion can be a proinvasive phenotype. However, beyond mere degradation of adhesion molecules, cells may control the scope of degradative activity by binding the soluble MMPs with cell surface adhesion molecules, thus limiting degradation to a zone in the immediate vicinity of the cell. On endothelial cells, the integrin $\alpha_v\beta_3$ binds MMP-2 through the MMP's hemopexin domain in response to angiogenic stimuli.[73] Similarly, CD44 has been shown to bind MMP-9 to the surface of breast carcinoma and melanoma cells.[44] Interestingly, stimulation of melanoma cells by antibodies to CD44 increased expression of MMP-2,[74] suggesting that cell surface receptors might not only provide a mechanism for localizing protease activity but also may serve to initiate an autocrine-stimulating loop mechanism for increased expression of MMPs.

## UNIT – V

## PART – A

**1. What is Gene therapy?**

Treating genetic diseases by inserting normal copies of genes into the cells of people who have defective, disease causing genes.

**2. What is DNA Micro Array?**

Tiny chip of glass or plastic that has been spotted at fixed locations with thousands of different single-stranded DNA fragments; used for monitoring gene expression.

**3. What is Dysplasia?**

Abnormal tissue growth in which cell and tissue organization is disrupted; may be an early stage in cancer development.

**4. What is hyperthermia?**

Raising the temperature of body tissues a few degrees to sensitize cancer cells to the killing effect of radiation used in cancer treatment.

**5. What is Radiation therapy?**

A form of cancer treatment that uses high –energy x-rays or other types of ionizing radiation to kill cancer cells.

**6. Write shortly about platinating agent.**

Highly reactive platinum – containing compounds, such as cisplatin, that are used in cancer chemotherapy; cross link DNA by forming chemical bonds between the platinum atom and DNA bases.

**7. What is Mannography?**

It is a screening technique for breast cancer that uses low-dose x-rays to create detailed pictures that reveal the internal tissues of the breast.

**8. What is Anti metabolites?**

Molecules that resemble substances involved in cellular metabolism and that interact with enzymes in place of the normal substance, thereby disrupting metabolic pathways; used in cancer therapy.

**9. What is Alkylating agents?**

Highly reactive organic molecules that trigger DNA damage by linking themselves directly to DNA; used in cancer chemotherapy, but many are also careinogenic.

**10. What is Antibiotic?**

Natural substance produced by a micro organisms, or a synthetic derivative, that kills or inhibits the growth of other micro organisms or cells.

**11. What is Brachy therapy?**

Type of cancer treatment that uses a radiation source, such as tiny pellets, that can be inserted directly within (or close to) the tumour.

**12. What are differentiating agents?**

Substances that promote the process by which cells acquire the specialized characteristics of differentiated cells.

**13. What is Chemotherapy?**

Chemotherapy uses drugs that circulate through the blood stream to reach cancer cells wherever they may reside. Most chemotherapeutic drugs act by disrupting DNA structure, interfering with DNA Replication, or blocking cell division.

**14. What is Anti-angiogenic therapy?**

Anti-angiogenic therapies, which target the blood vessels needed by growing tumours, are receiving considerable attention because of the success seen with this approach in animal studies.

**15. What is Immunotherapy?**

Immunotherapies exploit the ability of the immune system to recognize and attack cancer cells. BCG and cytokines are relatively non specific approaches that stimulate the overall activity of the immune system rather than targeting cancer cells specifically.

**16. What are the strategies used for Gene therapy?**

Many human cancers exhibit defects in the P53 gene. If these defects could be corrected, restoration of theP53 pathway might cause cancer cells to self-destruct by apoptosis. Viruses engineered to contain a normal copy ofP53 gene have therefore been used in gene therapy experiments to infect tumours and insert the normalP53 gene into the DNA of cancer cells.

**17. What is Adoptive-cell–Transfer Therapy?**

ACT therapy is an alternative to vaccination in which a patient's own lymphocytes are isolated and grown in the laboratory to enhance their cancer-fighting properties prior to injecting them back into the body. Before reintroducing the anti tumour lympocytes, the patient is treated with high-dose chemotherapy to destroy most existing lymphocytes and thereby make room for the incoming anti tumour lympocytes, which become a large portion of the person's immune system.

**18. What is Monoclonel Antibody?**

Purified antibody directed against a single antigen; obtained using a laboratory technique for producing and selecting cloned populations of antibody – producing cells called hybridomas.

**19. What are the different forms of therapy used in the cancer?**

The ultimate goal of traditional cancer treatments is the complete removal of destruction of cancer cells accompanied by minimal damage to normal tissues. This goal is usually perused through a combination of surgery (when possible) to remove the primary tumour, followed (if necessary) by radiation, chemotherapy, or both to destroy any remaining cancer cells.

**20. What are the types of cancers are very responsive to radiation?**

1. Hodgkin's disease
2. Non-Hodgkin's lymphomas
3. Seminoma (testicles)
4. Neuroblastoma
5. Retinoblastoma

**21. What are the types of cancers poorly responsive to radiation?**

1. Melanoma
2. Glioblastoma
3. Kidney cancer
4. Pancreatic cancer
5. Sarcomas

## PART – B

**1. What are the types of surgery method used in cancer treatment?**

1. By 1980, the first Mastectomy – that is, complete removal of the breast in women with breast cancer followed by the development of surgical techniques for removing tumors from virtually every organ of the body.
2. Laser surgery utilizes a highly focused beam of laser light to cut through tissue or vaporize certain cancers, such as those occurring in the cervix, larynx, liver, rectum or skin.
3. Electro surgery – which involves high frequency electrical current, is sometimes used to destroy cancer cells in the skin and mouth.
4. Cryosurgery: Involves the use of a liquid nitrogen spray or a very cold probe to freeze and kill cancer cells. This technique is utilized for the treatment of certain prostate cancers and for precancerous conditions of the cervix such as dysphasia.
5. High – intensity focused ultra sound (HIFU) is a technique that focuses acoustic energy at a selected location within the body, where the absorbed energy heats and destroys cancer cells with minimum damage to surrounding tissues.

**2. What are difficult arised in surgery treatment?**

When cancer is diagnosed before a primary tumour has spread to other sites, surgical removal of a tumour can usually cure the disease. In fact, most cancer cures are achieved in this way. But cancers arising in internal organs are difficult to detect in their early stages and have often metastasized by the time they are diagnosed. Some times the metastatic tumours formed at distant sites are large enough to also be detected and surgically removed; in other cases, the body has simply been seed4ed with tiny clumps of cancer cells, known as micrometastases that are too small to be detected. Because roughly half of all cancers, (excluding skin cancers) have started to metastasize by the time they are diagnosed, surgical removal of the primary tumour is frequently followed by radiation, chemotherapy, or both to attack any disseminated cells that were not removed during surgery.

**3. What is the upgraded surgical treatment for breast cancer?**

The standard treatment for breast cancer between 1900 and 1970 was the radical mastectomy, a drastic and disfiguring operation that involves complete surgical removal of the breast along with the underlying chest muscles and lymph nodes of the armpit. From 1970 to 1990, the most common procedure was the modified radical mastectomy, which involves removal of the breast and lymph nodes but not chest muscles.

Today more than half of all breast cancer patients are treated by partial mastectomy (lumpectomy), which removes just the tumour and a small amount of surrounding normal tissue

surgery is usually followed by radiation therapy to the breast to destroy any cancer cells that may remain in the area.

**4. How is the Radiation therapy kills cancer cells?**

One type of treatment is the radiation therapy, which uses high-energy x-rays or other forms of ionizing radiation to kill cancer cells, Ionizing radiation removes electrons from water and other intracellular molecules, thereby generating highly reactive free radicals that attack DNA.

High doses of radiation kill cancer cells in two different ways. First, DNA damage caused by the radiation treatment activates the P53 signaling pathway, which triggers cell death by apoptosis. Lymphomas and cancers arising in reproductive tissues are particularly sensitive to this type of radiation – induced apoptosis.

Lymphomas and cancers arising in reproductive tissues are particularly sensitive to this type of radiation-induced apoptosis. However, more than half of all human cancers have mutations that disable the P53 protein or other components of the P53 signaling pathways. As a consequence, P53-induced apoptosis plays only a modest role in the response of most cancers to radiation treatment.

Radiation also kills cells by causing chromosomal damage by that is so severe that it prevents cells from progressing through mitosis, and the cells die while trying to divide. Because this process of mitotic death only occurs at the time of cell division, cells that divide more frequently are more susceptible to mitotic death than cells that divide less frequently. This difference in susceptibility makes rapidly growing cancers more sensitive to the killing effect of radiation than slower – growing cancers and also helps protect non-dividing or slowly dividing normal cells in the surrounding tissue from being killed by the radiation.

**5. What are the methods used to improve the effectiveness in the radiation therapy?**

1. Radiation therapy is usually divided into multiple treatments administered over several weeks or months.
2. Brachy therapy – uses a radiation source that can be inserted directly within (or close to) the tumors ex. early stage prostate cancer is some times treated by implanting small radioactive pellets, about the size of a grain of rice, directly into the prostate gland. The pellets emit low doses of radiation for weeks or months and are simply left in place after the radiation has all been emitted. The advantages of this radiation is that most of the radiation is concentrated in the prostate gland itself, sparing surrounding tissues such as the bladder and rectum.

3. Hypoxic radio sensitizers:- Mimics oxygen and are taken up by cancer cells, which frequently tend to be hypoxic (deficient in oxygen). Radiation creates more cellular damage in the presence of adequate oxygen, so the uptake of these drugs by cancer cells increase the effectiveness of radiation treatments. Combining radiation treatments with certain anti cancer drugs, such as fluorouracil and platinum compounds, can likewise enhance the effectiveness of radiation treatment.
4. Hyperthermia – Raising the temperature of tumours tissue by a few degree – a technique known as hyperthermia – also sensitizes cells to the killing effect of radiation. The combination of radiation and hyperthermia is most effective for tumours that are located in relatively accessible regions of the body, where the applied head can thoroughly penetrate the tumour tissue. The main difficulty with this approach is finding ways of applying heat to hard-to-reach turmours located deep inside the body.

**6. What is chemotherapy? List out drugs used?**

Which involves the use of drugs that either kill cancer cells or interfere with the ability of cancer cells to proliferate.

Chemotherapy is especially well suited for treating cancers that have already metastasized because drugs circulate through the blood stream to reach cancer cells wherever they may have spread, even if the metastasizing cells have not yet formed, visible tumours. This also means, however, that the toxic side effects commonly associated with chemotherapy can occur anywhere in the body because most anticancer drugs, like radiation, are toxic to dividing cell is general.

Despite its various side effects, chemotherapy has been successfully applied to a wide range of cancers. In some cases, as with certain forms of leukemia, chemotherapy may cure cancer itself. Dozens of anticancer drugs are currently available and the best choice will vary, depending on the type and stage of cancer being treated. Based on differences in the way they work, the various drugs can be grouped into several distinct categories.

| S.No. | Class | Examples | Mechanism of actions |
|---|---|---|---|
| 1. | Antimetabolites | Methotrexate<br>Fluorouracil<br>Cytarabine<br>Mereap to purine<br>Thioquanine | Folio acid antagonist<br>Pyrimidine analog<br>Pyrimidine analog<br>Purine analog<br>Purine analog |
| 2. | Alkylating and platinating agent | Mechlorethamine (nitrogen mustard)<br>Cycle Phosphemide<br>Chlorambucil<br>Cisplatin | DNA Cross linking agen<br>“<br>“<br>“<br>“ |

| | | | |
|---|---|---|---|
| 3. | Antibiotics | Doxorubicin<br>Deunorubicin<br>Mitomycin<br>Bleomycin | Topo Isomerase II inhabitor<br>"<br>DNA cross linking agent<br>DNA strand breaks |
| 4. | Plant-derived drugs | Etoposide<br>Teniposide<br>Topotecan<br>Vinblastine<br>Vincristine | Topo isomerase II inhibitor<br>"<br>Topo Isomerase I inhibitor<br>Anti microtubule agent<br>" |
| 5. | Hormone therapy | Tamoxifen<br>Arimidex<br>Leuprolide<br>Flutamide<br>Prednisone | Blocks estrogen receptors<br>Aromatase Inhibitor<br>Inhibition of androgen production<br>Blocks androgen receptors<br>Gluco corticoid |

**7. Describe briefly about the chemotherapy.**

Anti metabolite, the first group of chemotherapeutic drugs that we will consider, are molecules that resemble substances involved in normal cellular metabolism. This resemblance causes enzymes to bind to anti metabolitics in place of the normal molecules, thereby disrupting essential metabolic pathways and poisoning the cell. Most of the antimetabolites used in cancer chemotherapy disrupt pathways required for normal DNA synthesis and repair.

1940 – Sidney Farber decided to treat some of his patient with Folic Acid analogs, which are chemical derivative of folic acid that can substitute for natural molecule.

Aminoptesis initially given, but it is temporarily induced desirable effect. This methatexate, a derivative of folic acid that efficiently binds to and inhibit the enzyme dihydrofolate reductase. Dihydrofolate reductase catalyzes the production of a reduced form of folic acid that is required for the synthesis of several bases found in DNA; Inhibition of dihydrofolate reductase by methotrexate therefore disrupts pathways involved in DNA synthesis and repair. Shortlyafter its discovery, methotrexate was shown to be an effective treatment for chorio carcinoma, a cancer arising from cells of the placental membrane that are some times left behind after child birth.

In addition pyrimidine analogs fluorouracil cytarabine and purine analogs mercaptopurine, thioquanine, the close resemblance of these substances.

**Alkylating agent:-**

They are highly reactive organic molecules that trigger DNA damage by linking themselves directly to DNA.

**Examples:-**

1. Chlorambucil
2. Cyclophosphamide

Another group of DNA – cross linking agents used in cancer chemotherapy contain the element platinum. The ability of these substances called platinating agents.

Antibiotics and plant – Derived drugs are two classes of natural substances used in cancer therapy.

Members of the streptomyces group synthesize several antibiolocs that have found their way into our arsenal of anti cancer drugs, including doxorubicin, deunorubicin, mitomycin and bleomycin. All these antibiotics target the DNA molecule, although their mechanism of action are some what different.

Doxorubicin and deunorubicin insert themselves into the DNA double helix and inhibit the action of topoisomerase, an enzyme that normally breaks and rejoins DNA strands during DNA replication to prevent excessive twisting of the double helix. In contrast, mitomycin is a DNA cross linking agent and bleomycin triggers DNA strand breaks.

Plats are another natural source of anti cancer drugs.

1. Vincristine
2. Vinblastine

Obtained from Madagascar Periwinkle. Block the process of microtubule assembly, whereas Taxol stabilizer microtubules and promotes the formation of abnormal microtubule bundles. In either case, the mitotic spindle is disrupted and cells cannot divide.

Hormones and differentiating agents are relatively non toxic tools for halting the growth of certain cancers.

In 1940, Charles Huggins believed that the proliferation of prostate cells is dependent on steroid hormones known as androgens. In an effort to eliminate the source of androgens in man with advanced prostate cancer, he surgically removed their testicles, which produce most of the testosterone, and also treated them with the female steroid hormone, estrogen, more than half of his prostate cancer patient improved and saw their tumor growth is reduced. Instead of removing testicles, block the production of androgen by peptide hormone called gonadotrophins, which are synthesized in pituitary gland. Leuprolide is an analogue of gonadotrophins inhibits androgen production by the testicles. Flutamide and bicalutemide are drugs that act by blocking androgen

receptors. Simillarly estrogen hormone responsible for breast cancer, for that tamoxifen used. When tamoxigen is administered to breast cancer patients whose tumours require estrogen, it binds to estrogen receptors in place of estrogen and prevents the receptors from being activated. Another group of drugs called aromatase inhibitors, inhibit one of the enzymes required for estrogen synthesis.

One advantage of hormone therapy is that their side effects tend to be mild because they do not destroy normal cells and because they only affect a selected group of target cells whose proliferation is controlled by a hormone in question.

Another relatively non toxic approach to cancer chemotherapy involves use of substance called differentiating agents. Differentiating agents promote the process by which cell acquire the specialized structural and functional traits of differentiated cells. When cells undergo differentiation, they also lose the capacity to divide. An example of differentiating agent used in cancer therapy is retinoic acid, a form of vitamin A employed in the treatment of acute promyelocytic leukemia.

**8. What are the methods used to detect cancer?**

The success rates for current treatments are strongly influenced by the stage at which the disease is diagnosed. When cancer is detected early and tumour cells are still localized to their initial site of origin, cure rates tend to be very high.

Tumours tend to produce few or no symptoms when they are small and localized, so it is difficult to come up with reliable guidelines to help people detect cancer early. American cancer society publicized a list of seven warning signs that are possible indicators of the presence of cancer.

1. Change in bowel or bladder habits.
2. A sore that does not heal.
3. Unusual bleeding or discharge
4. Thickening or lump in the breast or else where.
5. Indigestion or difficulty swallowing.
6. Obvious change is a wart or mole.
7. Nagging cough or horseness.

1. The pap smear illustrates that early detection can prevent cancer deaths.

To obtain cells for examination, a doctor inserts a small brush and spatula into the vagina and uses them to scrape cells from the surface of the uterine cervix. The cells are then smeared across a glass slide – hence the name pap 'smear' – and the slide is stained and examined with a

microscope. If the cells in the specimen are found to exhibit abnormal features, such as numerous cells undergoing mitosis, cells with large irregular nuclei, and prominent variations in cell size and shape. It is a sign that cervical cancer may be present. Abnormal cells detected in a pap smear could also be a sign of infection or dysplasia rather than cancer. To make accurate diagnosis Biopsy performed.

Quite recently, the pap smear has been further improved by combining it with DNA test for a human papilloma virus (HPV), which is linked to the development of cervical cancer.

2. Mammography is an Imaging Technique used in screening for early stage breast cancer, screening for early breast cancer is performed using an x-ray procedure called mammography. While employs low dose x-rays to create detailed pictures that reveal the internal tissues of the breast. The main advantage of mammography is its ability to detect small tissue abnormalities that cannot be felt, although the procedure is not accurate enough to prove the presence of absence of cancer. If mammography reveals the presence of an abnormal tissue mass, a biopsy must be performed to determine whether it is actually a cancer.

3. Colonoscopy, x-ray procedures, and the focal

a) Occult Blood test are used in screening for early stage colorectal cancers. Colonoscopy uses a version of this instrument called a colonoscope, which is long enough to visualize the entire length of the colon. If any abnormal growth are found during colonoscopy, they can be biopsied or, in the case of small polyps, removed entirely with a tiny instrument that is inserted through the colonoscope.
b) X-ray imaging techniques are also employed in screening for colorectal cancer. In this barium enema administered through anus and into the rectum and colon. The barium compound helps improve the contrast of the image when x-ray pictures are taken. A newer x-ray procedure called virtual colonoscopy, employs an x-ray scanner to take multiple pictures of the colon at various angles.
c) Another screening strategy involves biochemical testing of fecal samples for indication that might be growing in the colon or rectum. Because colorectal cancers tend to bleed intermittently, one useful indicator is the presence of blood is the feces. A screening test called the Fecal Occult Blood Test (FOBT) is designed to detect such blood, even when it is present in amounts that are too small to be seen. (Occult mears hidden)
d) Other substances present in fecal samples might also indicate the presence of cancer. Since colorectal cancer continually shed cells from the inner surface of the colon or rectum, small amount of DNA from these cells end up in the feces. Researchers have shown that techniques capable of detecting tiny amount of DNA can identify the presence of DNA containing APC mutations in fecal samples obtained from individuals with colorectal cancer.

4. Blood tests for cancer screening include the PSA Test for prostate cancer as well as experimental new proteomic techniques.

Men over the age of 50 are advised by many doctors to get a PSA test, which measures how much Prostate - Specific Antigen (PSA) is present in the blood stream. PSA is a protein produced by prostate gland that normally appears in only tiny concentration in the blood. If a PSA blood test reveals a high concentration of PSA, it indicates the existence of a prostate problem that might be an infection, hyperplasia or cancer. A biopsy therefore needs to be performed. A number of other cancers also produce proteins that are released into the blood stream in elevated amounts. Two of these proteins are alpha-feto protein, which is produced by some liver cancers and Carcino Embryonic Antigen (CEA), which is produced by some colon, stomach, pancreatic and lung cancers. Another example is CA125 a protein released into the blood stream by many ovarian cancers.

Proteomic analysis is recently used by researchers. (The Term proteomic refers to all the proteins produced by a cell or organism). The key to proteomic analysis is mass spectrometry, a high – speed, extremely sensitive method for identifying proteins based on difference is mass and electrical charge.

Imaging techniques utilize x-rays, magnetic fields, or ultrasound to form images. In the case of x-ray imaging, a variety of approaches are available. Conventional x-ray procedures (for example, a chest x-ray) may be useful for the initial identification of tissue abnormalities. The highest resolution images are produced by a Computed Tomography Scan (CT Scan), a technique in which an x-ray scanner moves around the body taking multiple pictures that are then assembled by a computer into a series of detailed cross sectional images.

Magnetic Resonance Imaging (MRI) involves a similar approach using strong magnets and radio waves instead of x-rays, thereby generating a more accurate image.

Ultrasound imaging uses sound waves and their echoes to produce a picture of internal body structures. High frequency sound waves are transmitted into the region of the body being studied, the echoes are picked up by a receiving instrument, and a computer converts the data into a visible image.

Cancer diagnosis includes information regarding the stage of the disease.

Tumour staging uses three main criteria to establish a stage number that reflects how early a cancer has been detected. 1. The size of the primary tumour and the extent of its spread into nearby tissues. 2. The extent to which cancer cells have spread to regional lymph nodes, and 3. the extent to which distant metastases are evident.

**9. Write about use of *Recombinant Vaccines in cancer Treatment.***

Whole tumor cells have been used as immunogens because the specific antigens recognized by T cells have been largely unknown. However, the cloning of several melanoma antigens recognized by T cells has opened new possibilities for active immunization strategies for cancer. Studies in murine models have demonstrated that antigens expressed at high levels in recombinant adenoviral, fowlpox, and vaccinia vector systems can induce a significant antitumor immune response against tumors bearing the same antigen. Recombinant viral vaccines can result in the *in vivo* production of high quantities of heterologous proteins. However, expression of native viral proteins by these vectors can also result in a host immune response against the vector itself, which thereby diminishes the effectiveness of repeated immunizations.

Immunization studies with naked DNA given intramuscularly or DNA administered on gold beads using the gene gun technique have resulted in significant antitumor effects. Because these methods do not use viral vectors, no irrelevant viral proteins are expressed, which thus allows repeated immunizations.

Current efforts are focused on enhancing immune responses through adjuvant exogenous cytokine administration or introduction of genes encoding cytokines or costimulatory molecules into the recombinant vectors

**10. What is the role of *Dendritic Cells in cancer Treatment?***

Another strategy to actively immunize a patient against cancer is to use potent antigen-presenting cells, such as dendritic cells. These cells are capable of stimulating immune responses from quiescent lymphocytes. Dendritic cells pulsed with tumor peptide or protein antigens have been shown to have significant antitumor effects in murine models and were reported to be effective in one study of patients with lymphomas. Nestle and colleagues treated 16 patients with metastatic melanoma with intranodal injections of dendritic cells pulsed with tumor lysates or peptides derived from known tumor antigens. Eleven of 16 patients demonstrated a positive delayed-type hypersensitivity reaction to peptide-pulsed dendritic cells, and clinical responses were observed in 5 patients.

Although antitumor responses can be obtained in murine models by administering dendritic cells pulsed with peptide antigens or whole proteins, this approach limits immune responses to specific defined MHC-binding epitopes within a given antigen or requires the production of recombinant proteins. For many tumor antigens, though, multiple epitopes have been described that bind to a variety of MHC molecules. One strategy that may enable the presentation of multiple, even undefined epitopes within a given tumor antigen is the introduction of an antigen gene into the dendritic cell. This may allow multiple epitopes to be

presented in the context of both class I and class II molecules as well as the constitutive expression of these antigens in transduced dendritic cells. Several strategies have been proposed to genetically modify dendritic cells with tumor antigens. One is to transduce bone marrow cells and differentiate the cells *in vitro* into dendritic cells, using GM-CSF. Another is the transient transfection of dendritic cells using cationic lipids, gene gun, adenovirus, or recombinant influenza virus.

***11. Write the effect of Tumor Suppressor Genes by Gene Theraphy.***

***Tumor Suppressor Genes***

Cancer can result from the abnormal expression of genes that control the cell cycle. Some genes, termed *tumor suppressor genes*, regulate cell growth, and their absence by mutation or deletion results in the malignant phenotype. One approach to treat tumors with deleted or mutated tumor suppressor genes is to replace these genes by *in vivo* gene transfer. Currently, gene transfer techniques do not exist that are capable of efficiently delivering these genes systemically to all tumor cells in the body, and significant technical improvements are required if this is to become a practical approach.

Because of this limitation in systemic delivery, several groups have attempted local gene delivery of tumor suppressor genes. Swisher and colleagues treated 28 patients with non–small cell lung cancer with intratumoral administration of an adenovirus vector containing wild-type p53 complementary DNA. Reverse transcriptase–PCR analysis of posttreatment biopsy specimens were positive for the presence of vector-specific p53 mRNA in 12 of 26 patients. Partial response of the injected lesion was observed in 2 of 25 evaluable patients (8%). Because local therapy is of limited usefulness in the face of metastatic disease, these studies highlight the need for improved vectors that would allow efficient, systemic gene delivery.

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of the injected lesion was observed in 2 of 25 evaluable patients (8%). Because local therapy is of limited usefulness in the face of metastatic disease, these studies highlight the need for improved vectors that would allow efficient, systemic gene delivery.

**12**. **Write about *Suicide Genes in Gene theraphy.***

Because retroviral vectors integrate preferentially into dividing cells, replicating tumor cells might be targeted with relative specificity compared to normal tissues. To test this hypothesis, Culver et al. implanted cell lines producing recombinant retrovirus containing the hTK gene into brain tumors in rats and then delivered systemic ganciclovir therapy. Ganciclovir is a nucleotide analog that is converted into a cytotoxic molecule by hTK but is a poor substrate for mammalian TK. When this method was used, tumors regressed, and significant toxicity to surrounding normal tissues was not observed. Because the hTK gene can only be delivered locally *in vivo*, this treatment approach is limited to those cancers whose primary morbidity is due to local, unresectable disease. Over 30 clinical trials using suicide gene therapy have been approved by the RAC and include studies involving patients with brain tumors, mesothelioma, prostate cancer, head and neck cancer, ovarian cancer, and colorectal cancer. These trials use retroviral and adenoviral gene transfer techniques to deliver the herpes simplex virus TK (HSV-TK) or cytosine deaminase suicide genes, followed by systemic therapy with ganciclovir (for HSV-TK) or 5-fluorocytosine (for cytosine deaminase).

A major limitation in the current application of *in vivo* gene therapy approaches for cancer, such as suicide gene strategies, is the absence of an adequate delivery system that can transfer the suicide gene to all cancer cells within a patient's body. Despite a potential "bystander effect" that may mediate the destruction of tumor cells surrounding those expressing the suicide gene, current gene transfer technology is too inefficient to allow the application of this strategy for the treatment of disseminated metastatic cancer. A second limitation to the use of suicide genes is that of specificity. For any cancer therapy to be effective, there must be greater toxicity for the tumor cells than for normal tissues. Retroviral vectors may theoretically be more selective for tumor cells than for normal tissues because retroviruses only infect proliferating cells. However, retroviruses are suboptimal for *in vivo* administration because of their low efficiency of transduction. Adenoviral vectors, on the other hand, show no selectivity for tumor cells, although a number of groups are attempting to modify surface receptors to engineer specificity.

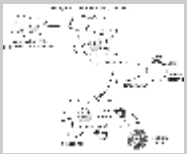
Enhanced tumor specificity might also be accomplished by using tumor-specific promoter and enhancer regions to direct transcription of the suicide genes. For example, the α-fetoprotein promoter is primarily active in hepatoma cells, whereas the tyrosinase promoter is specifically active in melanocytes and melanoma cells. This approach has also been suggested to target tumor vasculature, using promoters that are relatively specific for endothelial cells, such as the E-selectin, vascular cell adhesion molecule, and CD31 promoters. The use of tumor- or tumor-

vasculature–specific promoters might decrease destruction of normal cells, because the latter would not express the suicide gene product. Yet, for these approaches, the fundamental problem of inadequate gene delivery still remains.

**13. Write briefly about *Retroviral Vectors used in Gene therapy.***

Retroviruses are RNA viruses that are capable of stably integrating DNA within the host cell genome. The replication cycle of a retrovirus begins with viral attachment to a cell by a specific receptor. The virus enters the cell and the viral RNA is reverse transcribed to DNA by the virally encoded reverse transcriptase. The viral DNA is then transported to the nucleus, where it integrates into the host cell genome. The integrated viral DNA, termed the *provirus*, is transcribed, and then both spliced and unspliced transcripts are translated to form the viral proteins. Some of the unspliced transcripts are packaged, via a packaging signal sequence (Ψ), into viral capsids. The mature viruses then bud from the host cell membrane.

Retroviral vectors for gene transfer have been constructed by substituting the gene of interest in place of the viral protein coding regions, which, thus, makes these vectors replication incompetent. These vectors are packaged into retroviral particles using helper, or packaging, cell lines that contain the structural viral protein genes in *trans* (i.e., from another site in the packaging cell genome). Because the retroviral vector contains the Ψsequences, it is packaged into the mature virus and is capable of infecting target cells but incapable of replication due to the absence of the retroviral protein coding regions. The viral structural genes provided in *trans* are not packaged due to the absence of the Ψsequences.



View Figure

Figure 60-1
Production of retroviral vectors with packaging cells. The gene of interest is cloned into a retroviral vector and then transfected into a helper cell line, which provides the retroviral structural genes in *trans*. The retroviral structural genes cannot be packaged due to the absence of a packaging sequence (Ψ), whereas the retroviral vector can be packaged, which thereby produces a replication-incompetent retrovirus. LTR, long terminal repeats. (From **ref. 194**, with permission.)

Use of retroviral vectors is one of the most common methods of gene transfer in currently approved gene therapy protocols. Its advantages include the ability to stably integrate into the host genome and the absence of viral protein expression. Current disadvantages of retroviral vectors include low titers resulting in low levels of gene transfer efficiency, although new methods have been described to concentrate retroviral supernatants using ultracentrifugation.

Retroviral packaging cells can use a variety of envelope genes from other viruses. Because the envelope protein is the primary determinant of the host range of the retrovirus, the particular envelope, or pseudotype, used can have a profound impact on transduction efficiencies. lists a number of envelope genes along with their relative host ranges. The recent development of packaging cell lines using alternative envelope genes has resulted in significant advances in the ability to transduce primary lymphocytes. Retroviral vectors produced from the PG13 packaging cell line, which uses the gibbon ape leukemia virus envelope (GALV), are capable of transducing B cells and T cells with significantly higher efficiencies than are those derived from amphotropic packaging cell lines. By combining the use of PG13-packaged vectors with a 1-hour centrifugation at 1000 g, Bunnell et al. were able to obtain transduction efficiencies of primary T cells in the 40% range.

The vesicular stomatitis virus (VSV) G glycoprotein can also be used to pseudotype retroviral vectors. Unlike other envelope proteins, the VSV-G protein confers enhanced physical stability to retroviral particles, which allows concentration with ultracentrifugation to titers of $10^9$ or higher. VSV-G pseudotyped retroviral vectors have a wide host range and have been used successfully to transduce primary T cells.

In addition, future retroviral vectors may contain improved promoters for higher and regulatable levels of gene expression.

**14. Discuss about the *Nonviral Methods of Gene Transfer.***

Cationic lipids, complexed to DNA, have been developed that are capable of mediating high gene transfer efficiencies *in vitro* for some cell types. Cationic lipids such as DOTMA (1,2-dioleyloxypropyl-3-trimethyl ammonium bromide), the prototype cationic lipid, along with a neutral lipid such as DOPE (dioleoyl-phosphatidyl-etanolamine) can be complexed with DNA and added to cultured cells. New formulations of lipids are currently under development that may allow improved *in vitro* gene transfer as well as *in vivo* administration. Cationic lipid formulations have already been used to deliver genes to the lung *in vivo* as well as intratumorally. More recently, DNA-gelatin nanospheres have been used to allow the slow release of DNA *in vivo*. In a murine model, intramuscular injection of nanospheres containing the β-galactosidase marker gene led to higher expression levels than injection with naked DNA or DNA-lipid complexes.

Physical methods of gene transfer include the "gene gun," which propels gold beads coated with DNA into cells. Although this method results in low transfection efficiencies, gene transfer to epithelial cells can be performed *in vivo*, which gives it a potential role in immunization strategies.

The direct *in vivo* injection of DNA alone into selected tissues, such as muscle and thyroid, can result in gene transfer. This approach, termed *naked DNA*, has been used primarily in studies attempting to immunize against the products of the encoded genes.

Nonviral methods of gene delivery are more convenient and have obvious safety advantages over viral methods. However, the majority of current nonviral gene transfer methods result in transient gene expression, and the efficiency of gene transfer is lower than with most viral methods. To address these issues, several strategies are being studied. Ultrasonography has been demonstrated to enhance cell membrane permeability to DNA. Increased gene expression was seen with application of ultrasound to tissue after DNA injection. Gene delivery may also be enhanced by complexing DNA to peptides or polymers. To obtain prolonged gene expression, stable integration of plasmid DNA by incorporating transposons or integrases is being evaluated.

***15. Discuss about the Hydrodynamic Gene Transfer.***

New techniques are emerging that allow efficient systemic expression of naked DNA constructs in murine models. Wolff's group has demonstrated that high levels of DNA expression in hepatocytes, and to a lesser extent in other organs, can be obtained after the rapid intravenous injection of plasmid DNA in a large volume (10% of body weight). Gene expression was dependent on the speed of injection as well as the injection volume. Expression of specific genes was promoter dependent and could be detected for several days. This technique, termed *hydrodynamic gene transfer*, has proven useful for studies requiring gene expression in the liver. In addition, studies of secreted proteins, in which the liver is used as an ectopic site to produce large amounts of material for continuous systemic delivery, have been performed. This allows the study of novel proteins such as new cytokines without the need to produce large amounts of recombinant material. With dependence on such large volumes, this technique is confined to use in animal models. However, it is conceivable that this technique could be used in patients with delivery of lower volumes via occlusion catheters introduced into the portal vein.

**16. Write briefly about Antiangiogenic Gene Therapy.**

The discovery by Folkman[173] in the 1970s that tumors produce substances which stimulate the growth of their vasculature has led to intensive investigation into methods to inhibit tumor neovessel formation. Production of proangiogenic cytokines, including VEGF and fibroblast growth factor, results in angiogenesis, which is required for tumor growth.[174] One gene therapy strategy, therefore, has been to inhibit the production or function of proangiogenic cytokines. A second strategy involves the delivery of genes that encode inhibitors of angiogenesis.[175] A number of endogenous proteins have been described that are capable of inhibiting angiogenesis, and their expression *in vivo* may result in antitumor activity through interference with tumor blood supply.

### *Genes That Inhibit Proangiogenic Cytokines*

VEGF is a proangiogenic cytokine that can bind to two high-affinity receptors (VEGFR-1 or Flt-1, and VEGFR-2 or KDR) expressed on vascular endothelial cells. An endogenous, alternatively spliced soluble form of Flt-1 (sFlt-1) has been identified[176] that is capable of inhibiting the effects of VEGF on vascular endothelial cells *in vitro*. Systemic administration of recombinant adenovirus expressing sFlt-1 resulted in tumor inhibition in mice with preexisting lung or liver metastases.[177] In another preclinical model using GS-9L gliosarcoma cells in rats, intracerebral or subcutaneous intratumoral injection of retrovirus-producing cells encoding a dominant-negative VEGFR-2 resulted in tumor inhibition that was associated with decreased vessel density within tumors.[178]

Antisense approaches are also being tested as a means to inhibit VEGF. Use of a recombinant adenovirus encoding antisense VEGF for intratumoral injection of subcutaneous human gliomas in nude mice resulted in the inhibition of tumor growth.[179] The von Hippel-Lindau (VHL) gene[180] has been shown to down-regulate VEGF production by human renal cancer cells.[181] Therefore, gene therapy by introducing the VHL gene into tumors is a potential strategy to down-regulate VEGF expression *in vivo*. As with other approaches that depend on *in vivo* gene delivery into tumor cells, this approach is limited by the requirement for an efficient system that allows gene transfer to a majority of cancer cells *in vivo*.

Urokinase-type plasminogen activator (uPA) is a fibroblast growth factor that can result in endothelial cell proliferation. Soluble N-terminal fragments of uPA have been used to competitively inhibit the binding of endogenous uPA with its receptor. Systemic administration of recombinant adenovirus encoding a competitor fragment of uPA has been shown to inhibit liver metastases in an experimental model of human colon cancer in nude mice.[182]

Tie-2 is an endothelium-specific receptor tyrosine kinase that plays an important role in angiogenesis of embryonic vasculature through the interaction with its ligand, angiopoietin 1.[183] Systemic administration of a recombinant adenovirus expressing a soluble Tie-2 receptor (sTie-2) capable of blocking Tie-2 activation resulted in growth inhibition of subcutaneous murine mammary (4T1) and melanoma (B16F10.9) cells. In addition, the sTie-2 recombinant adenovirus inhibited the development and neovascularization of pulmonary metastases.[184]

### *Genes with Antiangiogenic Properties*

A number of endogenous inhibitors of angiogenesis have been described and include antiangiogenic proteolytic fragments, interleukins, IFNs, thrombospondins, and tissue inhibitors of matrix metalloproteinases (TIMPs). Angiostatin is a 38-kD internal fragment of plasminogen, and endostatin is a 20-kD fragment derived from the C-terminal noncollagenous domain of the basement membrane constituent collagen XVIII.[185] Administration of angiostatin and endostatin

can lead to tumor dormancy and regression in murine models.[185] However, these proteins have been difficult to produce in large quantities for clinical use due to instability. Because it may be necessary to administer antiangiogenic agents chronically to maintain long-term tumor suppression, other strategies besides administration of recombinant protein may be required for these approaches to be effective. Gene therapy using genes encoding these proteolytic fragments may be an attractive alternative to exogenous dosing.

Systemic administration of recombinant adenovirus-expressing angiostatin resulted in dose-dependent inhibition of the establishment and growth of C6 rat gliomas in nude mice. Using intravenous delivery of complexes of cationic liposomes and plasmid DNA encoding angiostatin, Liu et al. demonstrated reduced B16F10 melanoma metastases in a 7-day tumor model.

Feldman et al. demonstrated inhibition of subcutaneous MC38 murine colon adenocarcinomas using intravenous administration of recombinant adenovirus expressing the endostatin gene. In this model, circulating levels of endostatin were as high as 2038 ng/mL in nude mice after injection of adenovirus.

Preclinical gene therapy approaches have been investigated using genes encoding a number of other antiangiogenic agents, including IL-4, IL-10, IL-12, IFN-β, thrombospondin-1, TIMP-1, TIMP-2, and platelet factor-4. In addition, the combination of ionizing radiation and intratumoral administration of a recombinant adenovirus expressing TNF-α resulted in tumor suppression in a murine xenograft model mediated by the destruction of tumor microvasculature

**R 8357**

**B.E./B.TECH. DEGREE EXAMINATION, NOVEMBER/DECEMBER 2006.**

**SEVENTH SEMESTER**

**INDUSTRIAL BIOTECHNOLOGY**

**IB 046 – CANCER BIOLOGY**

**PART – A**

1. Give any two mechanisms by which proto-oncogenes are converted to oncogenes.
2. What role yeast genetics played in the understanding of cancer?
3. What is tumor suppressor? Give two examples.
4. Which proteins of adenovirus are responsible for cellular transformation?
5. What is focus forming unit?
6. Define: (a) Teratogen; (b) Mutagen.
7. What is physiological basis of radiation therapy for treating cancer?
8. How cancer may be detected in situ? State True or False, give reasons.
9. Transformed cells produce more fibronectin.
10. Metastasized tumors are primarily found in the brain.

**PART – B**

11. (a) What different strategies are used towards the treatment of solid and metastasized tumors?

Or

(b) How estrogens or their analogs could be used in the treatment of cancer? Describe the mechanism and give some specific examples.

12. (a) Transformed cells often secrete plasminogen activator. How does this help the cells to metastasize.

Or

(b) Describe the different morphological stage of tumor development.

13. (a) What are direct and indirect carcinogens? Classify the following as per the above criterion: (i) ethyl methyl sulfonate; (ii) aflatoxin; (iii) benzopyerene; (iv) nitrogen mustard.

Or

(b) How can you determine the effect(s) of an uncharacterized carcinogen using an in vitro assay?

14. (a) (i) What is mitotic index? How is it measured? What does it indicate?

(ii) What is labeling index? What does it signify? How is it measured?

Or

(b) What is the influence of diet on cancer? Provide any one example associating prevalence of a particular type of cancer in a community but not in others.

15. (a) What is the role of helper virus in the transduction process? Give some examples of transducing and helper viruses.

Or

(b) Compare and contrast secreted and nuclear oncogens. Give two examples of each.

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**C 3081**

**B.E./B.TECH. DEGREE EXAMINATION, MAY/JUNE 2007.**

**SIXTH SEMESTER**

**BIOTECHNOLOGY**

**BT 1009 – CANCER BIOLOGY**

**PART – A**

1. What are cyclin dependent kinase inhibitors (CDKI)? Give any two examples.
2. Differentiate between hereditary cancer and sporadic cancer.
3. What is Knudson postulate?
4. Define Relative Biological Effectiveness (RBE)?
5. Write any two RNA viruses and its associated cancer.
6. Write short notes on oncogene with serine-threonine specific protein kinase activity.
7. Explain briefly on the role of proteinase in the tumor invasion.
8. WRITE the soil and seed hypothesis of Paget.
9. Write the different types of cancer therapy?
10. What is meant by Chemotherapy? And classify them.

**PART – B**

11. (a) Explain the role of cyclin and cyclin dependent kinase in the regulation of cell cycle with suitable diagram.

Or

(b) Describe the identification of earlier stage of cancer development by using Polymerase Chain Reaction (PCR) technique with suitable examples.

12. (a) (i) Discuss the different categories of carcinogenic compounds with their chemical structure.

(ii) Write the biochemical conversion of polycyclic aromatic hydrocarbons to potent carcinogenic agent?

Or

(b) Illustrate the molecular mechanism of X-ray induced carcinogenesis.

13. (a) Discuss the biochemical role of Ras, Gsp, Gip proteins in normal and transformed cells.

Or

(b) Write an essay on:

(i) Molecular mechanism in the activation of proto-oncogene.

(ii) DNA transfection assay.

14. (a) Describe the altered cell surface properties and biochemical process of metastatic cell from tumor cell in details.

Or

(b) Explain the different stages of metastatic process in detail.

15. (a) Explain the stages involved in the delivery of radiation therapy. Discuss the complications of radiation therapy.

Or

(b) What are monoclonal antibodies (MAbs)? Write the application on MAbs in the detection of cancer with suitable example.

**J 3080**

**B.E./B.TECH.DEGREE EXAMINATION, MAY/JUNE 2009.**

**SIXTH SEMESTER**

**BIOTECHNOLOGY**

**BT 1009 – CANCER BIOLOGY**

## PART - A

1. What are tumor markers? Give two examples.
2. Write the role of $p^{53}$ in normal cell and malignant cell.
3. Define relative biological effectiveness RBE.
4. Distinguish between the modes of transformation of normal cell by DNA virus and RNA virus.
5. What are the phenotypic characters modified during metastasic process?
6. Write a note on soil and seed hypothesis.
7. Explain briefly the application of PCR in mutation.
8. What is mean by brachy therapy?
9. How is serine-therionine protein kinase involved in tumor cell progression?
10. Give any two vaccines that are used in cancer treatment.

## PART – B

11. (a) Explain in detail how cyclin dependent kinase inhibitors control the cell cycle with neat flow diagram.

    Or

    (b) Discuss the genetic and morphological differences between normal and cancer cell in detail.

12. (a) Describe the different group of chemical carcinogens and their molecular mechanism of carcinogenesis.

    Or

(b) Write in detail about the DNA damage occurred by X ray and gamma ray at molecular level.

13. (a) Discuss the mode of transformation of normal cell by growth factors and its receptors.

Or

(b) Describe the role of primary and secondary messenger molecules in cancer development.

14. (a) How does malignant cell become metastasic cell? Explain in detail.

Or

(b) Explain in detail the genes that are activated during metastasic development.

15. (a) Illustrate the detection techniques and modalities of therapy in cancer treatment.

Or

(b) Describe the pharmacological and adverse effects of anti metabolites and natural products in cancer therapy.

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## Q 2083

## B.E./B.Tech. DEGREE EXAMINATION, NOVEMBER/DECEMBER 2009.

## SIXTH SEMESTER

## BIOTECHNOLOGY

## BT 1009 – CANCER BIOLOGY

## PART – A

1. Define cell cycle regulation.
2. List the molecular tools for Early diagnosis of cancer.
3. Write the principle behind X-Ray induced mutation.
4. Give the concept of metabolism.
5. How do you identify oncogenes?
6. How is kinases activated?
7. State three step theory.

8. Define tumor cell invasion.
9. List out various forms of therapy.
10. How do you predict aggressiveness?

## PART – B

11. (a) Briefly discuss on mutation of tumor suppressor genes.

Or

(b) Explain the transformation of normal cell to tumor cell.

12. (a) With suitable theory explain the chemical carcinogenesis.

Or

(b) Enumerate the mechanisms of radiation carcinogenesis.

13. (a) Explain the role of growth factors in transformation.

Or

(b) What are signaling molecules? Explain in detail.

14. (a) Explain the clinical significances of invasion.

Or

(b) Explain, in detail, the basement membrane disruption.

15. (a) What is the role of tumor makers in predicting response to theraphy? Explain.

Or

(b) What are the possible approaches for tumor targeted therapy? Discuss briefly.

**Q 11192**

**B.E./B.Tech. DEGREE EXAMINATION,APRIL/MAY 2011.**

**SIXTH SEMESTER**

**BIOTECHNOLOGY**

**BT 2027 – CANCER BIOLOGY**

**PART – A**

Write Short notes on:

1. Antioxidants
2. Polycyclic Aromatic hydrocarbons
3. Human papilloma virus
4. Hybridoma
5. Extravasation
6. Angiogenesis
7. Chromosome translocation
8. DNA methylation
9. Protein kinase
10. Cancer vaccine

## PART-B

11. (a) What are six hall marks of cancer? Describe in detail.

Or

(b) What do you know of epigenetic factor? Explain the various aspects involved.

12. (a) Elaborate on one screening assay for carcinogen.

Or

(b) What are various etiological factors in the genesis of cancer? Discuss in detail.

13. (a) Write a alaborate note on telomeres and chromosome alterations.

Or

(b) Reactive oxygen species play an important role in defining biology of cancer: Discuss.

14. (a) Elaborate on different stages of metastasis.

Or

(b) Give an elaborate update on diagnosis and treatment of cancer.

15. (a) Discuss the role of RNA virus in cancer.

Or

(b) Where do you place the natural products in cancer therapeutic management? Discuss in detail.

# CBT 333-TISSUE ENGINEERING
# UNIT I

## 1. What is tissue engineering?

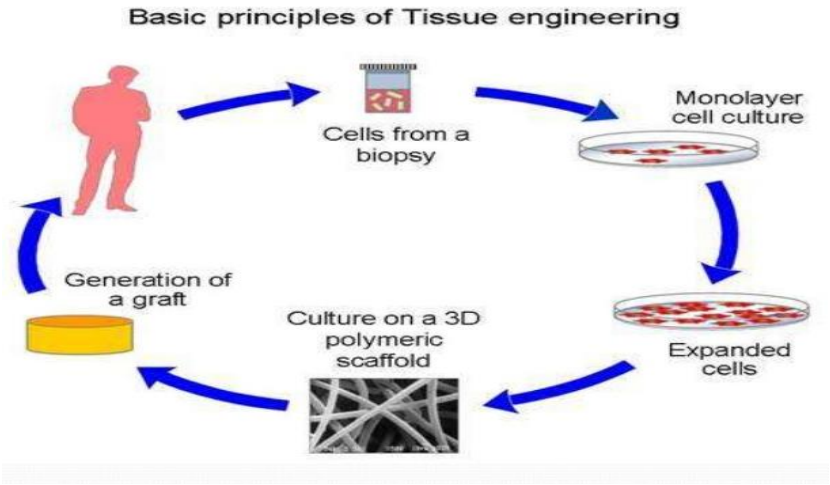
**Tissue engineering** is defined as "*the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain or improve tissue function"* **– Y.C. Fung**.

"*The application of biological, chemical, and engineering principles toward the repair, restoration, or regeneration of living tissues using biomaterials, cells, and factors alone or in combination*" – **C. T. Laurencin.**

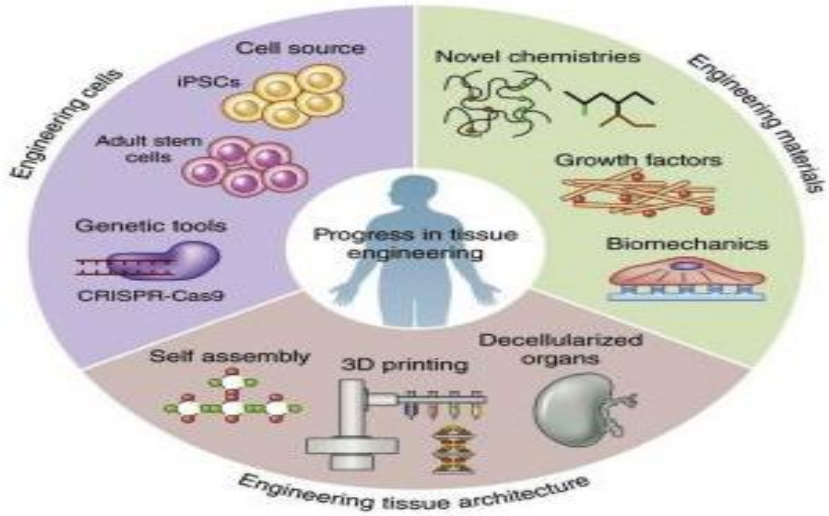
From these definitions, it is clear that tissue engineering has the potential to address the organ failure and tissue loss. This is because current clinical approaches for restoring the organ or tissue function are organ transplantation, surgical reconstruction, or use of prostheses. However, each treatment strategy has its own merits and demerits.

**For example,** organ transplantation is only treatment at the end stage of organ failure. This procedure involves mainly remove the failed organ from the patient and replaced with the procured organ for transplantation. With today's medical advancement, it can be done for any organ – liver, kidney, heart, etc. But this whole procedure should follow legal and ethical considerations. Organ transplantation society encourages the use of organs donated from the cadavers instead from the living donors in order to reduce the risk for living donors while transplantation. Although such therapies have merit to save the lives of thousands, shortage of donor organs and tissues as the patient waiting list number has increased per year tremendously; and requirement of immunosuppressant limits its potential to address organ failure crisis.

In case of surgical reconstruction, there is no possibility of immune rejection because of the use of patient's own tissue (autologous). However, autologous grafting chiefly requires surgery at donor site; even limited supply, inadequate size and shape (Complaint mismatch) with donor site morbidity restrict its use towards tissue loss. In some cases of organ failure, say for example loss of hand; or loss of leg; patients are advised to use artificial prostheses. But they are biologically non functional and they do not behave physiologically as a true organs. Examples are artificial heart, heart valves, prosthetic hip, and artificial breast. But these materials are subject to fracture, wear, toxicity, inflammation, which could induce the long term complications and rejections at the later stage.
Hence limitations of existing therapies provoke the search of new technologies or therapies as tissue engineering to combat the organ failure and tissue lose crisis. Prof. Cato T. Laurencin defined Tissue Engineering as "*the application of biological, chemical, and engineering principles toward the repair, restoration, or regeneration of living tissues using biomaterials, cells, and factors alone or in combination*".




**Current Scope Of Development**




**2. Tissue Engineering Triad**

- Biomaterials,
- Cells and
- Growth factors

are known as the "**Tissue Engineering TRIAD".**


There are three different strategies that could be adopted for the regeneration of new tissues. In the first approach cells can be used as therapeutic agents to restore the functional tissue. This approach mainly involves the isolation of cells from different cell sources (autologous, allogenic; syngenic and xenogenic) and even use of stem cells

using a special technique called stem cell therapy, placing them in the site of interest for improving the tissue function.

The second approach involves the exogenous delivery of growth promoting substances like growth factors using the carriers (polymeric or lipidemic) can stimulate the endogenous stem/progenitor cells for the specific differentiation thereby replacing the lost cells or tissues.
The third approach is to use artificial 3-dimentional scaffolds or matrices for the growth of cells where cells can be either recruited from the host tissue *in vivo* or seeded *in vitro*.

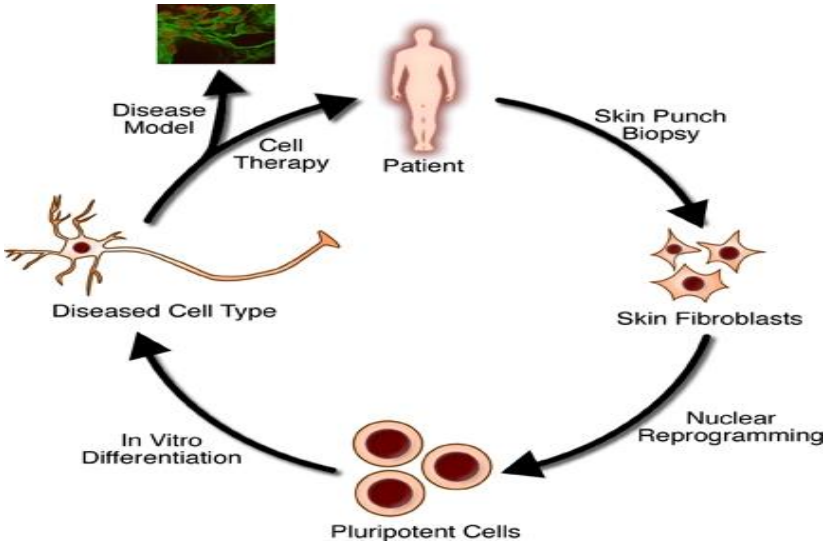
Even though these strategies can overcome the problems associated with the current clinical treatments, there are few scientific challenges to construct a tissue or organ using these approaches. For example, use of cells: limited supply of autologous cells; requirement of immunosuppressant for allogenic source; disease transmission associated with the xenogenic source; ethical considerations with the use of stem cells are the major challenges in case of first approach.

Specific differentiation of stem cells with desired functionalities with the appropriate growth factor delivery is still unresolved question in tissue engineering. Even the third approach of tissue engineering is the development of artificial extracellular matrix (ECM) analogue towards the regeneration of specific tissue. In this, attempt to make ECM analogue with respect to chemical environment surrounding the cells within a native tissue; architecture; dimensions with signals is really tricky. Moreover construction of matrix should not provoke any immune response; toxicity i.e. it must be compatible to the host tissue; whatever the scaffold that we have prepared should control the cell fates such as cell adhesion, migration, proliferation, differentiation as like our native.

**Regenerative medicine**

- The term regenerative medicine is often used synonymously with tissue engineering, although those involved in regenerative medicine place more emphasis on the use of stem cells or progenitor cells to produce tissues.

  **A simplified overview of the general methods used in regenerative medicine**



**In 2003, the NSF published a report entitled "The Emergence of Tissue Engineering as a Research Field", which gives a thorough description of the history of this field**

**Examples**

- **Bioartificial windpipe:** The first procedure of regenerative medicine of an implantation of a "bioartificial" organ.
- **In vitro meat:** Edible artificial animal muscle tissue cultured in vitro.
- **Bioartificial liver device:** several research efforts have produced hepatic assist devices utilizing living hepatocytes.
- **Artificial pancreas:** research involves using islet cells to produce and regulate insulin, particularly in cases of diabetes.
- **Artificial bladders:** Anthony Atala (Wake Forest University) has successfully implanted artificially grown bladders into seven out of approximately 20 human test subjects as part of a long-term experiment.
- **Cartilage:** lab-grown tissue was successfully used to repair knee cartilage.
- **Scaffold-free cartilage:** Cartilage generated without the use of exogenous scaffold material. In this methodology, all material in the construct is cellular or material produced directly by the cells themselves.
- **Doris Taylor's heart in a jar**
- **Tissue-engineered airway**
- **Tissue-engineered vessels**
- **Artificial skin constructed from human skin cells embedded in a hydrogel, such as in the case of bioprinted constructs for battlefield burn repairs.**
- **Artificial bone marrow**
- **Artificial bone**
- **Laboratory-grown penis**
- **Oral mucosa tissue engineering**
- **Foreskin**

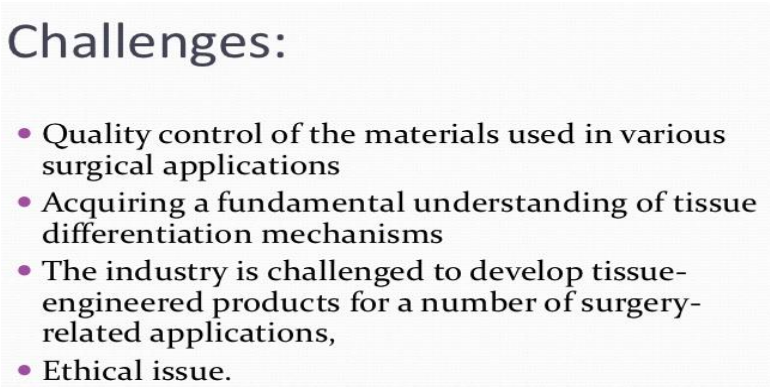
**What part of the body tissue engineering replace?**

- Skin
- Vascular tissue
- Thyroid gland
- Adipose tissue
- Liver
- Nerve
- Kidney
- Pancreas
- Esophagus
- Cartilage
- Bone
- Bladder

### 3. Challenges in tissue engineering

In conclusion, there are few challenges to be answered for the reconstruction of functional tissues. They are:

1. How do we develop a close approximate biological replica?
2. Should the tissue be produced *in vitro* implanted *in vivo*?
3. What type of scaffold should we choose?
4. How do we manufacture?
5. What cells are to be used?
6. Under what conditions can cells expand without affecting its phenotype?
7. What regulators do we use to stimulate the cell proliferation and differentiation?




**Future Scopes of Tissue Engineering**

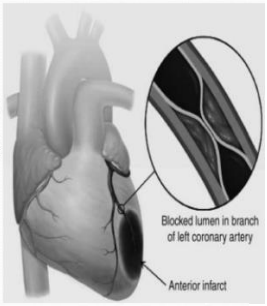
At present, tissue engineering plays a relatively small role in patient treatment. Artificial skin, valves, and cartilage are examples of engineered tissues that have been approved by the FDA. However, currently, they have limited use in the humans.

Supplemental bladders, small arteries, skin grafts, cartilage, and even a full trachea have been implanted in patients, but the procedures are still experimental and very costly. While more complex organ tissues like heart, lung, and liver tissue have been successfully recreated in the lab, they are a long way from being fully reproducible and ready to implant into a patient.

These tissues, however, can be quite useful in research, especially in drug development. Using functioning human tissue to help screen medication candidates could speed up development and provide key tools for facilitating personalized medicine while saving money and reducing the number of animals used for research.

## Applications of Tissue Engineering

- Therapeutic cloning for tissue repair
- Coronary Heart Disease
  - Myocardial Infarction
  - Congestive Heart Failure
  - Dysfunctional Heart Valves
  - Peripheral Vascular Disorders
  - Abdominal Aortic Aneurysms




- Neurological
  - Stroke
  - Parkinson's Disease
  - Alzheimer's Disease
  - Epilepsy
  - Traumatic Brain and Spinal Cord Injury
  - Multiple Sclerosis


- Neurological
  - Stroke
  - Parkinson's Disease
  - Alzheimer's Disease
  - Epilepsy
  - Traumatic Brain and Spinal Cord Injury
  - Multiple Sclerosis


- Orthopedic
  - Non-union Fractures
  - Cartilage Damage and Repair
  - Ligament Damage
  - Vertebral Disc Damage
  - Bone Graft Materials
- Urological
  - Incontinence
  - Kidney Disease
  - Bladder

- Dental
  - Missing teeth
  - Periodontal disease
- Skin/Integumentary
  - Burns
  - Diabetic Ulcers
  - Venous Ulcers
  - Plastic Surgery
- Ophthalmology
  - Cornea
  - Retina

## Organ Transplantation

- **Bioartificial liver device**:
- **Artificial pancreas**:.
- **Artificial bladders**:
- **Cartilage**:
- **Artificial skin** constructed from human skin cells embedded in collagen
  -Artificial bone marrow
  -Artificial bone
  -Artificial penis
  -Oral mucosa tissue engineering

## Ethical problems

•Using Xenogenic cells: Species boundaries crossed
•Using Human embryonic cells: Unethical to use human embryo & aborted fetus.
•Use of human embryo with large scale cultivation for profit
•Rights of Tissue Donors:
-Profit making
-Information

## Ethical Problems

- Role of Cell banks:
  - Privacy of Donor
- Prolonging of life through Tissue engineering:
  Set goal for raising life span through tissue engineering
- Playing THEE!!!
- Organ trafficking
- Cost of using Technology.


**4. Can we use cells as therapeutic agents to restore the tissue function?**

Yes. Cells are the building blocks of tissues, which contribute to the respective functions. Cell based therapies are a distinct class where cells are utilized as therapeutic agents to treat the pathological conditions. However, cell-based therapies are not new because for many decades people are practicing blood transfusion to anemic patients to restore the oxygen supply. Similarly, platelet transfusion for blood clotting defects; bone marrow transplantation for cancer patients is widely practiced now.

Cell based therapies can also be used to repair the cartilage. Hepatocytes and kidney cells are also used extracorporeal support devices to carryout the functions of the liver and kidney respectively. Similarly, beta-islet cells for diabetes, sheets of fibroblasts for ulcers and burns, genetically modified myocytes for muscular dystrophy have also been used clinically with varying degrees of success. We will look in to how cells are used to treat various defects in a short while.

***Sources of Cells***

There are different cell sources available. They are:

**Autologous:** Cell can be taken from the donor and introduced back to donor itself i.e., where donor and recipient are same. Limited supply and the donor site surgery are the major drawbacks.

**Allogenic:** Cells can be transferred from donor to recipient of same species. However, difficult to reduce the patients risk against the transplant antigens.

**Syngeneic:** Cells can be transplanted between genetically identical twins. Using this method, only twins can benefit but not other living beings.

**Xenogenic:** Cells transplantation is between species. Where transmission of new diseases and immune rejection concern.

TABLE 1.1

Types and Sources of Human Stem Cells

| Origin | Types of Stem Cells | Sources of Isolation |
|---|---|---|
| Adult | Mesenchymal stem cells | Bone marrow |
| | Hemopoietic stem cells | Bone marrow and peripheral blood |
| | Neural stem cells | Neural tissue |
| | Adipose-derived stem cells | Adipose tissue |
| | Muscle-derived stem cells | Muscle |
| | Epidermal-derived stem cells | Skin, hair |
| | Umbilical cord blood stem cells | Umbilical cord blood |
| | Umbilical cord matrix stem cells | Wharton's jelly |
| Embryonic | Embryonic stem cells | Inner cell mass of 5–7 day blastocyst |
| | Embryonic germ cells | Gonadal ridge of 6–11 week fetus |

**5. How do we use cells as therapeutic agent for various tissue defects?**

To answer this question, we should first know what are the different types of tissue defects based on the functions? Tissue defects can be classified as mechanical, metabolic, synthetic, communication and combination defects based on the tissue functions. For example the major organ is affected in mechanical tissue defect is cartilage or bone since the major function of these tissues are providing the mechanical strength to the body. Similarly in case of metabolic defects, liver is the chief organ for metabolism. Hence is there any abnormality in the liver function leads to metabolic defect.

Pancreas is the prime organ for synthesis of hormone like insulin, which is a primary regulator for glucose metabolism. Thus any defect in the insulin production or dysfunction of pancreas will lead to synthetic defects. Nerve is the only organ used to communicate as well as coordinate all body functions. Hence, the dysfunction of nerve due to any injury will promote the communication defects. Skin is largest organ performing multiple functions including immunological barrier; vitamin metabolism; maintain homeostasis, and so on. Thus the defect in skin tissue is coming under combination defects.

**Tissue defects can be classified as**

- **Mechanical,**
- **metabolic,**
- **Synthetic,**
- **Communication and**
- **Combination defects based on the tissue functions.**

TABLE 1.2

Classes of tissue defects treated by TEMPs.

| Defect | Organ | Function |
|---|---|---|
| Mechanical | Cartilage/Bone | Resists compression |
| Metabolic | Liver | Nitrogen metabolism |
| Synthetic | Pancreas | Insulin production |
| Communication | Nerve | Coordination |
| Combination | Skin | Prevents $H_2O$ loss, immunologic barrier, vitamin metabolism |

## Cells as therapeutic agent

- ***5.1. Cells used to restore the mechanical defects***
- ***5.2. Cells used to restore the metabolic defects***
- ***5.3. Cells used to restore the synthetic defects***
- ***5.4. Cells used to restore the communication defects***
- ***5.5. Cells used to restore the combination defects***

### *5.1. Cells used to restore the mechanical defects*

Let us take an example of cartilage tissue defect. First of all the major challenge in the functional cartilage restoration relies in the native structure of this tissue. That is, they are avascular (no blood vessels for nourishment), aneural (no innervations), and alymphatic tissue (absence of lymphatic capillaries). This tissue is an unusual biphasic tissue, which is made up of solid matrix called the extra cellular matrix (ECM) and fluid phase (synovial fluid).

The major component of cartilage is ECM where terminally differentiated chondrocytes will be dispersed at low densities. This architecture promotes frictionless surface with pain free motion. In aged population, osteoarthritis is the common problem where there is a deterioration of cartilage including loss of chondrocytes and ECM architecture. This deterioration cannot be repaired due to the lack of blood vessels resulting in acute pain during movement. Cartilage defects in the knee can be treated by autologous transplantation. Briefly, biopsy is collected outside the affected area from the patients and subjected to enzymatic treatment where the chondrocytes will be harvested. These chondrocytes are sub-cultured for expansion and then injected back to patient at the lesion. About 200,000 patients are undergoing this type of cellbased therapy every year.

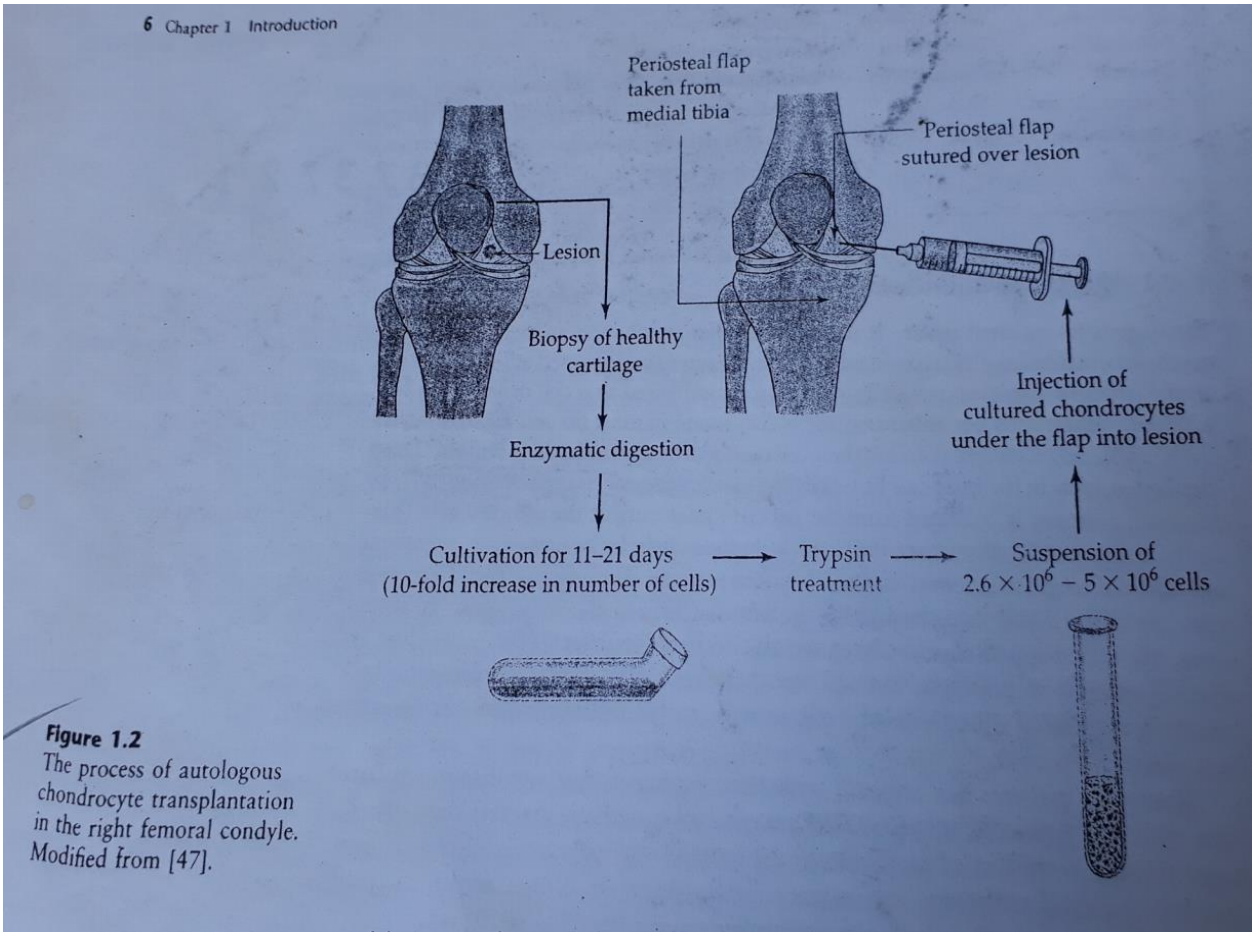



**Figure 1.2**
*The process of autologous chondrocyte transplantation in the right femoral condyle. Modified from [47].*

### *5.2. Cells used to restore the metabolic defects*

Let us take liver as an example. Liver plays a major role in metabolism (protein, fats and carbohydrates), detoxification of foreign compounds, production of vital serum proteins (albumin and clotting factors) and production of bile for digestion. Loss of liver function is mainly by hepatitis C, cirrhosis or excessive alcoholism or even cancer. Whole organ transplantation can use to treat liver defect at the end stage. However, this has many disadvantages like immune reactions/rejections, limited supply of donor organs. Hence there are other some temporary approaches can be used mainly non-biological approach (Charcoal resins and dialysis) and biological approach such as blood exchange and animal organ perfusion. Extracorporeal hepatocyte based bioreactors are even used to culture human hepatocytes and can able to maintain the liver function in the form of bioartificial liver (BAL).

Hepatocytes are the chief cells present in the liver, which are highly proliferative cells. But the major problem is associated with the hepatocyte *in vitro* culture is transdifferntiation, that is the capacity of hepatocytes to differentiate into fibroblast. Thus, the phenotype of hepatocytes is lost within a week and then it become functionally inactive. Usually, a bioreactor with hollow fiber cartridge, charged with the immobilized hepatocytes (intraluminally or extraluminally) is used. This device is connected outside the body to blood or plasma circulation of patient. The hepatocytes can also be embedded in a collagen hydrogel to avoid hydrodynamic damage and then injected in the hollow fibers. Due to the hydrogel nature of the collagen, it contracts thus forming a lumen, which helps in the diffusion of medium and plasma. This type of

extracorporeal device can improve the detoxification and synthetic and even regulatory function of diseased liver.

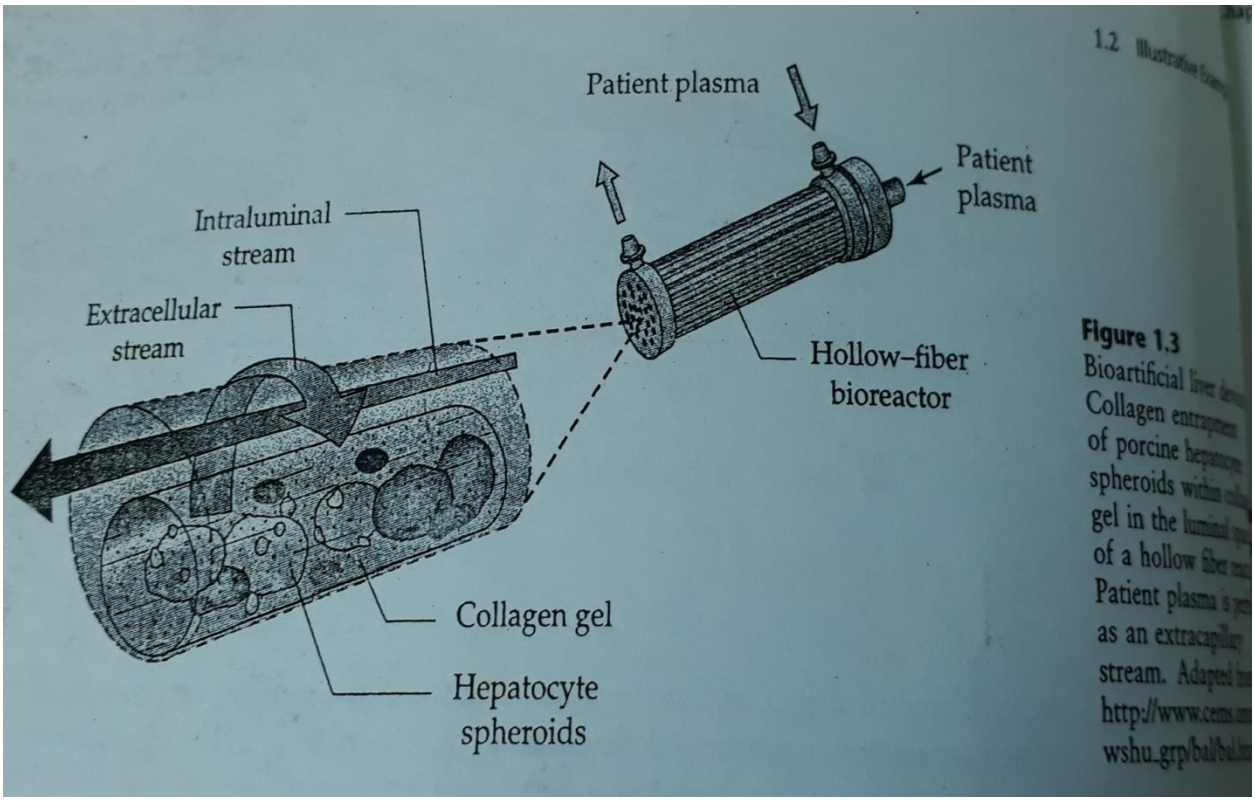


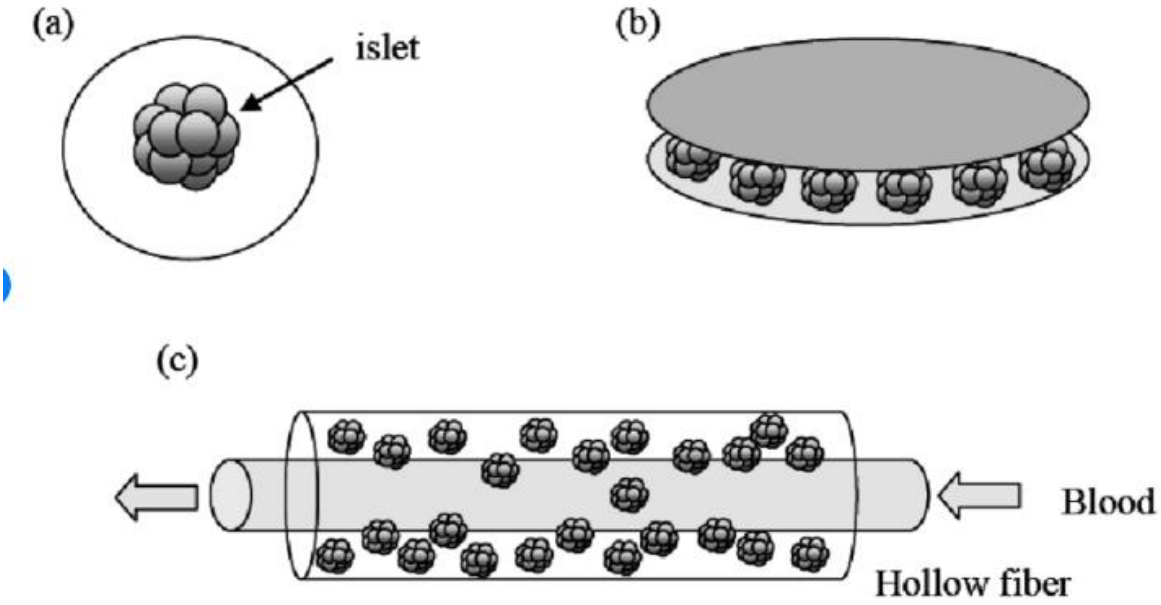

**Figure 1.3**
Bioartificial liver de...
Collagen entrapment ...
of porcine hepatocyte ...
spheroids within ...
gel in the luminal ...
of a hollow fiber ...
Patient plasma is ...
as an extracapillary ...
stream. Adapted ...
http://www.cems...
wshu_grp/bal/bal...

***5.3. Cells used to restore the synthetic defects***

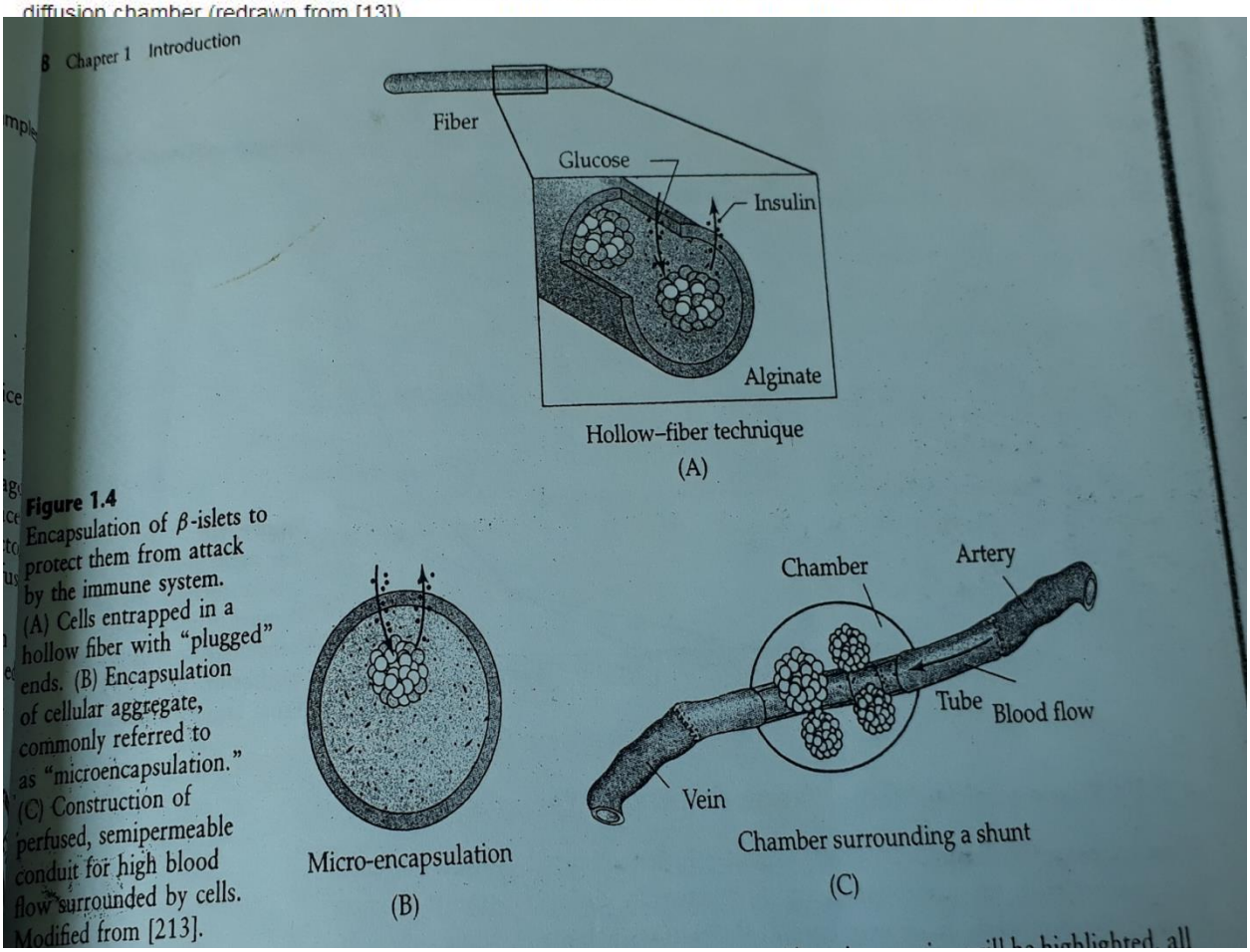
Let us discuss about prime organ for synthesis, which is none other than pancreas / beta islet cells. Pancreas is located behind the lower part of the stomach and the major clusters of cells in the pancreas is called islets of Langerhans, which make insulin and enzymes that help our body to digest food. In insulin dependent diabetic patients, pancreas loses its ability to secrete insulin.

Allogenic cells are used (cells isolated from the cadaverous pancreas), and injected into the liver portal vein thus enabling the pancreas to secrete insulin. However, since the source of islet cells is allogenic, there will be immediate rejection due to recurring autoimmunity, insufficient transplanted β-cell mass and inflammation. Even the use of immunosuppressive agents provokes toxicity. Similarly, in case of xenogenic islet transplantation, porcine islets are used since its glycemic regulation is similar to humans. However, complications such as long-term survival of porcine grafts, lack of biocompatibility, poor immune-protective properties, hypoxia and chances of disease transmission could limit the use.

Hence, physical separation of donor's cells from the immune system is mandatory to avoid immune rejection. This could be achieved by the encapsulation of donor cells using semi permeable membrane, which selectively allow the passage of nutrients and insulin while preventing the passage of antibodies and other biomolecules that could damage the implanted cells as shown in the cartoon below.




The conventional bioartificial pancreas types. (a) microcapsule, (b) diffusion chamber, and (c) hollow fiber-based diffusion chamber (redrawn from [13])




### *5.4. Cells used to restore the communication defects*

How do we use neural cells to restore the function of nerves? Nerve injury creates a gap or cavity due to the loss of myelin sheath, thereby loss of impulse transmission. Regenerating nerve, especially in the central nervous system is really challenging since

in the native environment axons in the central nervous system do not regenerate substantially due to the surrounding inhibitory environment. Hence, major aims of the cellular therapeutic interventions in the restoration of communication defect are:
i. To bridge the cavities
ii. To replace the dead cells and
iii. To create a permissible environment for axon regeneration
Cells such as Schwann cells, olfactory enshealthing cells can be used for achieve the above. The advantage of using Schwann cells is mainly because it can produce ECM, cell adhesion molecules, and neurotrophins that lead the peripheral axon to distal stump and synapse formation.

However, complications such as unfavourable interaction with glial scar components in CNS, inability to remyelinate the axon beyond the injury is leading to be big challenge. In addition, Schwann cells are known to exacerbate the chondroitin sulphate production, which hinders the regeneration further in astrocyte rich region. Hence, this can be replaced with the use of olfactory ensheathing cells because of its structural similarity to Schwann cells and astrocytes. Use of olfactory ensheathing cells can produce neurotrophins and cell adhesion molecules and migrate through the growing axons and induce the sprouting of axonal outgrowth. Hence, this provides a permissive environment for axonal growth and effectively supports the axonal outgrowth through glial scars. But again while harvesting the cells from the olfactory bulb one should ready to compromise the host's sense of smell.

- **Major aims of the cellular therapeutic interventions in the restoration of communication defect are:**

i. To bridge the cavities
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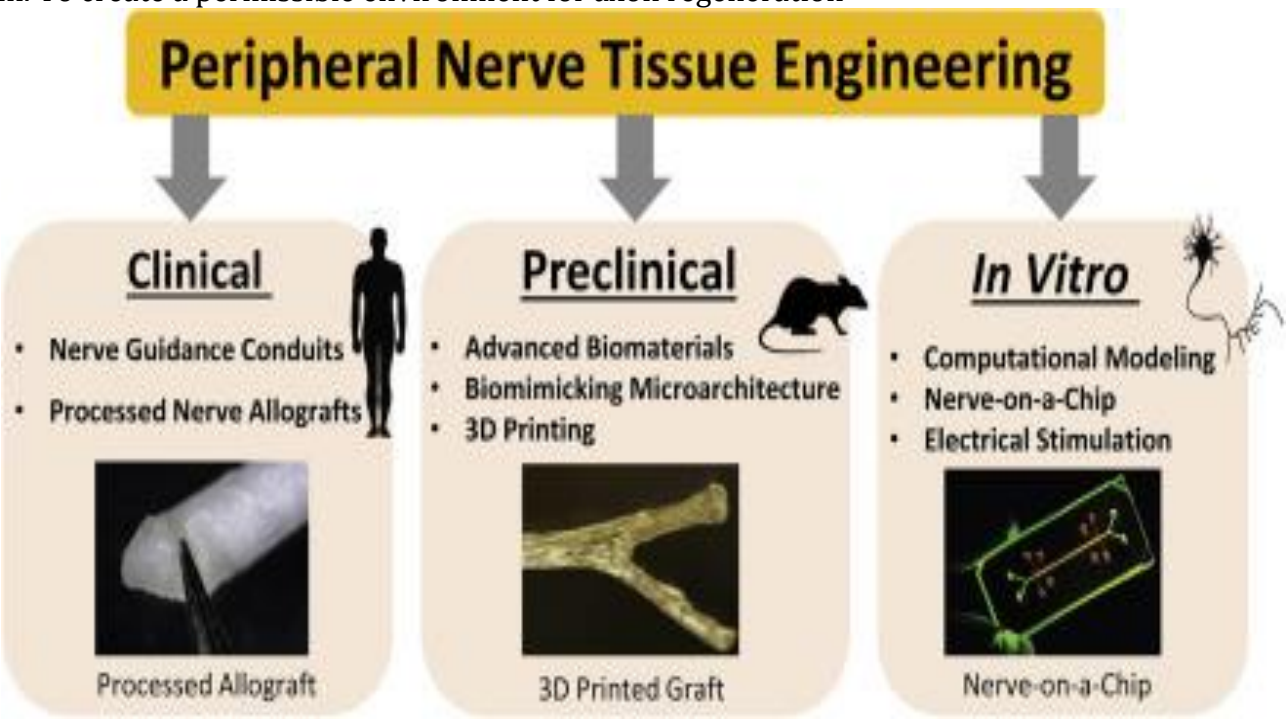


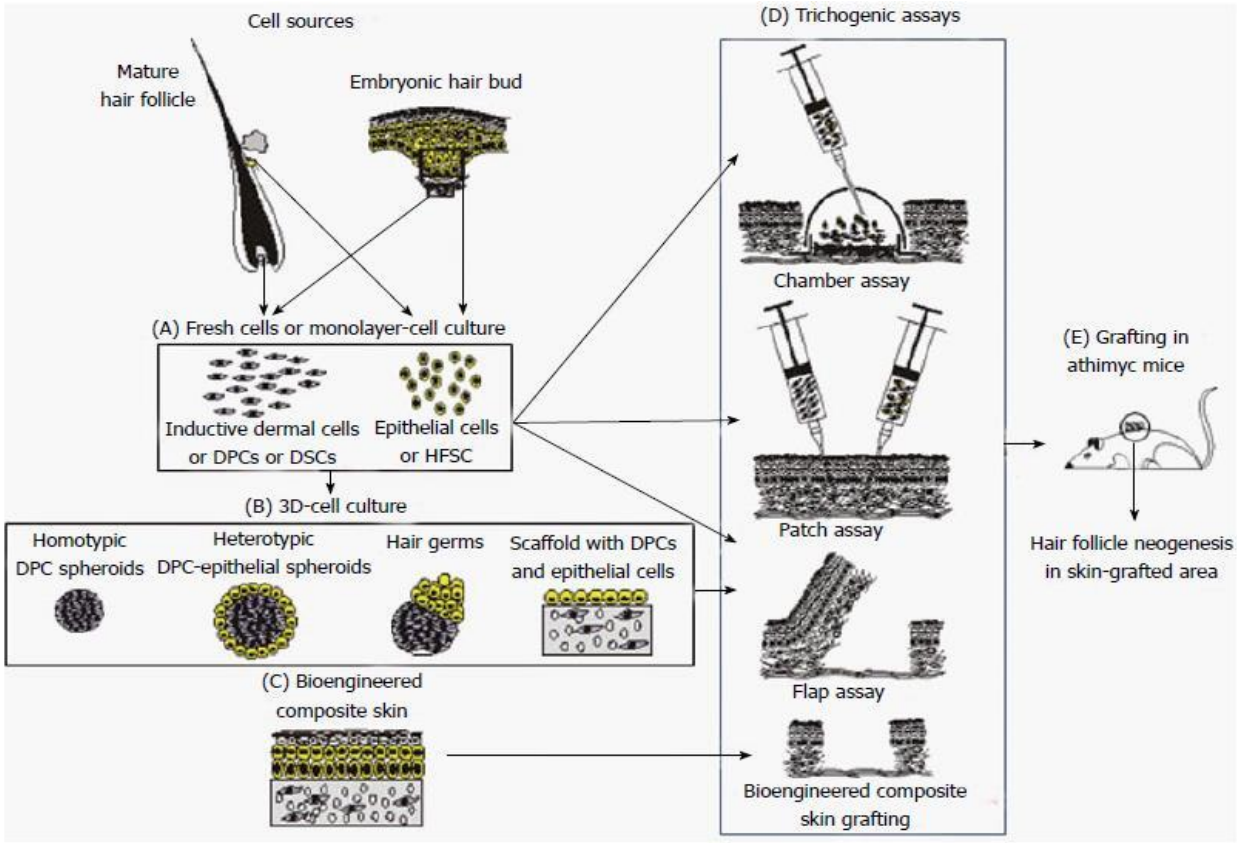

***5.5. Cells used to restore the combination defects***

Skin is the very good example for multifunctional organ. Skin is one of the most prolific tissues in the human body. It consists of two layers dermis consisting of fibroblasts and the epidermis consisting of keratinocytes. Variety of skin ulcers and burns are treated by the transplantation of fibroblasts and keratinocytes with no immune rejection. This therapy is almost well developed and lots of clinical products are now commercially available.

Normally ability of the skin to heal itself is noteworthy but when the injury is very severe, medical interference is essential to improve the healing process. Also treatment strategies should protect the wound area from the infectious agents and limit fluid loss in the intervening time.

Classic example is a burn injury. Burn injury is classified as first, second and third degree burns based on the severity of the injury. In first degree burn, the epidermis alone is injured. Though it is painful, it can heal very rapidly and does not require too much medical attention. Second degree burns damage both layers of the epidermis and the dermis while third degree burns destroys full depth and also underlying tissues.

During skin grafting procedure, health skin is taken from a different region, meshed and placed at the site of injury. This helps in the regeneration of the affected skin. Though, skin grafting is used widely, a secondary surgery is required to graft the skin resulting in donor-site morbidity and risk of infection at the sites of injury. In addition, the patient has to ay for the associated cost involved in the procedures. Thus, there is a requirement to develop synthetic materials which could be used a grafts to regenerate skin.



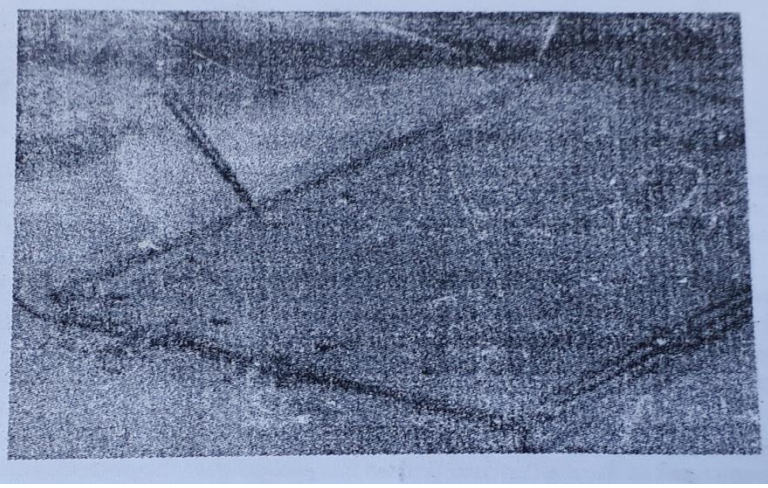


**Figure 1.5**
A specialized cell culture chamber for the production of the dermal layer for skin-grafting applications. From Smith & Neqhew, UK

**Bone-marrow transplantation**

Bone marrow is the most prolific organ and produces 40 billion myeloid cells daily. Most of the chemo and radiotherapy used for treating leukemia affects the rapidly dividing cells, bone marrow are susceptible to myoablative damage and hence it is necessary to protect the patients from hematopoietic failure.

Hematopoiesis is a process by which immature precursor cells develop into mature blood cells. Hence before performing radio and chemotherapy, the bone marrow harvested from the patient is cryopreserved for some time.

Once the therapy is finished, the stored bone marrow cells are put into circulation where there is homing of hematopoietic stem cells, which helps in regaining the bone marrow function. The process takes about two weeks during which the patients should be immuno-compromised and are at the risk of opportunistic infections.

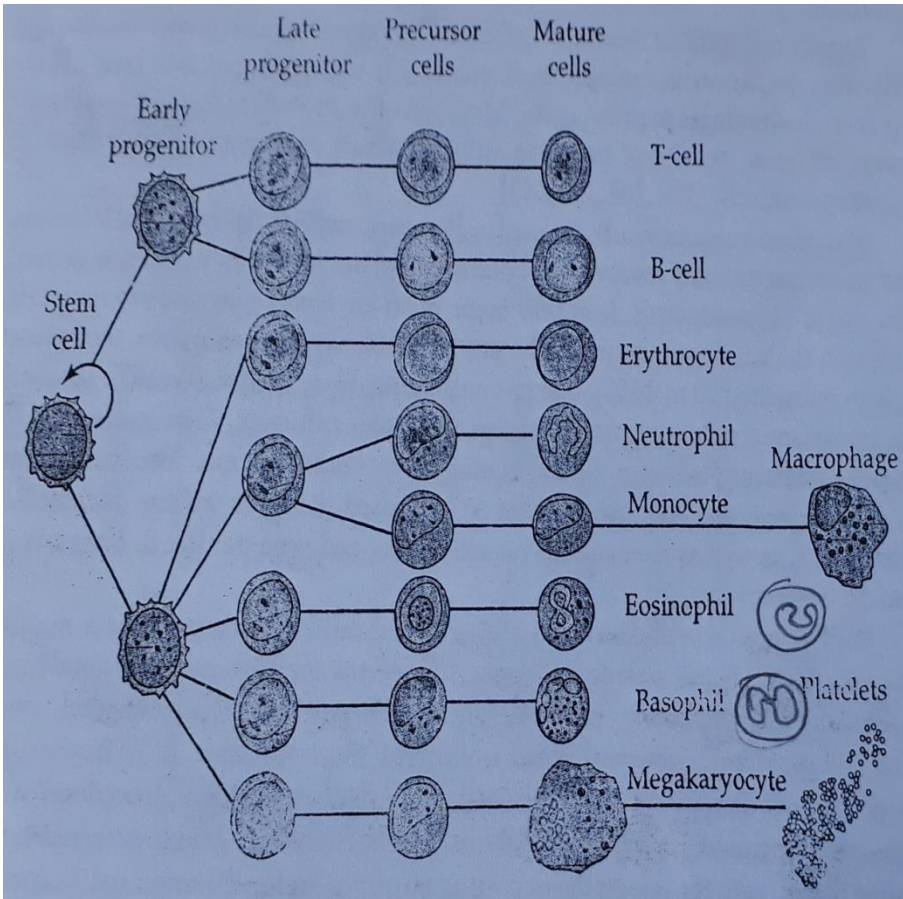



**Figure 1.6**
The process of blood-cell production (called hematopoiesis). The production fluxes through the lineages can be estimated based on the known steady-state concentration of cells in circulation, the total volume of blood, and the half-lives of the cells. Note that the 400 billion cells produced per day arise from a small number of stem cells. From [206].

## 1.5 | Summary

- Cell-based therapies involve the use of human cells as therapeutic agents.
- Red cell, platelet transfusions, bone-marrow transplantation, and retinal cornea replacements represent practiced cell-based therapies.
- Skin, cartilage, and $\beta$-islets as tissue-engineered medical products therapies are emerging.
- To implement these therapies, tissue function needs to be engineered and cells produced in therapeutic quantities.
- Therapeutic cell numbers range from 50 million to 5 billion cells.
- Engineering tissue function requires a quantitative understanding of tissue dynamics, stem-cell biology, cell communications, biomaterials, physico-chemical-rate processes and bioengineering design. Mastering all these subjects and integrating them into novel designs represents the overall challenge to the tissue engineer.

**6. Cell numbers and growth rate**

The cell density in the human tissue is in order of 1-3 billion cells per mL, hence human body consists of about 100 trillion cells. But if you consider an organ, it consists of few hundred million functional subunits. The number of cells is determined by the functional ability of each subunit. For example in kidney the number of nephrons (functional subunit) is determined by the maximal clearance of toxic byproducts and also the clearance ability of each nephron. For cell-based therapies certain number of cells is required and it varies depending on the type of tissue that is affected. For example, chondrocyte transplants require few tens of millions of cells; lymphocyte therapies require half a billion of cells, liver support requires 10 billion hepatocytes and bone marrow transplantation requires few billion cells.

There are certain limitations in the production of primary cells. A single cell can produce $10^{10}$ to $10^{15}$ cells in culture. However, this depends on the type of the cell also. Because fibroblasts and skeletal myocytes grow well in culture while hepatocytes and beta islet cells do not. Usually primary human cells can undergo 30 to 50 doublings in culture based on the age of the donor and the doubling is determined by the Hayflick limit. Primary cells vary in growth rate. For example, the doubling time of hematopoietic cells are 11-12 hours while dermal fibroblasts require 15 hours. Adult chondrocytes require 24-48 hours for doubling while hepatocytes do not grow in culture at all.

Cells are cryopreserved with suitable cryoprotectants like glycerol and dimethylsulphoxide. However, solution effects have to be taken care to avoid intracellular ice formation during freezing. Viability of cells after the cryopreservation process may vary from 40% to 95%. For treatment the cells are usually injected into the portal vein or it can be encapsulated in a suitable polymeric scaffold and then delivered to the patient.

**Importance of determination**

- Cell-based therapies certain number of cells is required and it varies depending on the type of tissue that is affected.
- For examples
  - chondrocyte transplants require few tens of millions of cells;
  - lymphocyte therapies require half a billion of cells,
  - liver support requires 10 billion hepatocytes and
  - bone marrow transplantation requires few billion cells.

TABLE 1.3

Cell numbers in tissue biology and tissue engineering: orders-of-magnitude.

| | |
|---|---|
| **Cell numbers *in vivo*** | |
| Whole body | $10^{14}$ |
| Human organ | $10^{9}$ to $10^{11}$ |
| Functional subunit | $10^{2}$ to $10^{3}$ |
| **Cell production *in vivo*** | |
| Maximum from a single cell (Hayflick limit) | $2^{30}$ to $2^{50} < 10^{15}$ |
| Myeloid blood cells produced over a life time | $10^{16}$ |
| Small intestine epithelial cells produced over a lifetime | $5 \cdot 10^{14}$ |
| **Cell production *ex vivo*** | |
| Requirements for a typical cellular therapy | $10^{7}$ to $10^{9}$ |
| **Expansion potential of human tissues *in vitro*** | |
| Hematopoietic cells | |
| Mononuclear cells | 10-fold |
| CD34 enriched | 100-fold |
| 2 or 3 antigen enrichment | $10^{6}$ to $10^{7}$-fold |
| T-cells | $10^{3}$ to $10^{4}$ -fold |
| Chondrocytes | 10-20 fold |
| Muscle, dermal fibroblasts | $> 10^{6}$ fold |

**Fundamental Limitations for production of primary cells**

- A single cell can produce $10^{10}$ to $10^{15}$ cells in culture. However, this depends on the type of the cell also.
- Because fibroblasts and skeletal myocytes grow well in culture while hepatocytes and beta islet cells do not.
- Usually primary human cells can undergo 30 to 50 doublings in culture based on the age of the donor and the doubling is determined by the Hayflick limit.

Primary cells vary in growth rate.

For example, the doubling time of

- Hematopoietic cells are 11-12 hours while dermal fibroblasts require 15 hours.
- Adult chondrocytes require 24-48 hours for doubling while hepatocytes do not grow in culture at all.

**Hayflick limit or Hayflick phenomenon**

- The number of times a normal human cell population will divide before cell division stops.
- The concept of the Hayflick limit was advanced by American anatomist Leonard Hayflick in 1961, at the Wistar Institute in Philadelphia, Pennsylvania, US.

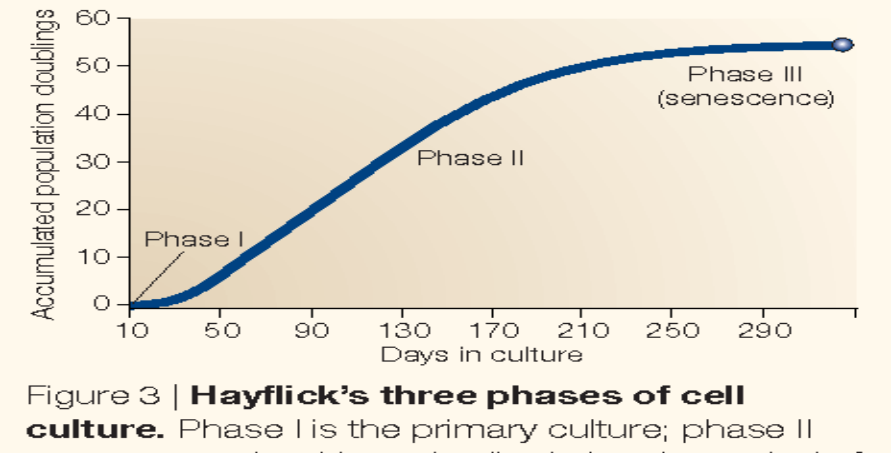
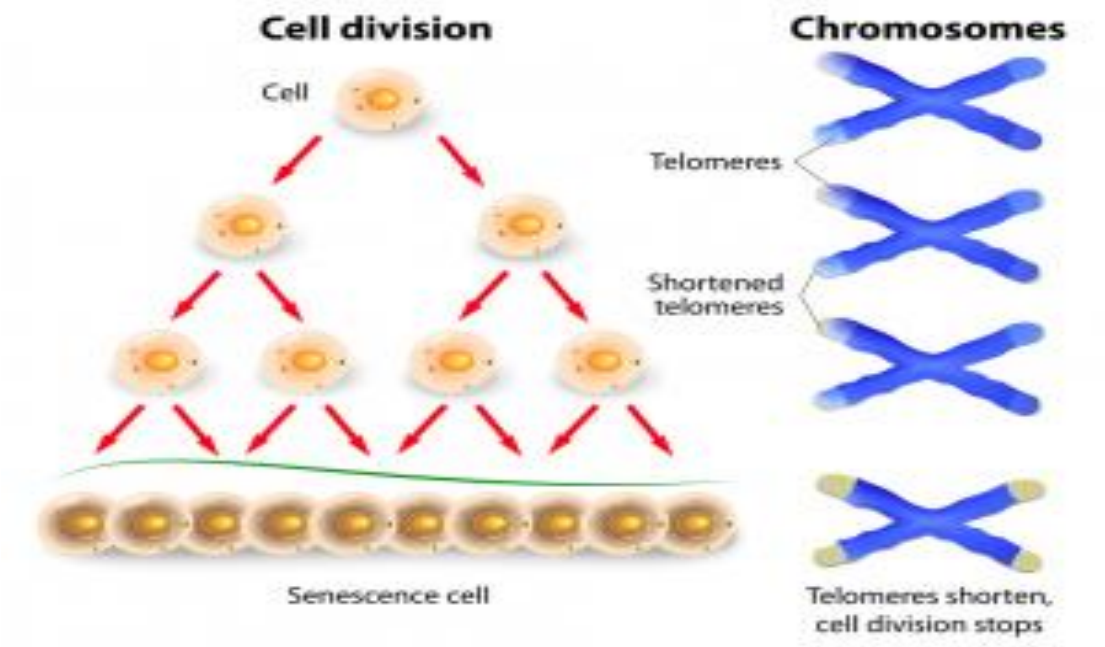



Figure 3 | **Hayflick's three phases of cell culture.** Phase I is the primary culture; phase II

Figure 3 | Hayflick's three phases of cell culture. Phase I is the primary culture; phase II represents subcultivated cells during the period of exponential replication. Phase III represents the period when cell replication ceases but metabolism continues. Cells may remain in this state for at least one year before death occurs.

❖




**Cells Preservation**

- Cells are cryopreserved with suitable cryoprotectants like glycerol and dimethylsulphoxide.
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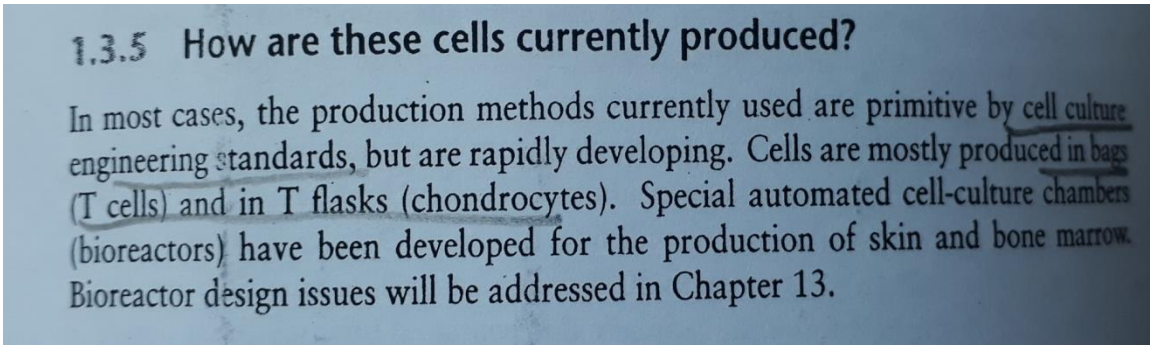


### 1.3.5 How are these cells currently produced?

In most cases, the production methods currently used are primitive by cell culture engineering standards, but are rapidly developing. Cells are mostly produced in bags (T cells) and in T flasks (chondrocytes). Special automated cell-culture chambers (bioreactors) have been developed for the production of skin and bone marrow. Bioreactor design issues will be addressed in Chapter 13.

**7. Challenges in tissue engineering**

Tissue engineers face challenge in the implementation of the cell-based therapies, which include

1. Proper reconstitution of the micro-environment for the proper functioning of the tissues
2. Scaling up to benefit the clinical society
3. Automation of system operation at a clinically meaningful scale
4. Implementation of the automated device in a clinical setting

First and foremost thing in tissue engineering is to create a suitable microenvironment, which is characterized by neighboring cells, extracellular matrix, cytokine, chemokine, hormone signaling, cell geometry and provision of nutrients and the removal of metabolic byproducts. Thus it is complex to create a micro-environment with all the above mentioned characteristics.

For proper reconstitution, it is necessary to mimic the dynamic, chemical and geometric variables. These microenvironments are connected via microcirculation and most of the metabolically active cells lie in few microns from the capillary. Thus these capillaries provide a perfusion environment thereby connecting the cells to the source of oxygen and sink for carbon dioxide and so on.

Bioreactors are used nowadays to simulate respiratory, gastrointestinal and renal functions, since they provide the microenvironment and also the perfusion characteristics, which are very important to maintain the tissue function.

**There are few challenges to be answered for the reconstruction of functional tissues.**

They are:

1. How do we develop a close approximate biological replica?
2. Should the tissue be produced in vitro implanted in vivo?
3. What type of scaffold should we choose?
4. How do we manufacture?
5. What cells are to be used?
6. Under what conditions can cells expand without affecting its phenotype?
7. What regulators do we use to stimulate the cell proliferation and differentiation?

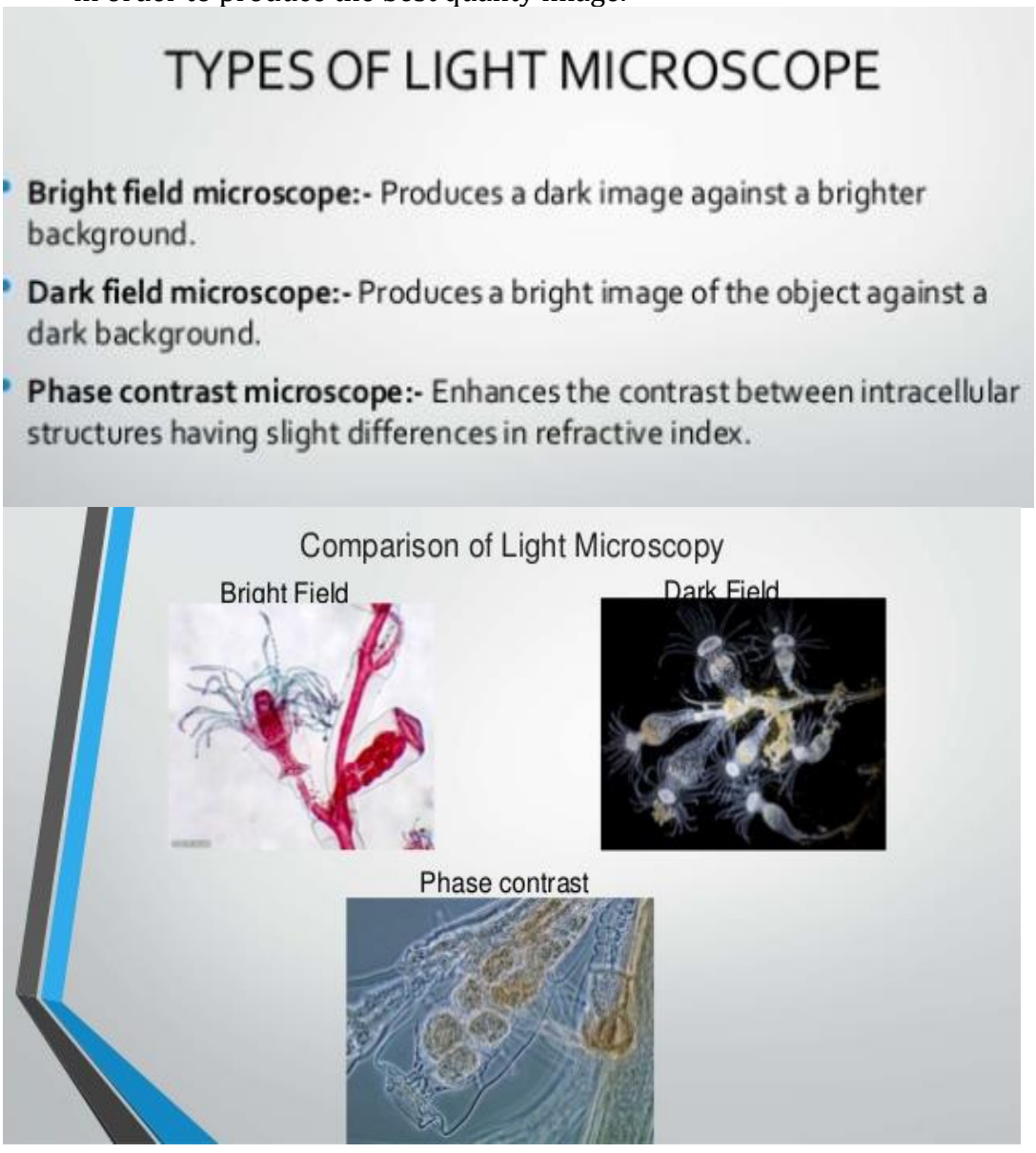
**8. Measurement of cell characteristics**

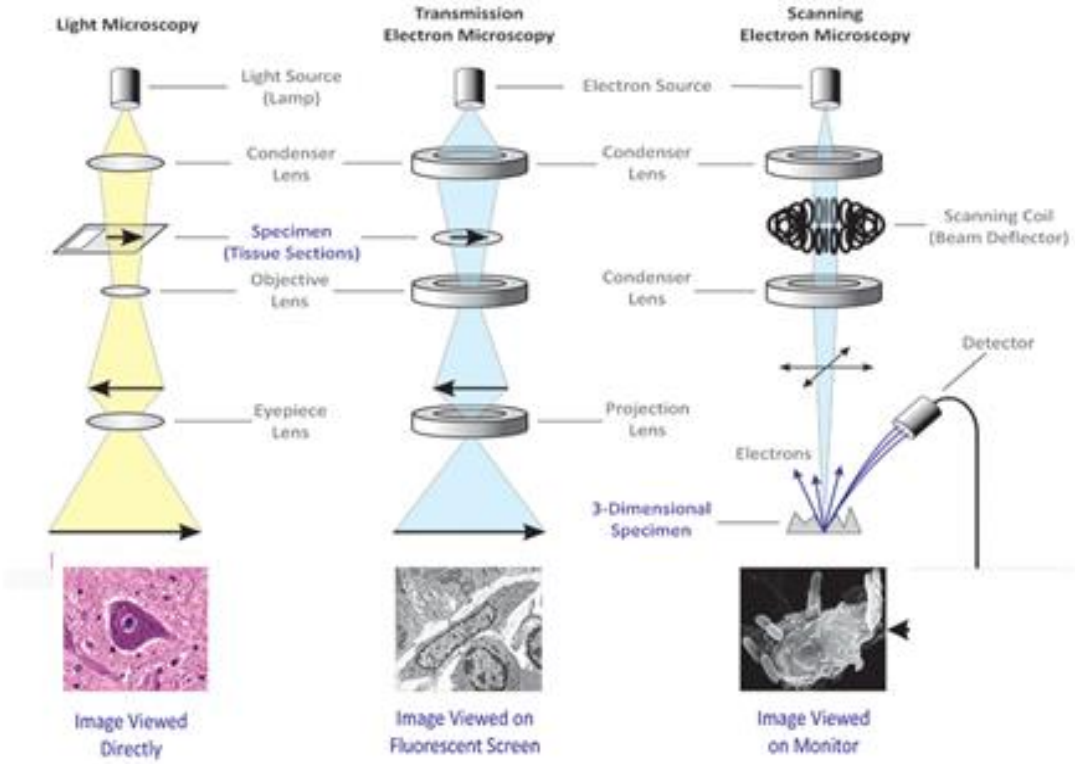
- **Basic tools**
  - ❖ Microscopy
    - Light Microscopy
    - Phase contrast microscopy
    - Time lapse microscopy
    - Deconvolution microscopy
    - Fluorescent microscopy
      - Confocal microscopy
    - Electron microscopy

- ❖ Detection of biochemical components
  - Antibody based techniques - fluorescent or enzymatic labelling or radioactive coupling
  - Non Antibody based techniques
    - Green fluorescent protein
    - Gene of β-lactamase
- ❖ Invivo imaging
  - Ultra sound, x rays, MRI, Computer Assisted tomography(CAT scan).

**Principle of Light Microscopy**

- Basic light microscope operation begins with bringing light to the sample and ensuring that the light source is of the correct intensity, directionality, and shape in order to produce the best quality image.

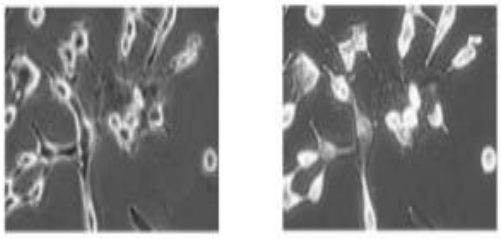


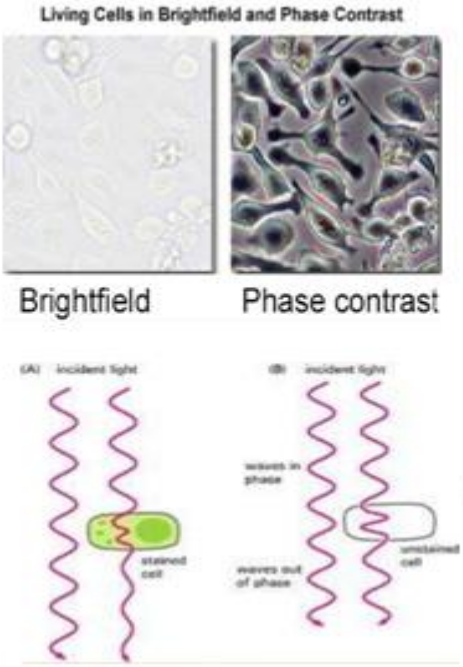


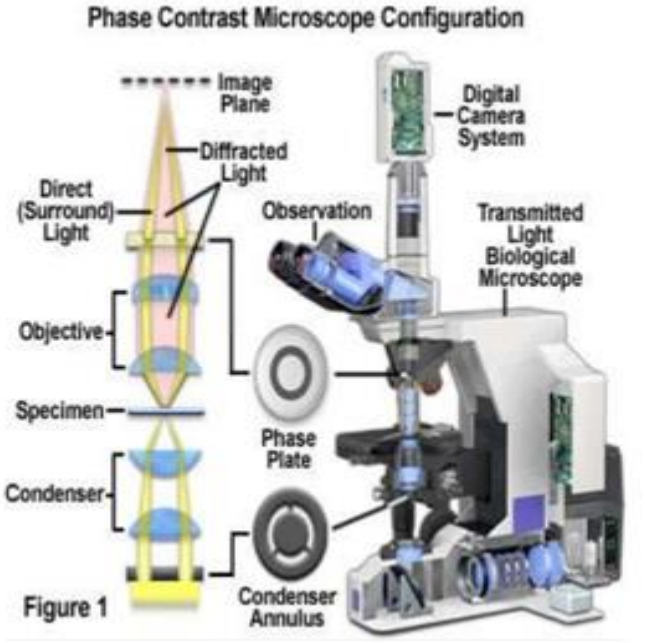

**Phase contrast microscopy**

- It is the first microscopic method which allow the observation of living cell.
- It was invented by Frits Zernike and was awarded noble prize in 1953.

- **Positive phase contrast** reveals medium to dark gray images on a lighter grey background; these images often have a bright halo along the edge of the sample.
- **Negative phase contrast** is the opposite. The specimen appears lighter with a dark background; they also have a dark halo outlining the image.





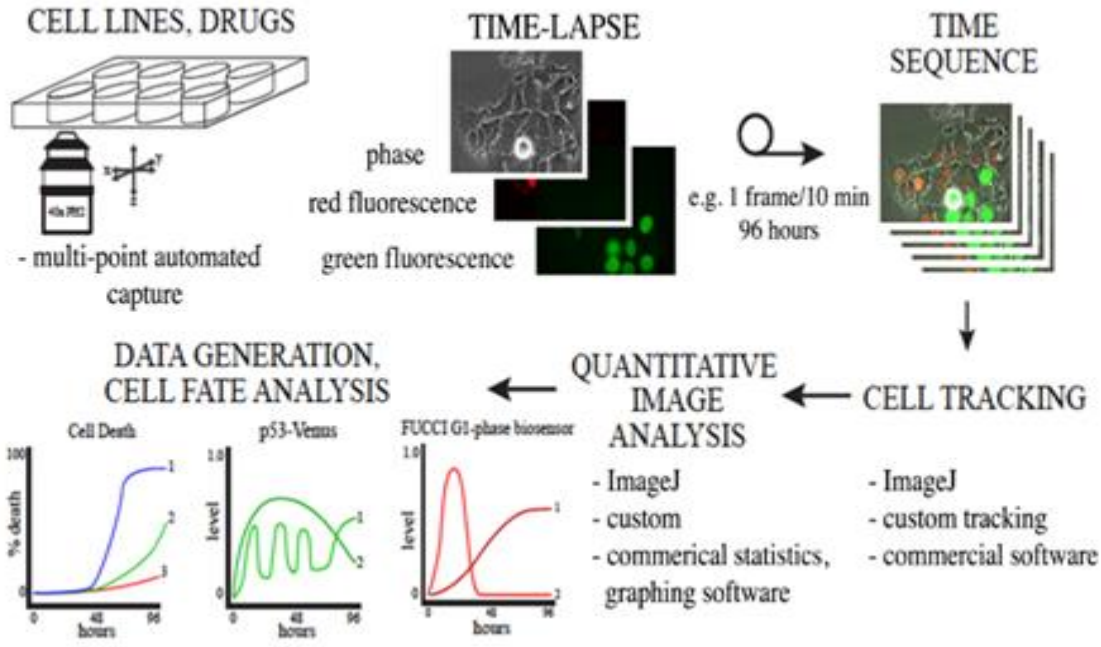

**Applications of Phase contrast microscopy**

- Applications for phase contrast microscopy equipment range from the study of living biological specimens, medical applications, study of live blood cells, and other biological and science applications
- Most commonly used to provide constrast of transparent specimens such as living cells or small organisms.

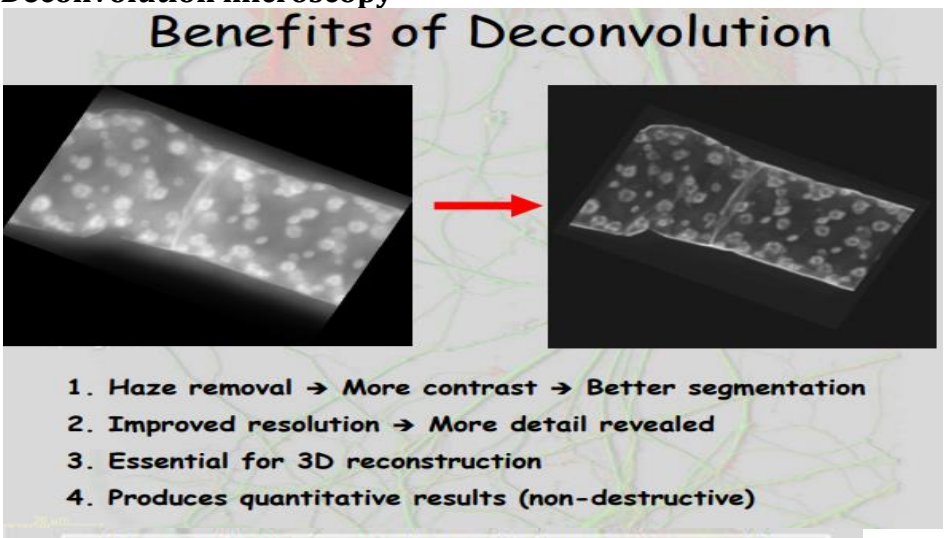
**Time lapse microscopy**

- Time-lapse microscopy is time-lapse photography applied to microscopy.
- Microscope image sequences are recorded and then viewed at a greater speed to give an accelerated view of the microscopic process.

| | |
|---|---|
| **Other names** | (Time-lapse) microcinematograph, (Time-lapse) video microscope, Time-lapse cinemicrograph |
| **Uses** | Observation of slow microscopic processes |
| **Inventor** | Jean Comandon and other contemporaries |
| **Related items** | Time-lapse photography, Live cell imaging |




**Deconvolution microscopy**

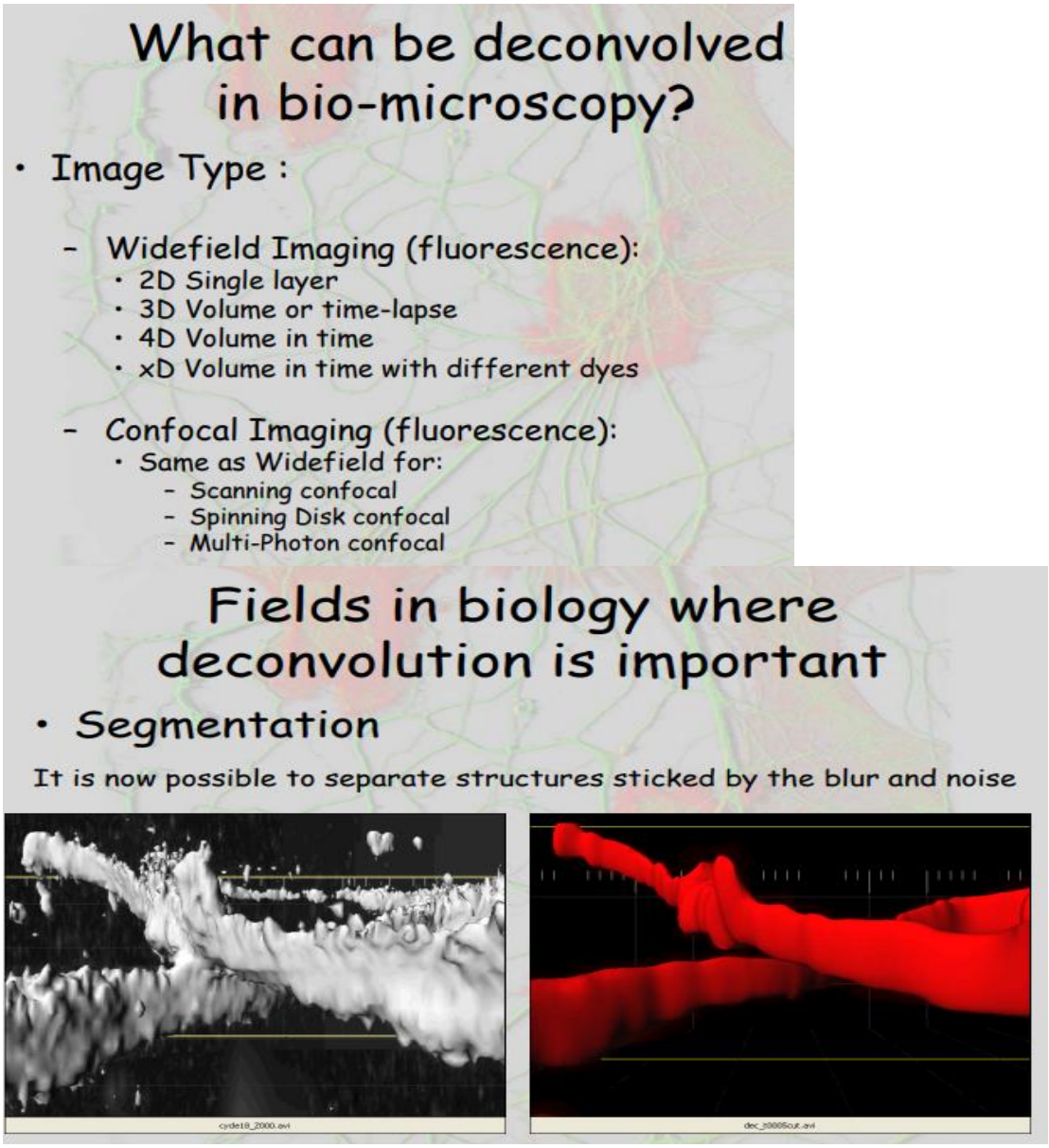



Deconvolution is a mathematical operation used in image restoration to recover an object from an image that is degraded by blurring and noise.

In fluorescence microscopy the blurring is largely due to diffraction limited imaging by the instrument; the noise is mainly photon noise.

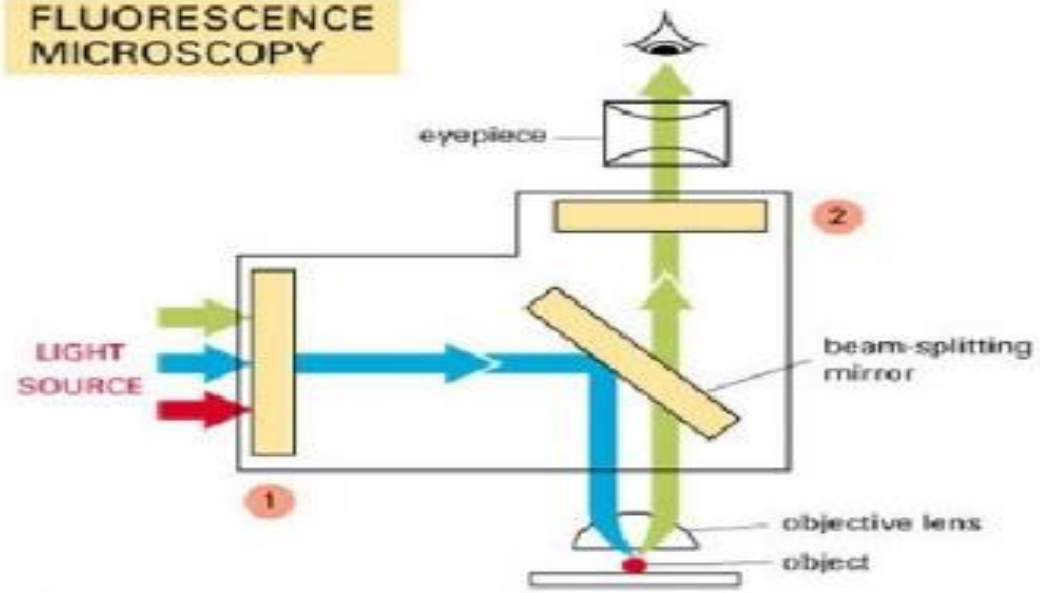
Usage:

- Microscopy: biomedical research, ophthalmology, medical diagnostics, nuclear imaging (CT), food processing, forensics, skin care/beauty product development, etc.
- Astronomy: telescope imaging, satellite imaging, aerial imaging (cartography), etc.
- Industrial: Quality control for printed circuit boards and semiconductors, etc.




**Fluorescent microscopy**

# FLUORESCENCE MICROSCOPY




Fluorescent dyes used for staining cells are detected with the aid of a *fluorescence microscope*. This is similar to an ordinary light microscope except that the illuminating light is passed through two sets of filters. The first ( 1 ) filters the light before it reaches the specimen, passing only those wavelengths that excite the particular fluorescent dye. The second ( 2 ) blocks out this light and passes only those wavelengths emitted when the dye fluoresces. Dyed objects show up in bright color on a dark background.

A fluorescence microscope is basically a conventional light microscope with added features and components that extend its capabilities.

**conventional microscope**

✓ uses light to illuminate the sample and produce a magnified image of the sample.

**fluorescence microscope**

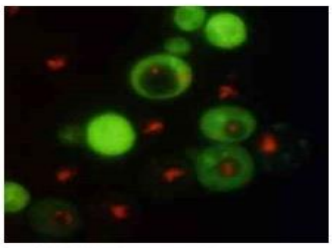
✓ uses a much higher intensity light to illuminate the sample

✓ This light excites fluorescence species in the sample, which then emit light of a longer wavelength.

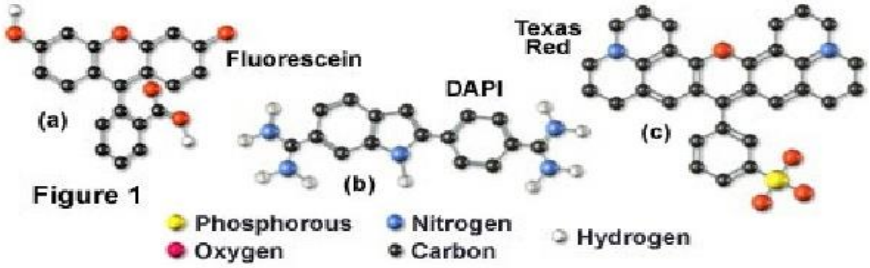
✓ A fluorescent microscope also produces a magnified image of the sample, but the image is based on the second light source

# TYPES OF FLUOROPHORES USED

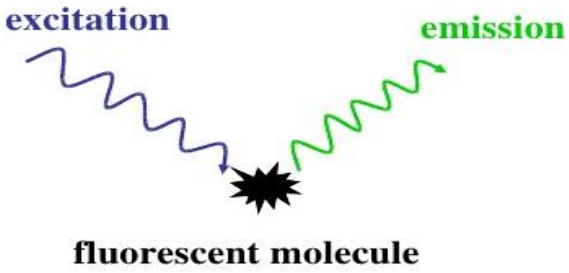
- fluorescein,
- DAPI,
- propidium iodide
- green fluorescent protein (GFP)
- Texas Red

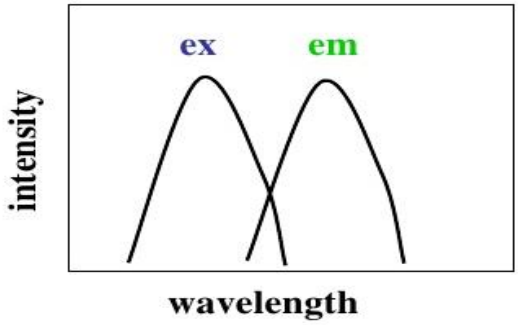


**Common Fluorophores in Widefield and Confocal Microscopy**




## 3) Fluorescence microscopy




| Fluorochrome | Excitation wavelength | Emission wavelength |
|---|---|---|
| Fluoroscein | 490 - blue | 520 - green |
| Rhodamine | 550 – green | 580 - red |
| Hoechst (stains DNA) | 345 - UV | 455 - blue |

Confocal microscopy : (having the same focus )

- An optical imaging technique for increasing optical resolution and contrast of a micrograph.
- Radiations emitted from laser cause sample to fluoresce.
- Uses pinhole screen to produce high resolution images.
- Eliminates out of focus.
- So images have better contrast and are less hazy.
- A series of thin slices of the specimen are assembled to generate a 3-dimensinal image.
- Is an updated version of fluorescence microscopy.

## WORKING MECHANISM

Confocal microscope incorporates 2 ideas:

1. Point-by-point illumination of the specimen.
2. Rejection of out of focus of light.

Light source of very high intensity is used—Zirconium arc lamp in Minsky's design & laser light source in modern design.

a)Laser provides intense blue excitation light.

b)The light reflects off a dichoric mirror, which directs it to an assembly of vertically and horizontally scanning mirrors.

c)These motor driven mirrors scan the laser beam across the specimen.

d) The specimen is scanned by moving the stage back & forth in the vertical & horizontal directions and optics are kept stationary.

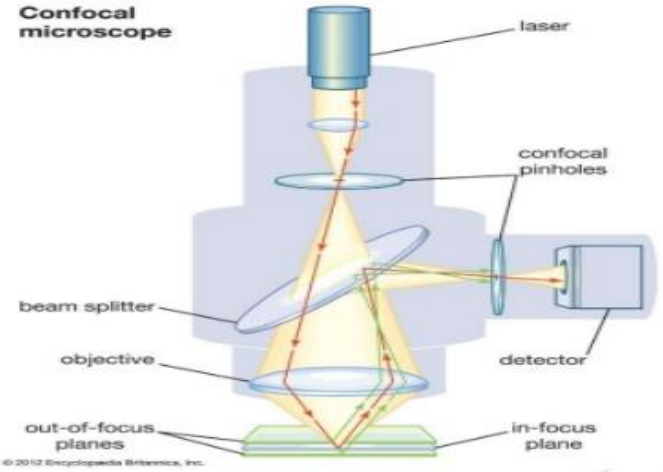
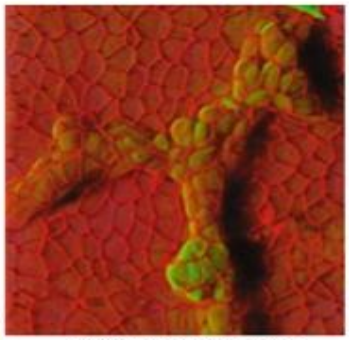



fig: schematic diagram confocal microscope

## APPLICATIONS

**1.Confocal microscopy allows analysis of fluorescent labelled thick specimens without physical sectioning.**



3D shadow projection

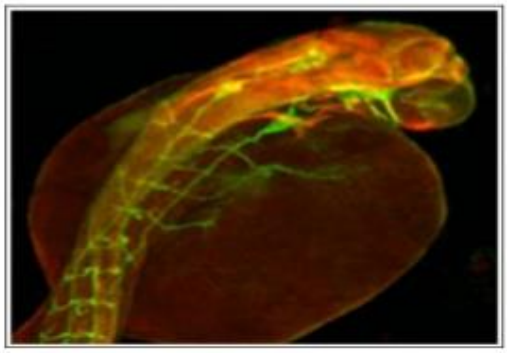


Fig:Zebra fish embryo whole mount
Neurons (green)
Cell adhesion molecule (red)

2. Three-Dimensional Reconstruction of Specimen

## APPLICATIONS

4. Improved Resolution

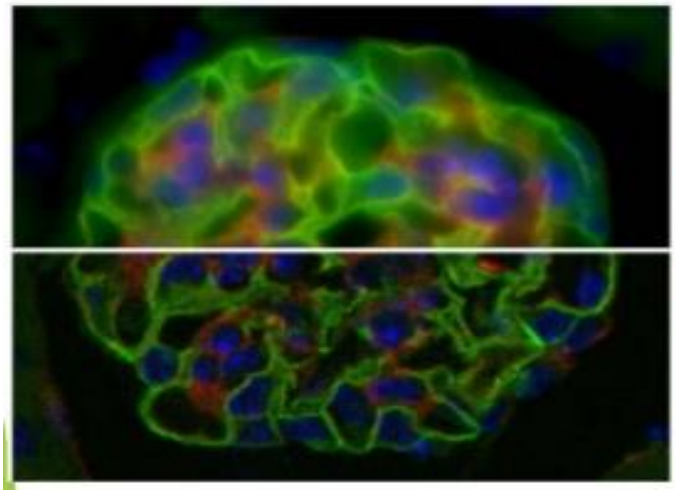


Fig: Kidney cells (fluorescence vs Confocal microscope

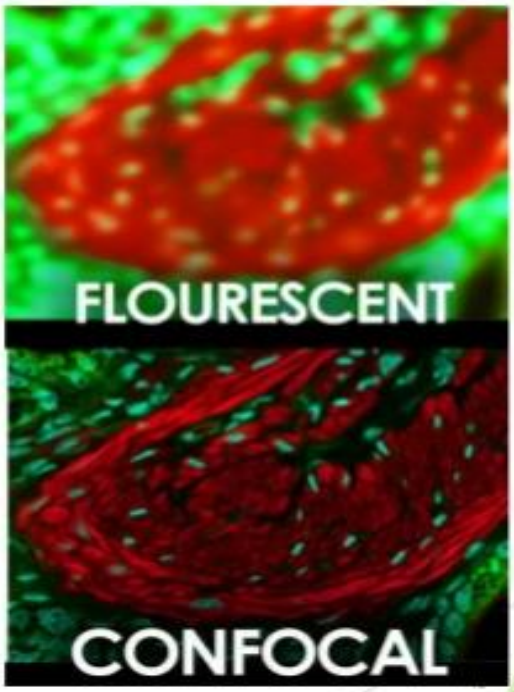



Fig:An Intestine Section

## ELECTRON MICROSCOPE

- Electron Microscopes uses a beam of highly energetic electrons to examine objects on a very fine scale. This examination brings the details about the observant.

- **Topography**
- **Morphology**
- **Composition**
- **Crystallographic Structure**

## TYPES OF ELECTRON MICROSCOPE

- Scanning Electron Microscope (SEM)
- Transmission Electron Microscope (TEM)

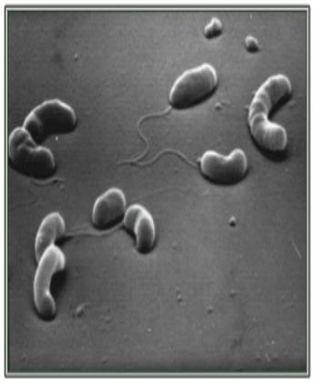
## Applications Of TEM

- It can be used for the study of:
  - To examine a small column of atoms.
  - Cancer research
  - Virology
  - Pollution Nanotechnology
  - Semiconductor research
  - Chemical Identity
  - Crystal orientation
  - Electronic structure

## SCANNING ELECTRO MICROSCOPE

- SEM focuses on the sample's surface and its composition
- Scan a gold-plated specimen to give a 3-D view of the surface of an object which is black and white.
- Used to study surface features of materials, cells and viruses.
- Scanning Electron microscope has resolution 1000 times better than Light microscope.

### SEM Images (Micron Range)



*Vibrio cholerae* with polar Flagella

## Advanced MICROSCOPES

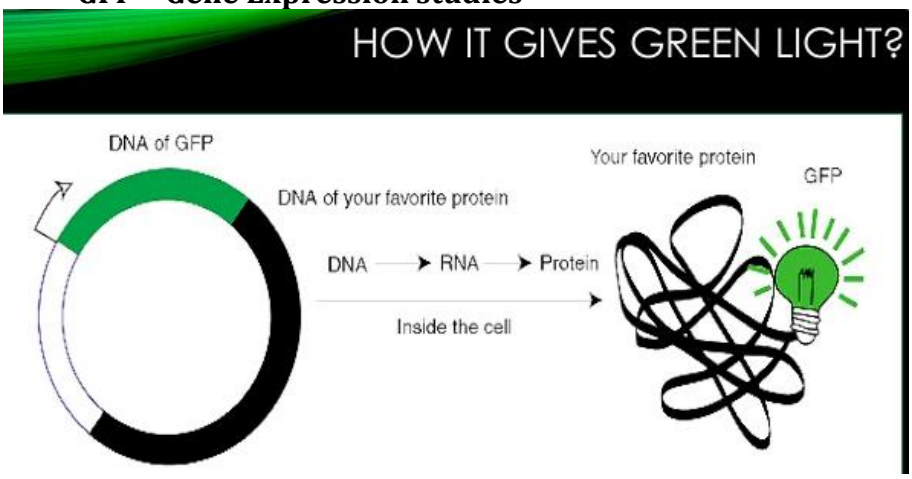
- **SCANNING PROBE MICROSCOPE** -Class of Microscope that measures surface features by moving a sharp probe over object surface. Used to visualize atoms and molecules in more distinctly.
  - Scanning Tunneling Microscope (STM)
  - Atomic Force Microscope (AFM)

**DETECTION OF BIOCHEMICAL COMPONENTS**

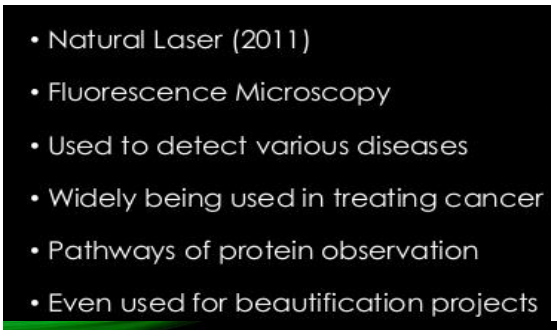
- **Antibody based techniques** - fluorescent or enzymatic labelling or radioactive coupling
- **Non Antibody based techniques**
  - Green fluorescent protein
  - Gene of β-lactamase
- **Future –Quantum dots**

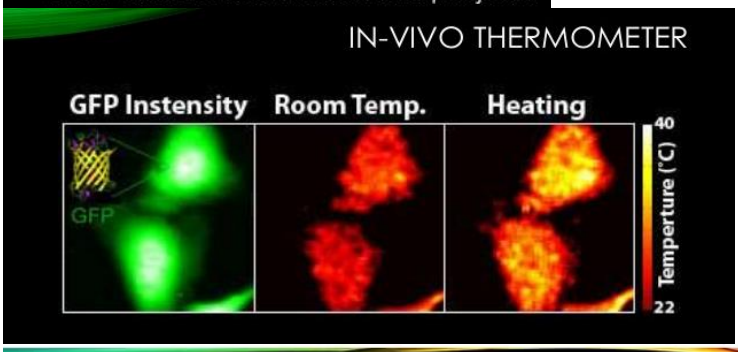
**Non Antibody based techniques**

- **GFP – Gene Expression studies**

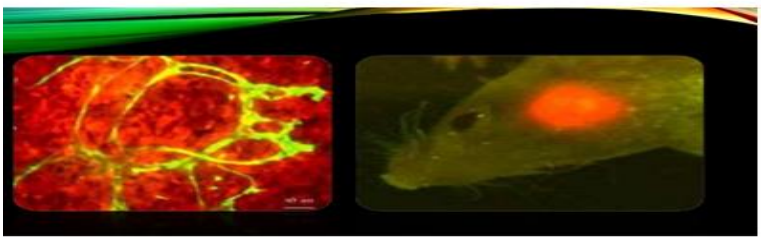



**APPLICATIONS**

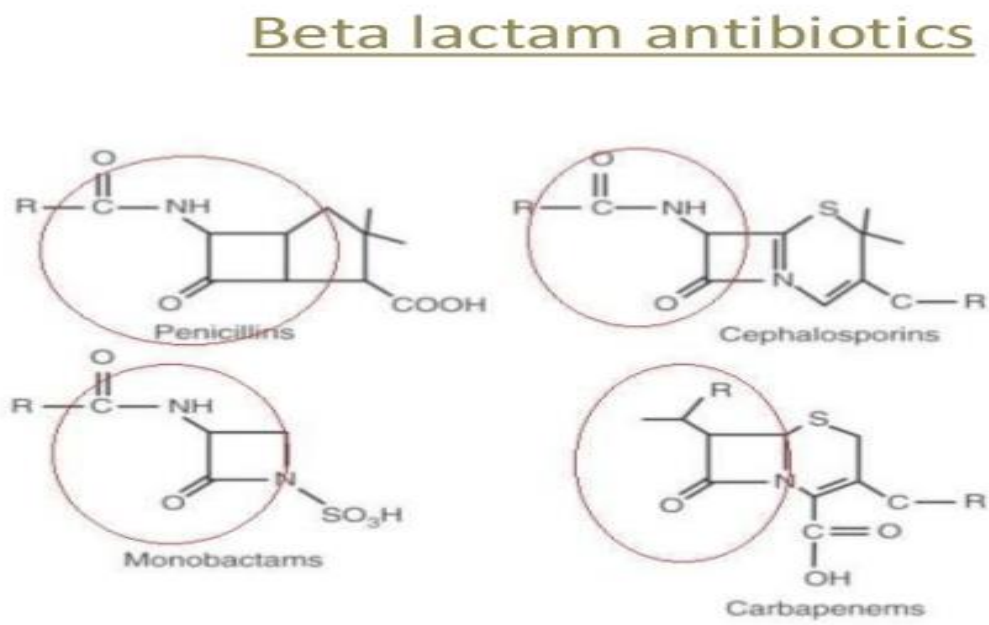






**Gene of β-lactamase - Gene Expression studies**

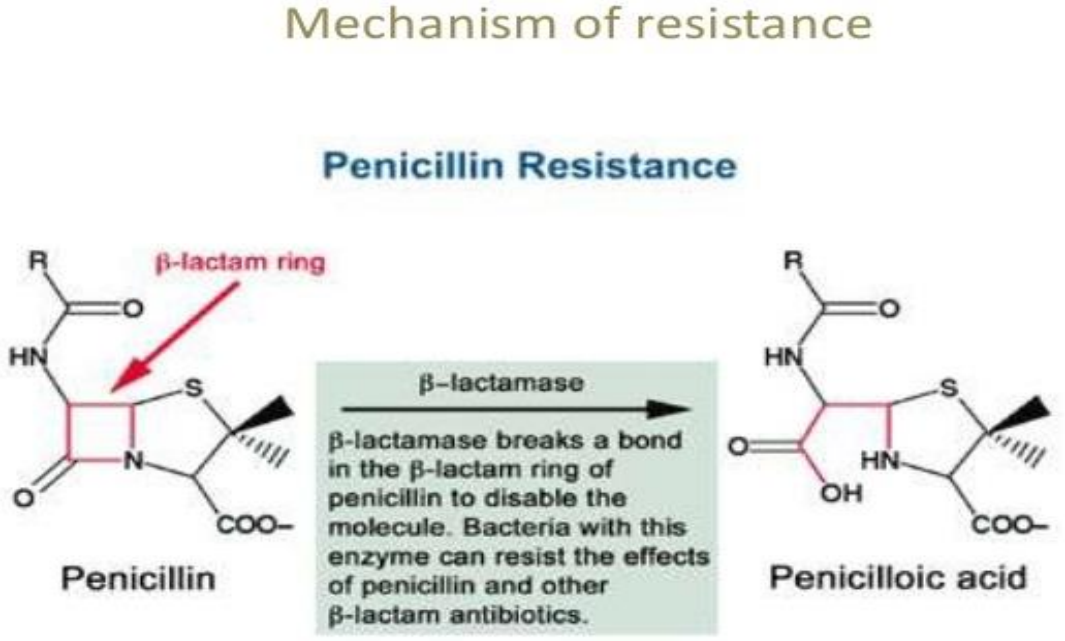



**Beta-lactamases**

**Beta-lactamases are enzymes (EC 3.5.2.6)** produced by bacteria that provide multi-resistance to β-lactam antibiotics such s penicillins, cephalosporins, cephamycins, and carbapenems (ertapenem), although carbapenems are relatively resistant to beta-lactamase. **Beta-lactamase** provides antibiotic resistance by breaking the antibiotics' structure. These antibiotics all have a common element in their molecular structure: a four-atom ring known as a β-lactam. Through hydrolysis, the lactamase enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties.

**Beta-lactam antibiotics** are typically used to treat a broad spectrum of Gram-positive and Gram-negative bacteria.

**Beta-lactamases** produced by Gram-negative organisms are usually secreted, especially when antibiotics are present in the environment.



## Methods of detection

### Organisms tested for beta lactamase

- Staphylococcus aureus
- CONS
- Enterococci sp.,
- Neisseria gonorrhea
- Hemophilus influenzae
- Moraxella catarrrhalis

**Phenotypic method**:

- Acidometric method
- Iodometric method
- Nitrocefin method
- penicillin disc diffusion test
- Penicillin broth microdilution test
- Penicillin zone edge test

**Genotypic test:**

- PCR for blaZ gene

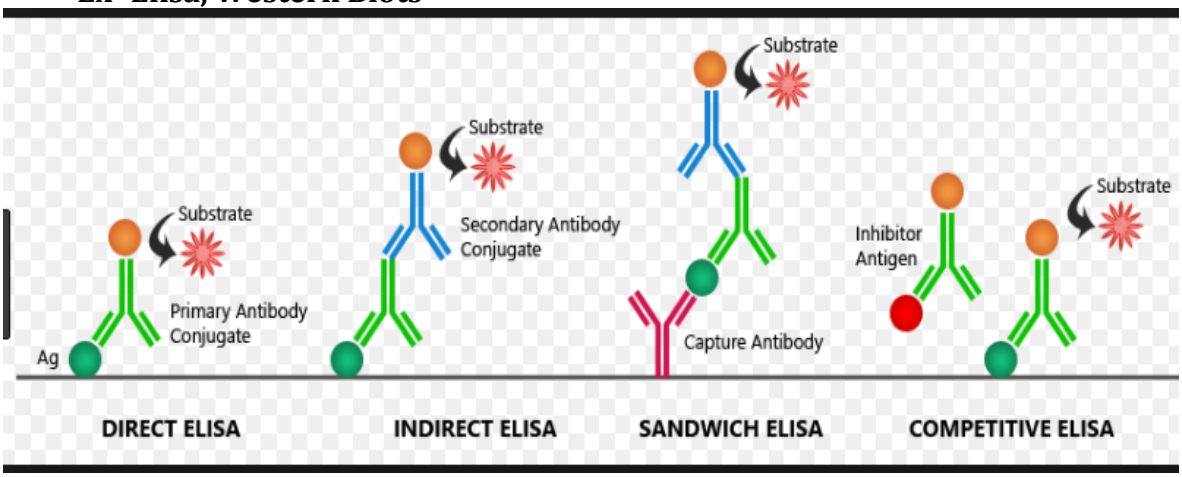
## TREATMENT

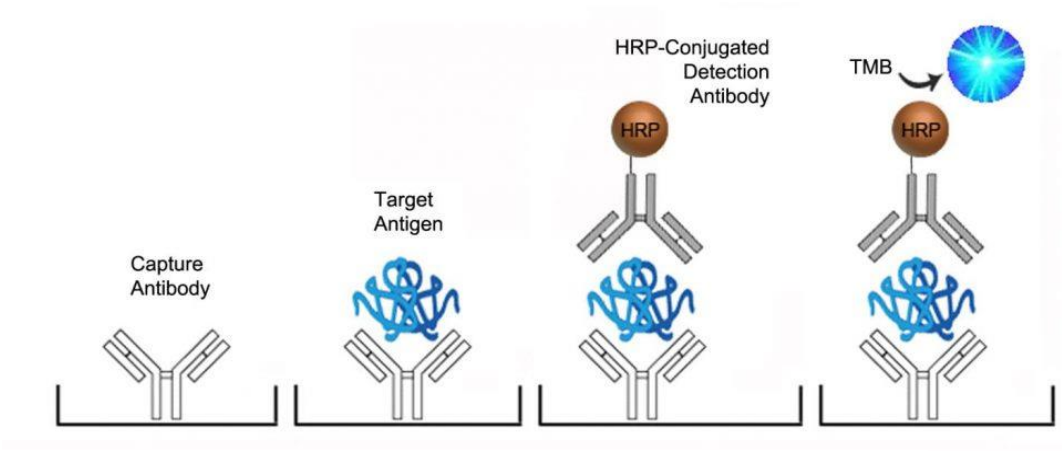
- Beta lactamase resistant semisynthetic penicillin( methicillin, nafcillin, cloxacillin,dicloxacillin)

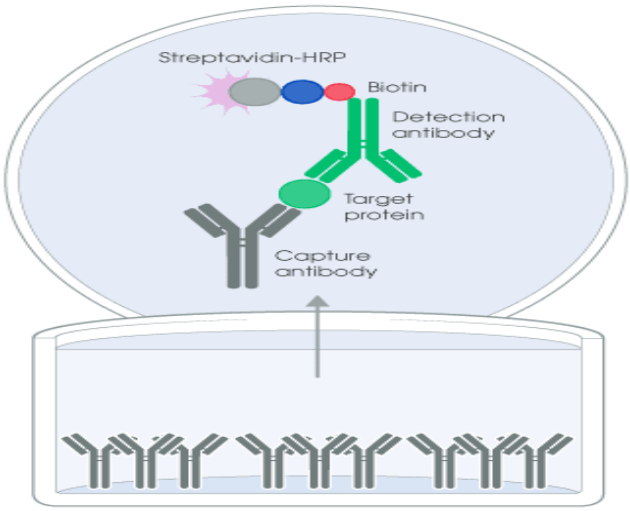
- Beta lactam – betalactamase inhibitor combinations ( eg: amoxicillin clavulunate, Ampicillin sulbactam, Piperacillin tazobactam)

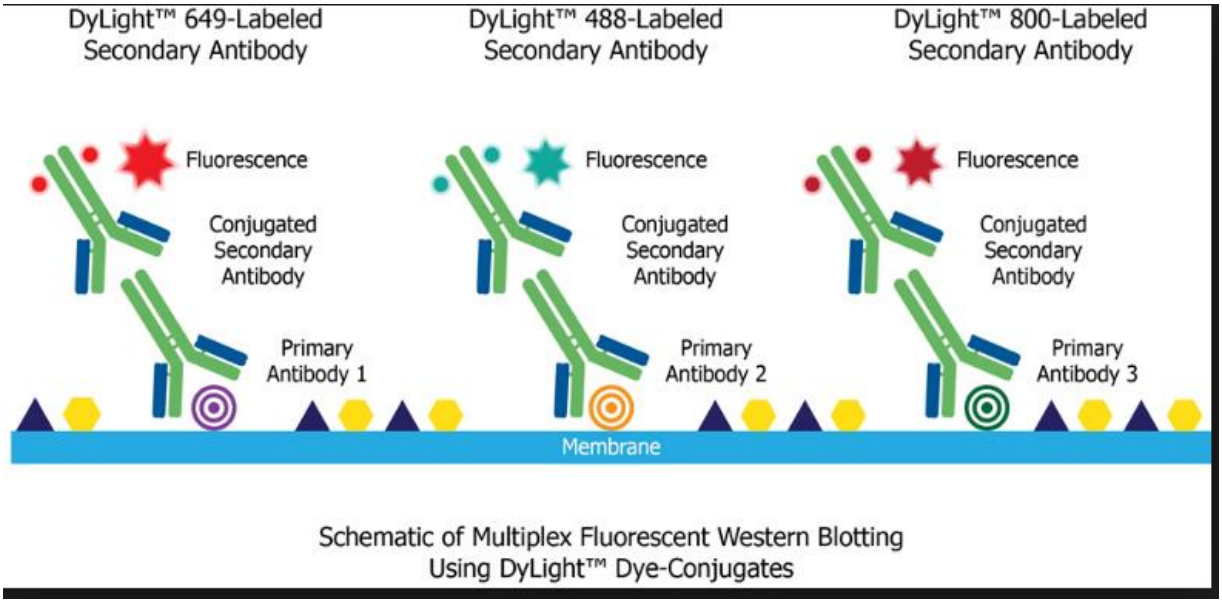
**ANTIBODY BASED TECHNIQUES - FLUORESCENT OR ENZYMATIC LABELLING OR RADIOACTIVE COUPLING**

- **Ex- Elisa, Western Blots**






**INVIVO IMAGING**

- **Ultra sound,**
- **x rays,**
- **MRI,**

❖ **Computer Assisted tomography(CAT scan).**

## Cell Morphology

- Fibroblastic (elongated and branched)
- Epithelial (rounded pattern)
- Projected surface area (PSA)

Perfect circle- 1 (Shape index)
Line- 0

### Cell Culture Morphology

- Morphologically cell cultures take one of two forms:
  - Anchorage independent cells (Suspension culture)
  - Anchorage dependent cells (Adherent Culture)

Regularly examining the **morphology** of the cells in culture (i.e., their shape and appearance) is essential for successful cell culture experiments.

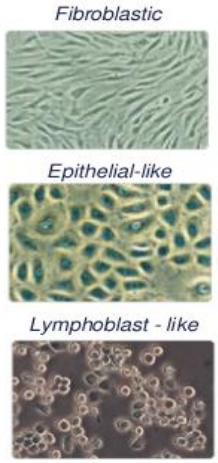
- This examination will help:
  - Detect any signs of contamination early on and to contain it before it spreads to other cultures around the laboratory.
  - Identify signs of deterioration in cells include granularity around the nucleus, detachment of the cells from the substrate, and cytoplasmic vacuolation.

**Suspension Cell Lines**

| Name | Species and Tissue of Origin | Morphology |
|---|---|---|
| NS0 | Mouse Myeloma | Lymphoblast |
| U937 | Human Hystiocytic Lymphoma | Lymphoblast |
| HL60 | Human Leukemia | Lymphoblast |
| WEHI231 | Mouse B-cell Lymphoma | Lymphoblast |
| YAC1 | Mouse Lymphoma | Lymphoblast |
| U266B1 | Human Myeloma | Lymphoblast |
| Jurkat | Human T-cell Leukemia | Lymphoblast |
| THP-1 | Human Monocyte Leukemia | Lymphoblast |

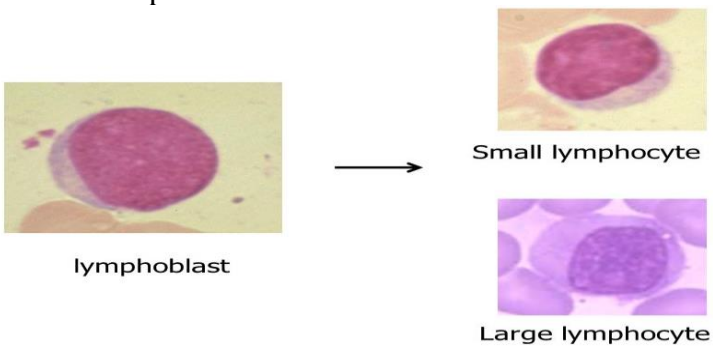
## Cell Morphology Categories

- Most mammalian cells in culture can be divided in to three basic categories based on their morphology.
  - **Fibroblastic** (or fibroblast-like) cells are bipolar or multipolar, have elongated shapes, and grow attached to a substrate.
  - **Epithelial-like** cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.
  - **Lymphoblast-like** cells are spherical in shape and usually grown in suspension without attaching to a surface.




### Lymphoblasts

- Lymphoblasts are larger than the normal small lymphocytes present in blood.
- The nucleus is generally round but may be indented or convoluted.
- The chromatin is usually finely stippled but may be coarsely granular.
- One or more nucleoli are present in the nucleus, but they are often difficult to see in routinely stained blood films . The cytoplasm is more basophilic than is seen in most blood lymphocytes and sometimes contains vacuoles.
- Rare lymphoblasts may be observed in disorders with increased antigenic stimulation; but when several of these cells are found during a differential count, lymphoid neoplasia is suspected.

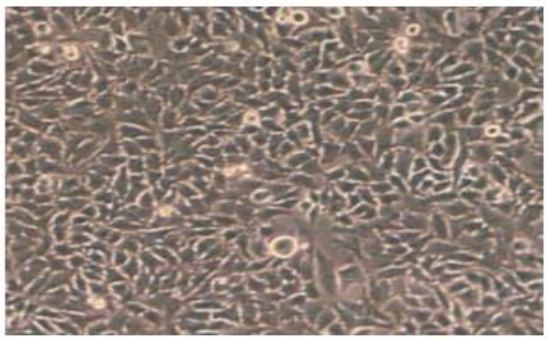



**Examples of attached cell types.**

### EPITHELIAL CELLS

- Epithelial cells are a type of cell that lines the surfaces of your body.
- They are found on your skin, blood vessels, urinary tract, and organs.
- An epithelial cells in urine test looks at urine under a microscope to see if the number of your epithelial cells is in the normal range.

Epithelial Cell Type

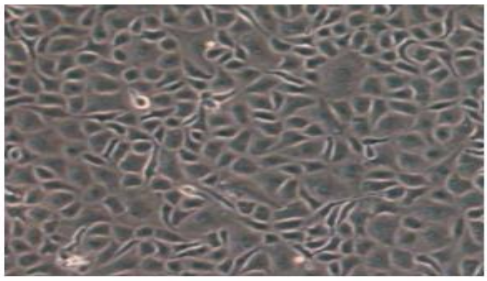


**ENDOTHELIAL CELL**

- Endothelium refers to cells that line the interior surface of blood vessels and lymphatic vessels, forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall.
- It is a thin layer of simple, or single-layered, squamous cells called endothelial cells.

  **Location:** Lining of the inner surface of blood vessels
  **System:** Circulatory system

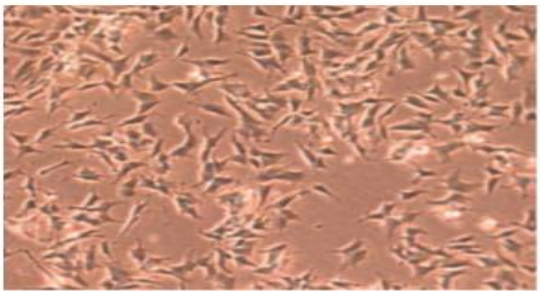
Endothelial Cell Type



**NEURONAL CELLS**

A neuron, also known as a neurone (British spelling) or nerve cell, is an electrically excitable cellnervous tissue that communicates with other cells via specialized connections called synapses.
It is the main component of

Neuronal Cell Type

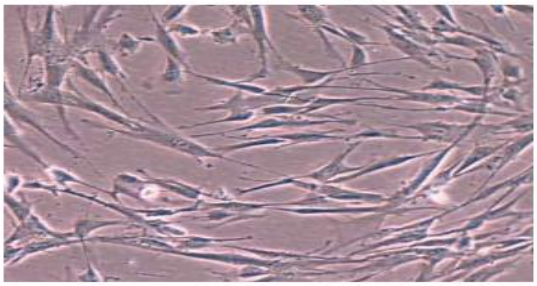


**FIBROBLAST CELLS**

- A fibroblast is a type of biological cell that synthesizes the extracellular matrix and collagen, produces the structural framework (stroma) for animal tissues, and plays a critical role in wound healing.
- Fibroblasts are the most common cells of connective tissue in animals.

  Function: Extracellular matrix and collagen cre...
  Location: Connective tissue

Fibroblast Cell Type



Comparison of Animal and Microbial Culture

| Features | Microbes | Animal Cells |
|---|---|---|
| Cell wall | Generally present | Generally absent |
| Cell membrane | Present | Present |
| Growth Rate | 10-50% per hour | 1-5% per hour |
| $O_2$ Requirement | High | Low |
| Nutritional Rqmt | Usually simple | Complex |
| $CO_2$ Requirement | Sometimes | Key for buffering |
| Environmental FX | Less affected | Very susceptible |
| Size | 100-2000 nm | 10000-100000 nm |
| Seeding density | 1 cell | $10^5$ cells/mL |
| Growth density | $10^9$-$10^{10}$ cells/mL | $10^6$ cells/mL |

**Cell morphology** is typically a qualitative and subjective measurement; however, quantitative information can be extracted using image analysis.
The projected surface area(PSA), for example is often used as an index of cell spreading.
The shape index, defined $= \frac{4\pi A}{P2}$

Where,
A- Area of the cell;
P-perimeter, provides a measure of shape

## CELL NUMBER AND VIABILITY

### Cell Counter

### Direct method of counting

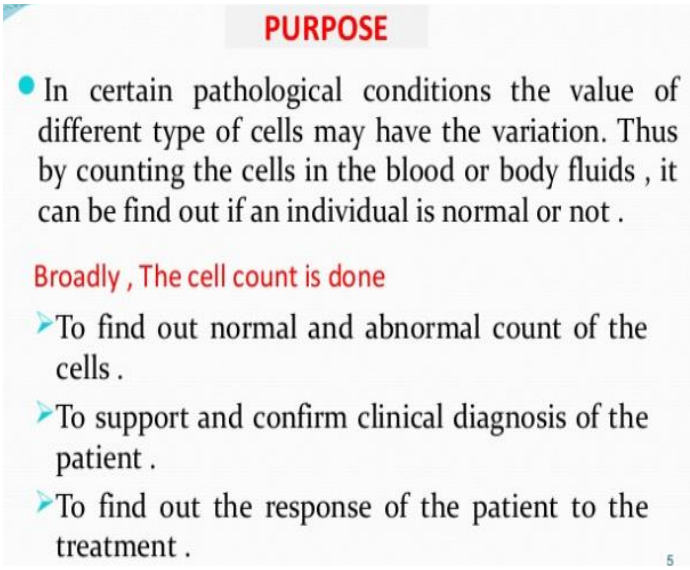
- **Hemocytometer**
- **Electronic particle counter or coulter counter**
  - Destructive assays or In-Direct method of counting
- **DNA content, protein content, metabolic activity**

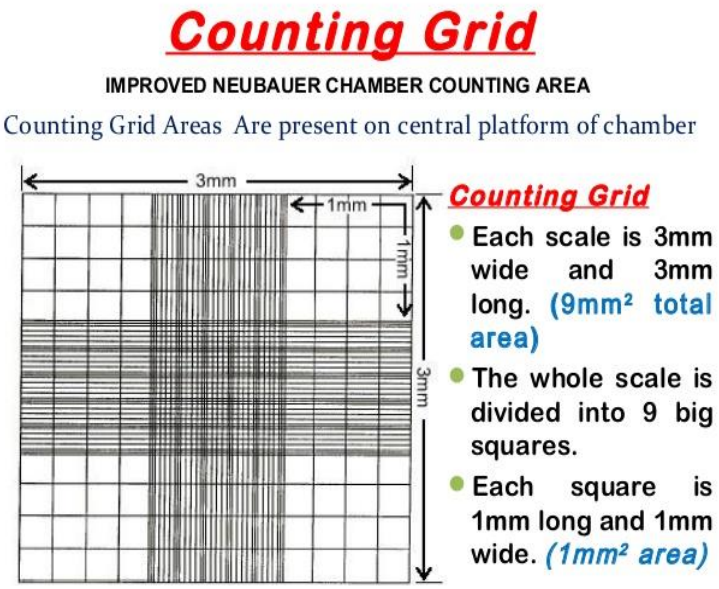
❖ Direct counting methods are rapid and simple.

- The morphology of cells can also be observed when they are counted under the microscope.
- The major disadvantage of these methods is that is gives a total cell count which includes both viable and non-viable cells.
- Accuracy also declines with very dense and very dilute suspensions because of clumping and statistical errors, respectively.
- Very dense suspensions, however, can be counted if they are diluted appropriately.

## Hemocytometer

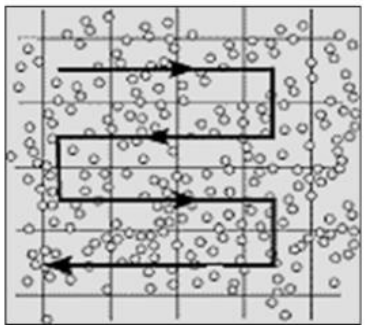
- The hemocytometer is a counting-chamber device originally designed and usually used for counting blood cells.
- The hemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber.

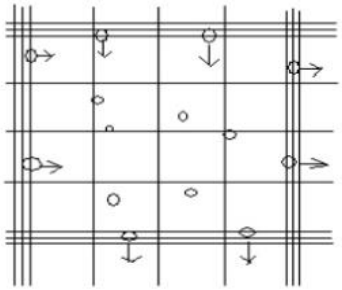


## Counting Rule

- Count the cells touching the triple lines of the left side and on the top of the square in the same square.
- Count the cells touching Bottom line, Right line in adjacent square.
  - This is to avoid double counting.





## Electronic particle counter or coulter counter

- A Coulter counter is an apparatus for counting and sizing particles suspended in electrolytes.
- It is used for cells, bacteria, prokaryotic cells and virus particles
- As fluid containing particles or cells is drawn through each microchannel, each particle causes a brief change to the electrical resistance of the liquid. The counter detects these changes in electrical resistance.

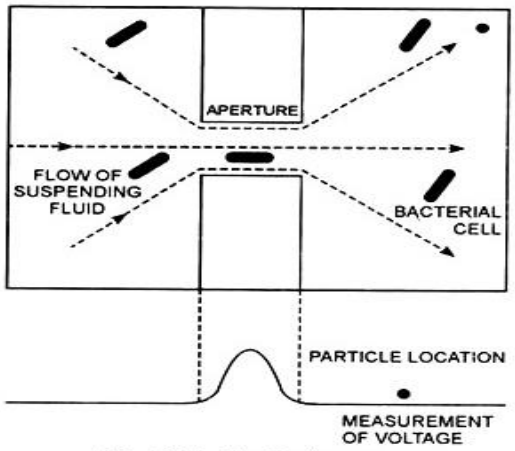



**Fig. 18.31.** The Coulter counter

- An **electronic instrument** called the Coulter counter can also be used for the direct enumeration of cells in a suspension. Fig. 18.31 demonstrates the principles of the Coulter counter.
- The instrument is capable of accurately counting thousands of cells in a few seconds.
- The suspending fluid, however, must be free of inaminate particles (e.g. dust), since smaller ones will score as cells and larger ones will plug the aperture through which the cells pass.

## Destructive assays or In-Direct method of counting

- **DNA content,**

- **protein content,**
- **metabolic activity - by Using**
- DNA fluorescent Dyes
- colorimetric protein binding Assay
- Enzyme conversion by mitochondrial activity in cell lysate

**DNA content**

- DNA can be stained by fluorescent dye.
- These DNA probes can stoichiometrically bind to DNA, which means the number of molecules of the probe bound is equivalent to the number of molecules of DNA found, so that the stained DNA can be quantitatively detected by flow cytometry due to its fluorescence.
- The most widely used fluorescent dye is propidium iodide (PI), which has red fluorescence and can be excited at 488 nm.
- PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA.

**Drawback**

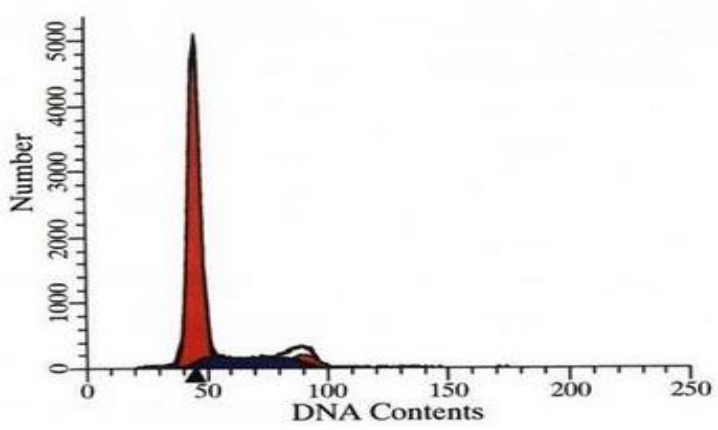
- It can not only bind to double-strand DNA, but also double-strand RNA. So RNAase should be added before staining. Otherwise, the dye is excluded by the plasma membrane so that the cells have to be fixed or permeabilised before adding the dye.

**Other fluorescent dye have also been used in DNA content analysis.**

- 4',6-diamidino-2-phenylindole (DAPI) can binds strongly to A-T rich regions in DNA and it can pass through an intact cell membrane therefore it can be used to stain both live and fixed cells.
- Besides,
- hoechst 33342,
- acridine orange,
- chromomycin A3,
- 7-aminoactinomycin D and
- Ethidium bromide can be used for this purpose, too.

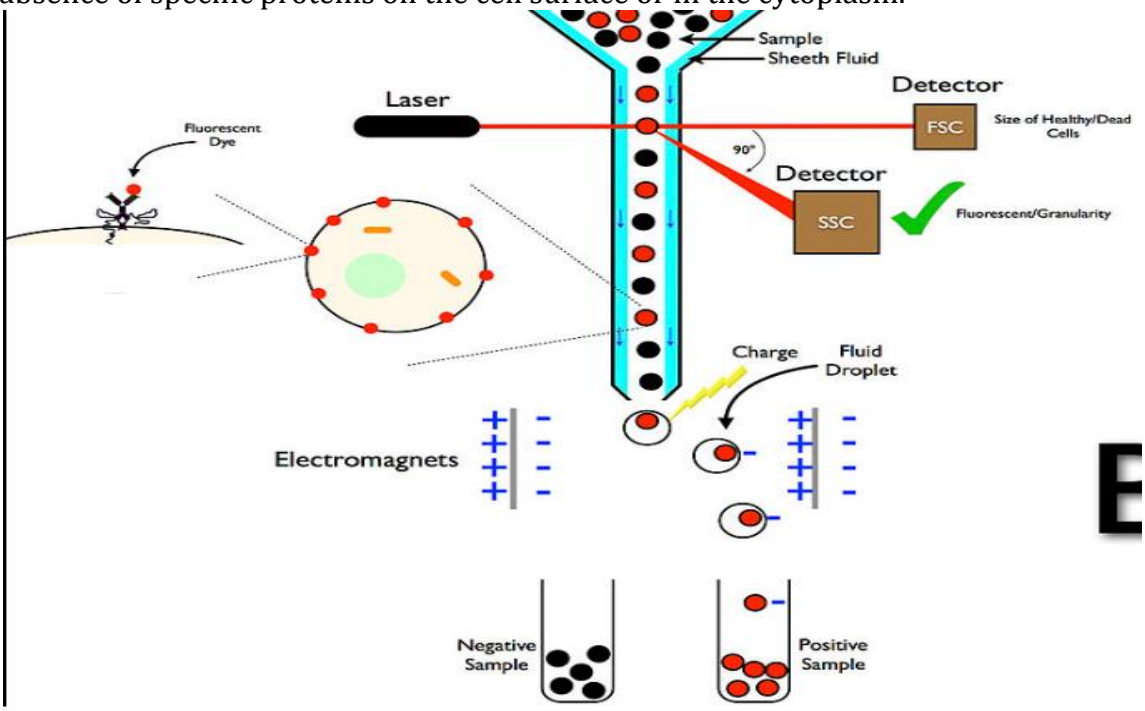
## Determination of the Number of Cells Indirectly by the Plate Count (Non-destructive method)

- The plate count is based upon the assumption that each organism trapped in on a nutrient agar medium will multiply and produce a visible colony.
- The number of colonies therefore is the same as the number of viable cells inoculated into the medium.
- In this procedure (Fig. 18.32) an appropriately diluted cell suspension is introduced into a petri dish.




**Flow cytometry**

- Flow cytometry is a technology that is used to analyse the physical and chemical characteristics of particles in a fluid as it passes through at least one laser.
- Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths.
- Cytometry is the measurement of the characteristics of cells.
- Variables that can be measured by cytometric methods include cell size, cell count, cell morphology, cell cycle phase, DNA content, and the existence or absence of specific proteins on the cell surface or in the cytoplasm.




- **Flow cytometry is routinely used in basic research, clinical practice, and clinical trials.**

  **Uses for flow cytometry include:**
- Cell counting
- Cell sorting
- Determining cell characteristics and function
- Detecting microorganisms
- Biomarker detection

- Protein engineering detection
- Diagnosis of health disorders such as blood cancers

**Protein content- Colorimetric protein binding Assay**

## Colorimetric protein assay selection guide

| | 660nm Protein Assay | BCA Assays | Bradford Assays | Modified Lowry |
|---|---|---|---|---|
| **Reaction scheme** | • Shift in absorption max to 660 nm when dye-metal complex binds protein<br>• Dye changes from reddish-brown to green | • Based on the reduction of copper followed by BCA chelation and complex formation<br>• Dye changes from green to purple (standard BCA assay) or from green to yellow (Rapid Gold BCA Protein Assay) | • Coomassie dye binds directly to protein<br>• Dye changes from brown to blue | • Based on the reduction of copper followed by biuret reagent chelation<br>• Dye changes from clear to blue (Folin-Ciocalteu reagent enhances blue color) |
| **Application** | • Ideal for measuring total protein concentration in samples containing both reducing agents and detergents<br>• Used for quick yet accurate estimation of protein<br>• Measures peptides as small as 2,500 Da | • Less protein-to-protein variation, compatible with most detergents<br>• Various accessory reagents and alternative versions of these assays accommodate many other particular sample needs<br>• Measures peptides as small as 2,000 Da | • Used for quick estimation of protein concentration<br>• Used to measure protein in the presence of reducing agents<br>• Measures protein of > 3,000 Da | • Substitute for "homemade" preparations of the Lowry reagents<br>• For the determination of a broad range of proteins after TCA precipitation<br>• Compatible with any published method for sample treatment modification of the Lowry Assay |

# Protein content

**Bradford protein assay**

- Colorimetric protein binding Assay

## Bradford protein assay

- The Bradford protein assay was developed by Marion M. Bradford in 1976.
- It is a quick and accurate spectroscopic analytical procedure used to measure the concentration of protein in a solution.
- The reaction is dependent on the amino acid composition of the measured proteins.

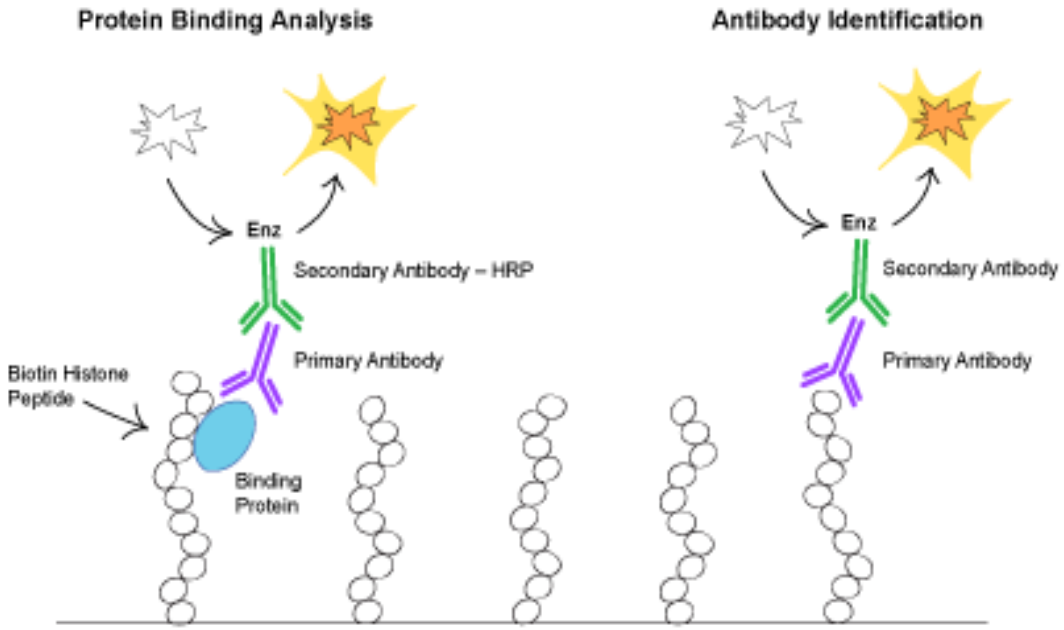
### Principle

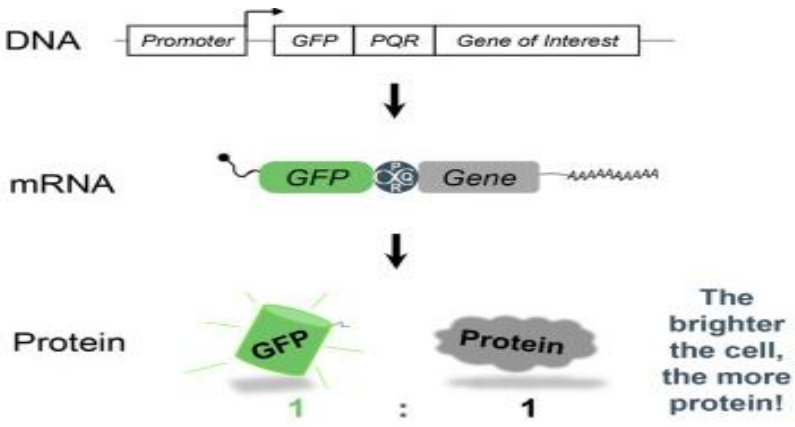
- The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250.
- The Coomassie Brilliant Blue G-250 dye exists in three forms: anionic (blue), neutral (green), and cationic (red).
- Under acidic conditions, the red form of the dye is converted into its blue form, binding to the protein being assayed.
- If there's no protein to bind, then the solution will remain brown.

# Bradford Method

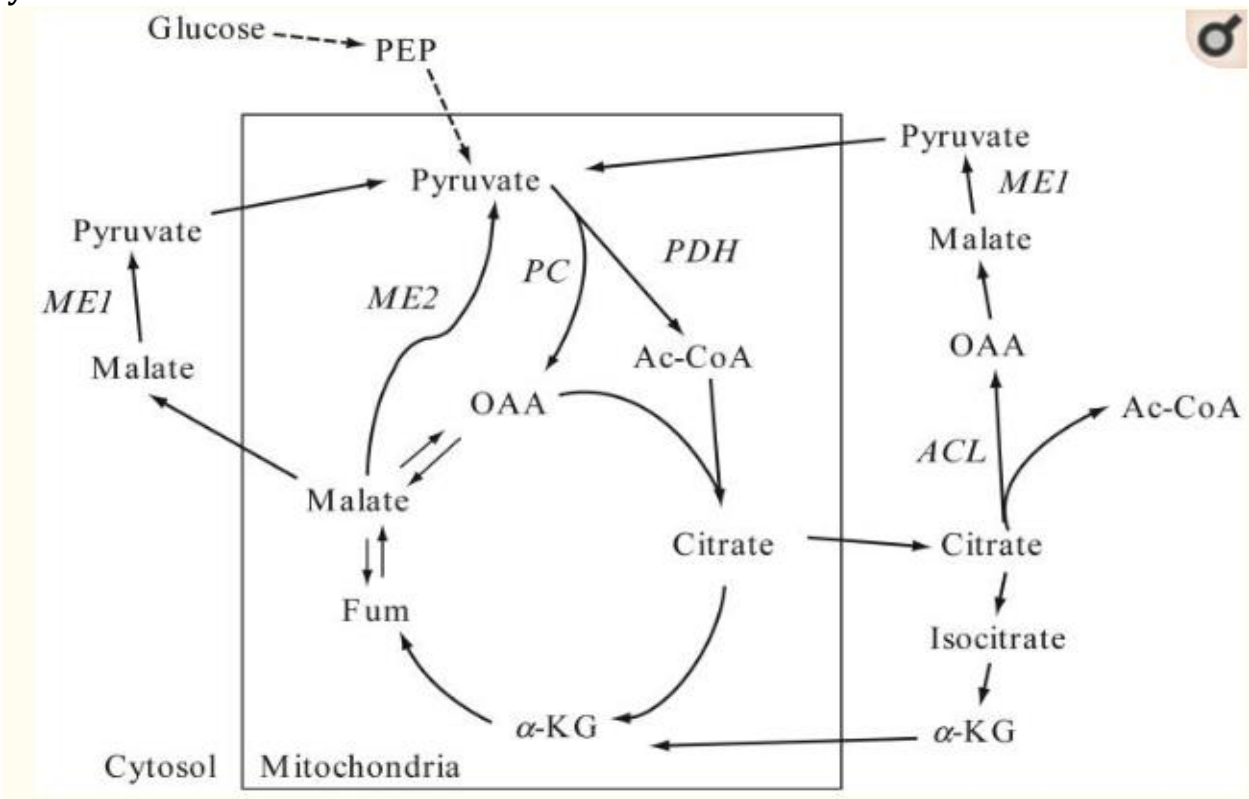
- It is the widely used method.
- The Bradford protein assay involves the addition of a acidic dye, Coomassie Brilliant Blue G-250 to the protein solutions.
- The dye binds to basic and aromatic amino acids resulting in a shift of the absorbance maximum from 465nm (brownish) to 595nm (blue).

- Bradford reagent contains **Coomassie Brilliant Blue G-250 dye** that binds to basic and aromatic amino acid residues (**arginine** (ARG), **fenylalanin** (PHE), **tryptophan** (TRY) a **prolin** (PRO)



**Metabolic activity- Enzyme conversion by mitochondrial activity in cell lysate**




**Anaplerotic substrate cycling pathways of insulin secreting cells:**

- insulinoma INS-1 cells and pancreatic islet $\beta$-cells.
- Pyruvate cycling can occur via several redundant and complementary pathways.
- Cytosolic malic enzyme cycles malate derived from the export of mitochondrial malate, or derived indirectly from the exported citrate that is converted to oxaloacetate (OAA) by ATP: citrate lyase (ACL), and then malate by malate dehydrogenase.
- Pyruvate cycling can also occur entirely within the mitochondria by the conversion of mitochondrial malate into mitochondrial pyruvate by malic enzyme 2 (ME2).
- An alternative cycling pathway exists for return of citrate to $\alpha$-ketoglutarate ($\alpha$-KG) via the conversion of exported citrate to isocitrate and then $\alpha$-KG.
- Each of these pathways can be considered a mechanism for the transfer of mitochondrial reducing equivalents to the cytosol in the form of NADPH.

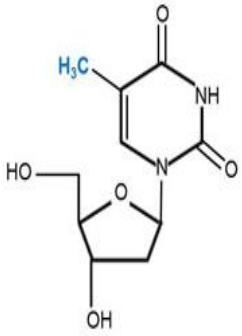
| Viability Criteria | Methods |
|---|---|
| Ability to attach and divide | Clonogenic assay |
| Plasma membrane integrity | Trypan blue assay<br>Polar nuclear stain<br>Fluorescent cytoplasmic stain |
| Metabolic activity | Mitochondrial potential stain |

| | |
|---|---|
| Presence of DNA | Non polar nuclear stain |

- **Cell fate processes**
  - Cell proliferation
  - Differentiation- ultrastructural and biochemical changes, expression profiling
  - Apoptosis or programmed cell death- labeling with terminal nucleotidyl transferase
  - Migration of cells – time lapse microscopy, migration chambers, micropatterning techniques

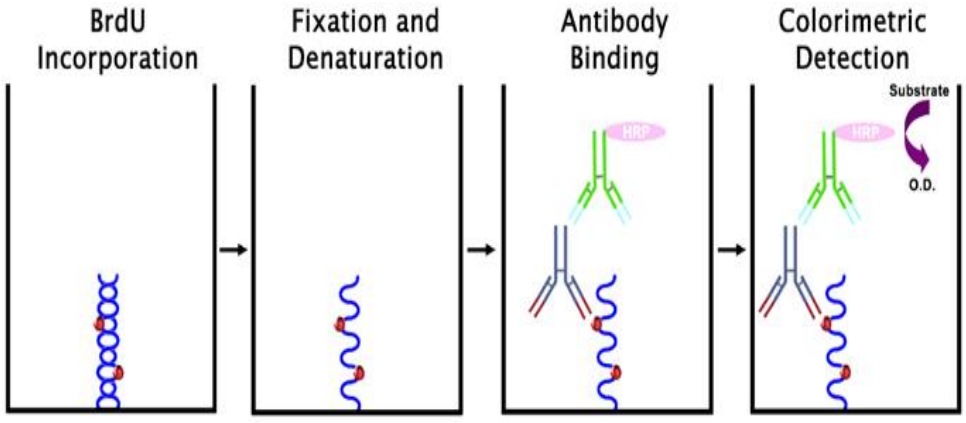
### 9.2.3 Cell-fate processes

The interacting cell-fate processes (proliferation, migration, apoptosis, and differentiation) can be measured *in vitro*. Cell proliferation can be measured by measuring cell number as described in the previous section, in suspension, *in situ*, or in a cell lysate. In addition, because cells often have a fixed number of nuclei, proliferative rates can be estimated by measurements of DNA synthesis rate. Labeled DNA precursors, which are preferentially incorporated into nucleic acids, are utilized to obtain a radioactive (3H-thymidine or 3H-deoxycytidine) or fluorescent (bromodeoxyuridine, BrdU) measurement of DNA synthesis. This technique allows quantitation of the number of cells entering S phase.

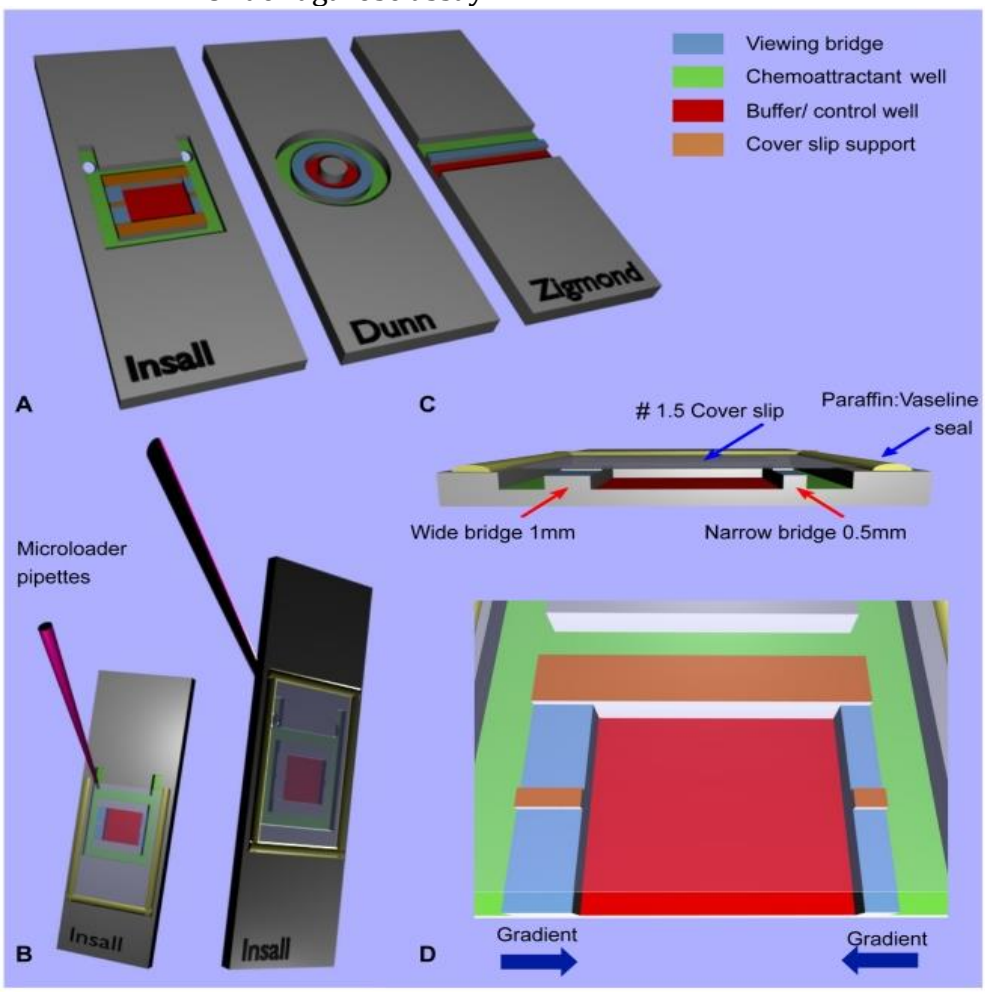



Thymidine


5'-bromo-2'-deoxyuridine (BrdU)



- **Measuring cell motility**
  - Boyden filter transmigration asssay
  - Zigmond chamber
  - Dunn chemotaxis chamber
  - Under agarose assay




- **Cell function**

**General methods to asses cell function are:**

| Function studied | Method |
|---|---|
| Protein synthesis and secretion | Incorporation of $^{3}H$ leucine, $^{35}S$ methionine, $^{3}H$ proline<br>Protein specific assays |
| DNA synthesis | Incorporation of $^{3}H$ thymidine, $^{3}H$deoxycytidine |
| Metabolic activity | Rate of consumption of glucose and aminoacids from medium, oxygen rate of production of $CO_2$ and lactate |
| Signal transduction | Intracellular calcium or pH dyes<br>Microphysiometer<br>Biochemical assays on cell extracts |

**TABLE 9.3**

**Example of tissue-specific functions assessed in two different cell types. From [34].**

| Function | Hepatocyte (Liver cell) | Vascular endothelial cell (Lining of blood vessel lumen) |
|---|---|---|
| Protein synthesis and secretion | Albumin<br>Transferrin | Tissue plasminogen activator<br>Surface adhesion molecules |
| Enzyme/metabolic activity | Ethoxyresorufin<br>Deethylase conversion | NO release<br>Conversion of angiotensin I to angiotensin II |
| Transport functions | Accumulation of fluorescein diacetate in bile canaliculi | Permeability of monolayers to albumin |

**9. MEASUREMENT OF TISSUE CHARACTERISTICS**

**General appearance**

- Size, shape, colour and weight
- Dia of blood vessel, roughness of cartilage
- Cellularity and homogeneity

Cell fate process like cell division, death and migration

- Cell orientation

Extracellular matrix component

- Function of cellular component
- Combination of interacting cell and ECM

Function

- Function of cellular component
- Combination of interacting cell and ECM

Mechanical measurements

- Passive elastic and viscoelastic properties
- Compressive and shear defromations to cartilage disks
- Tensile properties like young's modulus and ultimate tensile strength
- Mechanical loading in tissues invitro can be used to modify their properties

Physical properties

- Acoustic , optical , conductive , thermal properties
- Transport properties – radioactive, colorimetric or fluorescent

# UNIT II
# TISSUE ORGANIZATION

The human body consists of ten organs and each organ performs its own physiological functions. Each organ comprises of a number of tissues like epithelial, connective, nerve, muscle and glandular. The word tissue is derived from the French meaning “to weave”. A tissue is a collection of cells and extracellular matrix, which perform the function that are intended to.

## 1. Extracellular matrix

Tissue is made up of interconnected network of macromolecules known as extracellular matrix (ECM). ECM consists of mostly proteins and polysaccharides. ECM is synthesized, secreted, oriented and modified by the cellular components. In turn ECM controls the cell fate processes like adhesion, differentiation, migration and apoptosis via integrins (cell surface receptors). The components of the ECM vary with the tissue type. For example, basal lamina found at the base of the epithelial tissue consists of mainly collagen IV, laminin and heparan sulphate. On the other hand, the connective tissue mainly consists of collagen I, fibronectin, elastin and glycosaminoglycans.

### *1. Collagen*

Collagen is the chief constituent of extracellular matrix protein in the human body, which provides mechanical strength for various tissues and organs. Presently 27 types of collagen types with at least 42 distinct polypeptide chains have been identified. The collagen molecules are mainly made up of three polypeptide chains called α-chains. The triplet sequence is Gly-X-Y where X and Y represent amino acids such as proline and 4-hydroxyl proline other than glycine. The α-chain wind together to form a right handed triple helix. The role of glycine in every third position is small enough to fit into the restricted space in the centre of triple helix. The proline and hydroxyproline is mainly used to provide the stability for the triple helix.

***2. Glycosaminoglycans***

Glycosaminoglycan (GAG) is the most abundant heteropolysaccharide in the body and it is the long unbranched polysaccharides comprising repeating units of disaccharides either N-acetylgalactosamine (GalNAc) or N-Acetyl glucosamine (GlcNAc) with uronic acid (glucoronate or iduronate). The high negative charge molecules provide the extended confirmation, which imparts the viscosity to the solution. The high viscosity and low compressibility provides lubrication at the joints. These molecules are primarily found on the cell surface or in the extracellular matrix (ECM). Examples include hyaluronic acid, dermatan sulfate, heparin, heparin sulfate and keratin sulphate.

***3. Proteoglycans***

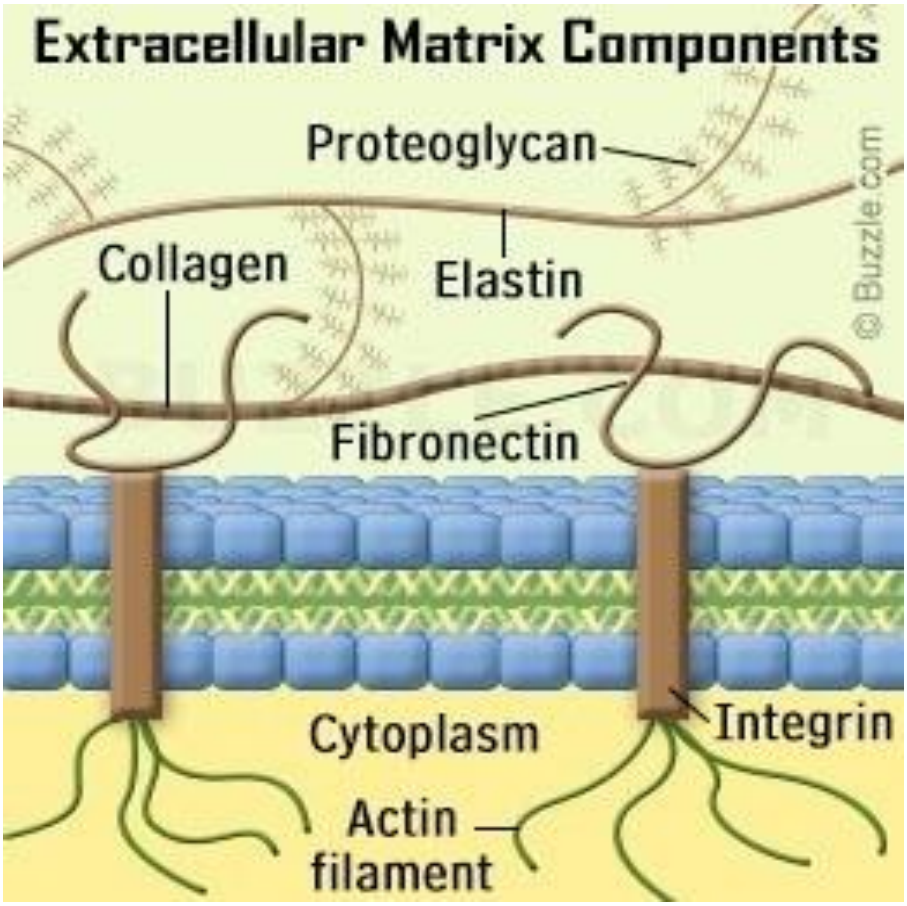
When GAGs are linked to core proteins via specific trisaccharide comprising of two galactose residues and a xylose residue, this molecule is collectively called as proteoglycans. The protein core of the proteoglycans allows the multiple GAG attachments.

***4. Fibronectin***

It is a cell adhesive protein found to involve in many cellular processes such as tissue repair, embryogenesis, blood clotting, and cell migration / cell adhesion. This is mainly found in two major forms - (i) as insoluble glycoprotein dimer serving as a linker in the ECM synthesized by fibroblast, chondrocytes, endothelial cells, macrophages and certain epithelial cells and (ii) as soluble disulphide linked dimer found in the plasma, synthesized by hepatocytes. The major function of such fibronectin is to anchor the cells to collagen or proteoglycan substrate.

***5. Elastin***

Elastin chains are cross-linked together to form elastic rubber like fibers offers elasticity of the tissues. Each molecule of elastin uncoils into more extended confirmations when the fiber is subjected to stress; but recoil spontaneously while the stretching force are relaxed.




ECM has six important characteristics and these features are critical to understand for tissue engineering applications.

1. These are very large molecules of molecular weight $10^5$-$10^6$ and hence it has poor diffusivities

2. They are multimodal meaning they can bind to the cell and also to the neighboring ECM molecules

3. They regulate cell fate processes including cell adhesion, differentiation, migration and apoptosis

4. They are sensitive to mechanical stress thereby changes its conformation and expose the active site for binding

5. ECM consists of variety of proteins like fibronectin, vitronectin, etc and the domains present in the proteins can be mimicked by small peptides

6. Bind to the cells via integrins

### *2. Cells*

There are over 200 different types of cells in the human body. The cell types fallinto two main categories – Epithelial and mesenchymal cells.

Epithelial cells: Epithelial cells are cuboidal in shape and are grown as twodimensionalsheets. These cells are polar and tightly interconnected with theneighboring cells and hence they cannot migrate individually. Basal side of thesheets is connected to the ECM molecules while the superior side is connectedto a vesicle or tubule. Early embryo consists of mainly epithelial cells whilemesenchymal cells occur as fillers.

Mesenchymal cells: Mesenchymal cells are loosely connected cells and usuallyexist alone and hence they can migrate independently. They are bipolar inshape with lamellipodial extensions. Fibroblast cells, which play a major role inmorphogenesis and wound healing, can grow readily in 2D surfaces. However,their growth is contact inhibited. Mesenchymal cells also have the potential todifferentiate into most specialized cells like osteoblasts, chondrocytes andfibroblast. Tissue types

The major tissue types include epithelial, connective, nerve, muscle andglandular, which perform variety of functions like covering, support, movement,control and production respectively.

### *1. Epithelial tissue*

Epithelial tissue consists of sheets of epithelial cells, which may be single layer ormulti-layered. In single layer, they are simple while in multi-layer they arestratified. They are very specialized cells to form lining of the all the internal andexternal body surfaces. The epithelial tissue is separated from the underlyingtissue by a thin sheet of connective tissue or basement membrane. Thebasement membrane provides structural support for the epithelium and there aredifferent types of epithelial tissue available based on geometric arrangements.

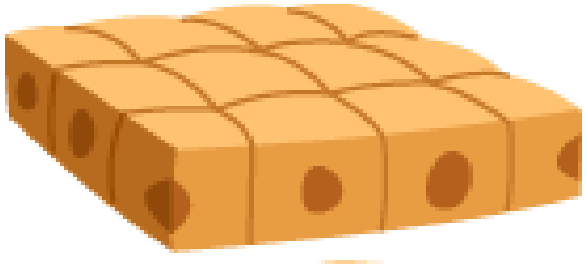
They are:

A. Squamous epithelium

The name squamous indicates that the cell width is greater than its height that isthey are thin, flat plates, having horizontal flattened elliptical nuclei (“fried eggshaped”) in its morphology. This is mainly located in the lining of cavities such asblood vessels, mouth, lungs and also outer layer of the skin.

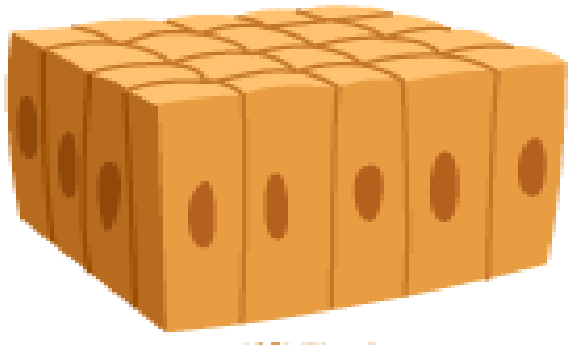
B. Simple cuboidal epithelium

They are roughly square shaped in structure (i.e.) cuboidal arrangement (widthand height are equal). Each cell has spherical nuclei in its centre and is mainlyfound in the glands, kidney tubule linings and also in the ducts of the glands.



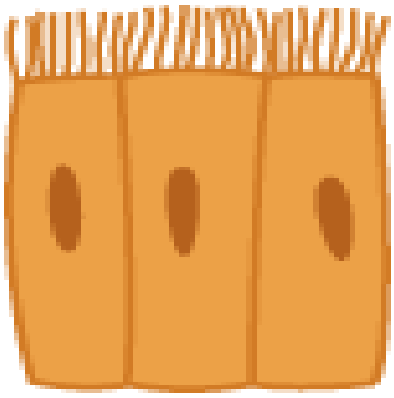
C. Simple columnar epithelium

Cuboidal as name indicates the height of the cell is greater than the width.Hence, cells are elongated and column shaped. The nuclei are elongated andlocated near the base of the cells and these are mainly present in the lining of thestomach and intestines.



D. Ciliated columnar epithelium

This type of epithelial cells possesses fine hair like projections, called cilia ontheir free surface. These structures are capable of rapid, rhythmic, wavelikebeatings in a certain directions. They are mainly located in the air passages likenose and also present in the uterus.



E. Glandular epithelium

Columnar epithelium with specialized cells called goblets cells are calledglandular epithelium. These are capable of synthesizing certain hormones orenzymes, sweat and even saliva. As the name indicating that this is located inthe lining of the glands.

F. Stratified Epithelium

This type of cells is composed of several layers of cells, which are mainly presentin the body linings where it has to withstand wear and tear. The top layer of cellsis flat and scaly and it may or may not be keratinized. This is mainly present inthe skin and also in the lining of the mouth cavity.

G. Pseudostratified epithelium

The epithelial layers appear stratified due to the different location of nuclei withrespect to basement membrane. Multiple cell layers can also be described byvarious shapes like stratified squamous epithelium like the one found inesophagus or it may be in the transition state between the cuboidal and columnarbased on the state of the organ as in bladder.The epithelial tissue has many functions in the body and is responsible forexcretion, diffusion, cleaning, reduces friction, protection, sensation, secretion,and also absorption.

***Connective tissue***

Connective tissue has variety of functions, which include mechanical support forbones and ligaments, storage of body's fluid like fat and blood, aid in immuneresponse and tissue repair mechanism. Connective tissue originates from themesoderm of the embryo. These include cells like fibroblasts, adipocytes,macrophages, plasma cells and also non-cellular components like collagen I andhyaluronic acid. Like epithelial cells,

there are various types of connectivetissues include loose connective tissue, fibrous connective tissue and specializedconnective tissues.

***Muscular tissue***

**T**he body consists of three types of muscle tissue – skeletal, cardiac and smooth.Skeletal muscles consist of striated and multinucleated myofibers that areresponsible for the movement. Cardiac muscle in heart helps to propel the bloodthrough the blood vessels. Cardiac muscle fibers are striated but contain onlyone nucleus and they branch and join at junctions called intercalated disks.These junctions are responsible for the propagation of action potential. Smoothmuscle fibers on the other hand are non-striated and are present in the holloworgans like intestine to squeeze the substances by alternate contraction andrelaxation. Different organization of muscle tissue is important to performfunctions ranging from slow, small magnitude contractions in the gut to the fast,co-ordinated depolarization of heart muscle

***Nervous tissue***

Nervous tissue is important for the function of the nervous organs like brain andspinal cord. The nerve co-ordinates the body functions and the nervous tissueconsists of mainly neurons and supporting cells like Schwann cells andastrocytes. Neurons are responsible for generating electrical impulses calledaction potentials. These electrical impulses are received by the soma (cell body)and are carried along axons to the other neurons via dendrites. Supporting cellshave no role in conducting electrical impulses, however, they play an importantrole in providing nourishment, insulation and protection.

***Glandular tissue***

Glandular tissue is mainly composed of polarized epithelial cells that produceand secrete the protein products from the precursors present in themicroenvironment. The protein products are usually secreted in endoplasmicreticulum and are packaged into secretory vesicles in Golgi apparatus and thenthey are exocytosed into the cytoplasm.

Glands are classified into exocrine and endocrine glands. Exocrine glandssecrete the products either onto the body surfaces like skin or the body cavitieslike digestive tube. These glands may be unicellular or multicellular and they arecalled ductless glands. Examples of exocrine gland are the sweat glands of theskin and the liver, which

secretes bile. On the other hand endocrine glands alsoknown as duct glands and they secrete hormones directly into the blood stream.Then these hormones are carried to the target site where they perform theirfunction.

## Tissue Dynamics

All tissues have their own characteristic replacement and production rates of cells and hence considered to be very dynamic. However the time scale of the tissue dynamics varies with the tissues and is relatively long. Bone marrow is the most prolific tissue in the human body, which is followed by the lining of the small intestine and epidermis. In all these three tissues, the cell turn over rate is in order of a few days while in other tissues it is longer. For example, hepatocytes replacement in the liver takes place for about a year.

### *Dynamic States of Tissues*

There are basically three dynamic states of a tissue which includes tissue homeostasis, tissue repair and tissue formation.

### Tissue Homeostasis

It is the normal steady state function of the tissue. Each tissue has distinct function and is necessary to maintain the steady state function of the body. Some tissues like bone marrow and skin produces cells as their main function, while glands produce a secreted product. Lungs and kidneys primarily carry out the mass transfer operations while liver act as biochemical refineries. Muscular hypertrophy and ventricular hypertrophy (hypertrophi – Increase in cell size) are the examples of how well tissue adapts to maintain homeostatic balance in accordance with the physiological demands.

### Tissue repair

Wound healing process is one of the common phenomenon exhibited by a biopsied tissue and the tissue grafts. Immediately after implantation of the tissue grafts, it initially shows a healing response, which is then followed by the integration of tissue grafts with the host tissue.

### Tissue formation

Tissue formation involves developmental biology and morphogenesis. Morphogenic process is very important in tissue dynamics and plays a very important role in tissue reconstruction, repair and engineering.

**Homeostasis in highly prolific tissues**

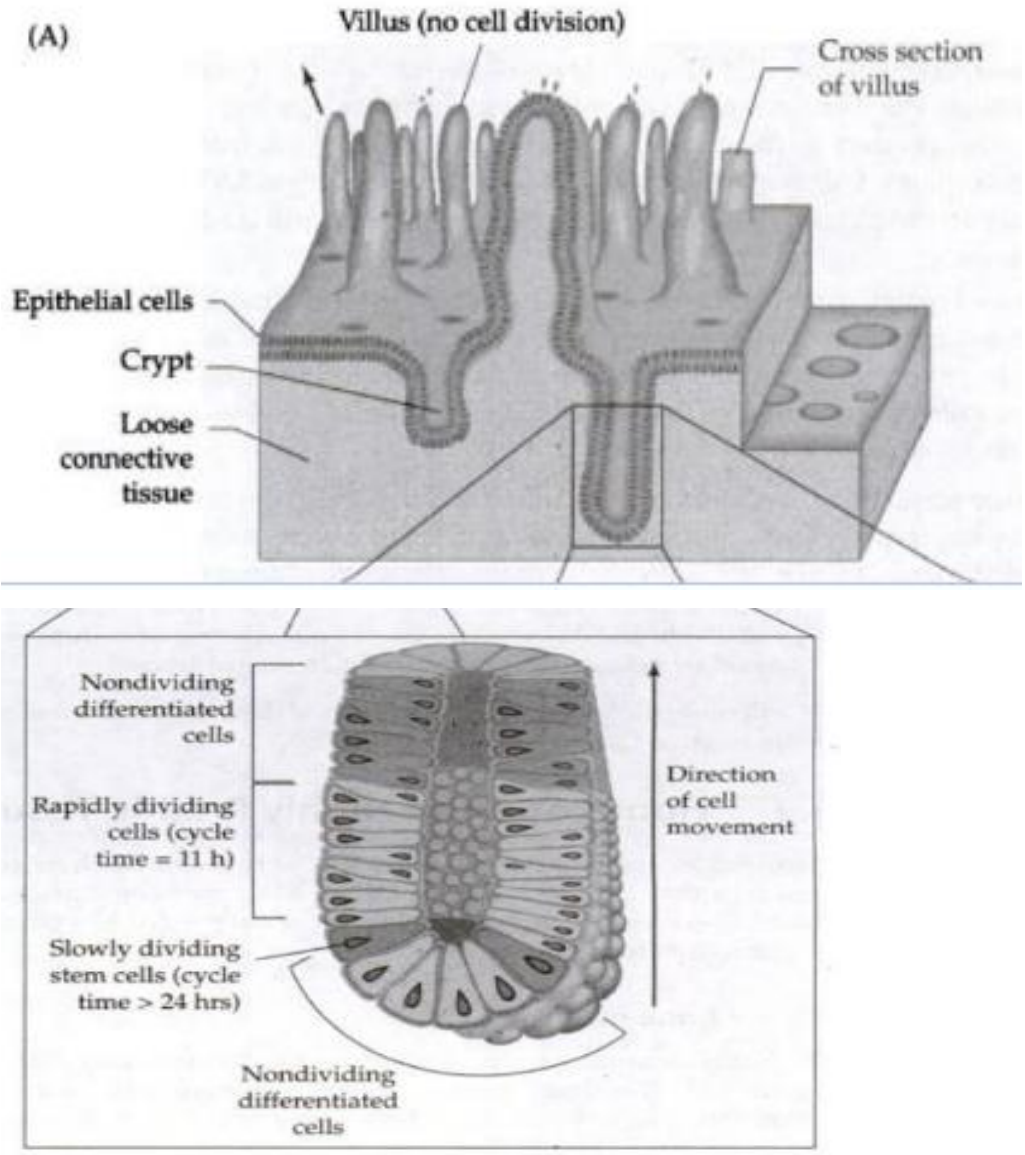
Tissues such as bone marrow, villi of the small intestine and skin are the three different examples for highly prolific tissues.

***Bone marrow***

Bone marrow is the most dynamic tissue in the human body. It produces about40 billion myeloid cells daily all of them are originated from the adult pluripotentadult stem cells. Bone marrow consists of 500 to 1000 billion cells and itproduces many cells within two or three days. It is the site of hematopoiesis.There are two different types of bone marrow includes red (vascular) and yellow(fatty) bone marrow. Red bone marrow forms all blood cells except lymphocytesand destruct old RBC. It is found mainly in hips, skull, ribs, and also ends of longbones. Yellow marrow stores fat and mainly fills the cavities of the bone. Atcritical conditions such as any severe blood loss, the yellow marrow can beconverted into red marrow. The major function of bone marrow is the productionof mature blood cells. Daily output of mature blood cells from bone marrow is 2.5billion erythrocytes; 2.5 billion of platelets; 50-100 billion granulocytes; and hugelymphocytes and monocyte production.

***Villi in the small intestine***

The lining of the small intestine consists of villi that help in the absorption ofnutrients. The outer layer of villi consists of epithelial cells and is highly dynamic.The turn over rate of the cellular contents is about five days approximately.Between villi, there are epithelial infoldings called cryptus, which are tubular in shape. The production of intestinal epithelial cells occurs in the crypt.The bottom of the crypt consists of slowly dividing tissue specific stem cells andthere are about 20 stem cells in the crypt. It then undergoes cell division and thedaughter cells that are formed moves up the crypt as it becomes cyclingprogenitor cell, which has the cycling time of 12 hours. These cells are alsoreferred to as transit amplifying cells, which then move up the crypt anddifferentiate. The mature cells leave the crypt and reach the base of the villi.

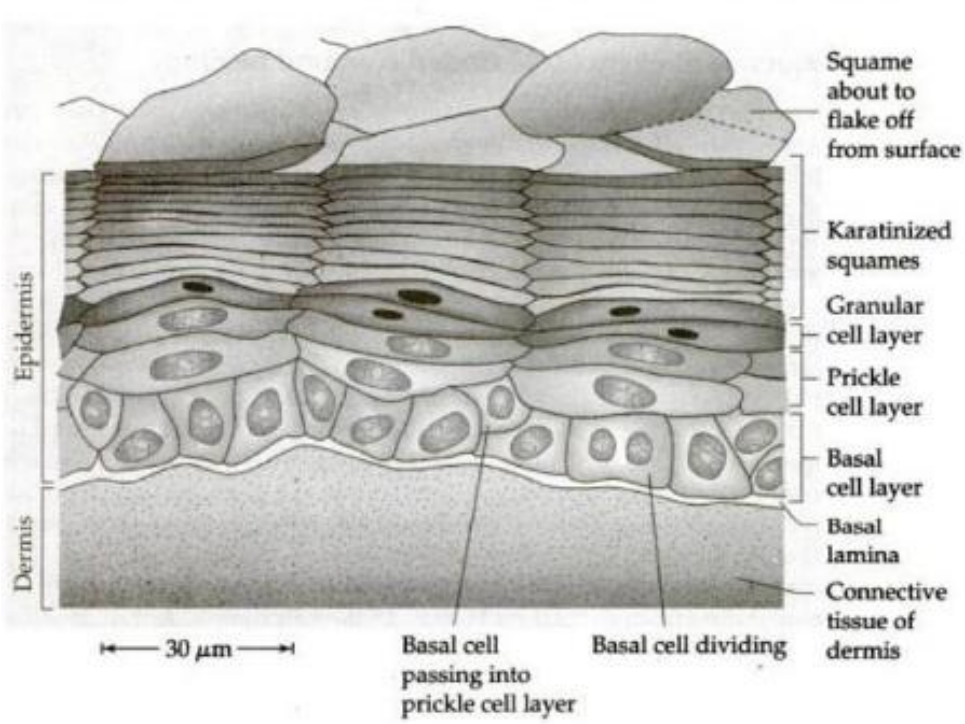



Then over a period of five days, they move from the base to the top of the villiwhere they die and slough off. As you see, the villi consists of tubular infoldingscalled crypts where the cells divide, differentiate into mature cells as they movefrom bottom to top. During their passage they perform the vital functions likeabsorption and digestion of nutrients that come from the lumen of the gut.

***Skin***

Skin consists of two layers – outer epidermis and inner dermis, which isseparated by basal lamina. The connective tissue dermis is made up offibroblasts while epidermis is made up of keratinocytes with the leastdifferentiated one located at the basal lamina. Skin is made up of squamouscolumnar epithelium and each column is about 30 micrometer in diameter. Thecells that are closer to the basal lamina replicate and

since the area available isconstant, the cells begin to move up. As they move up, they differentiate intoprickle cells, then to granular cells, which then develop into keratinizedsquamous cells that flake off. Thus only the cells that are present near the basallamina divides and later it differentiates into well-defined squamous cells. Thereare about 10-12 cells near the basal lamina that is known as epidermalprogenitor cells, which is responsible for the production of squamous columnarepithelial cells. The turnover rate of the cells is in the order of few weeks.




## Tissue repair

Wound healing is a highly co-ordinated process involving series of cellular eventsthat occurs in a damaged tissue and it varies with age. Fetal wound healing andadult wound healing differs in the time taken for healing and also the scars thatare formed during the healing process. Fetal wound healing is very fast andresults in scarless tissue, while on the other hand, adult wound healing is slowand results in the scar formation.

***Sequences of events that underlie wound healing***

Immediately after the injury to control the bleeding, platelets are activated andadhere to the injured site. They secrete the storage granules, which help inrecruiting more platelets to the site of injury to form thrombus. Release of agentsfrom the platelets causes vasodilation and increased permeability of bloodvessels. The clotting cascade

will be then initiated resulting in the cleavage offibrinogen to form fibrin plug. The fibrin plug along with the fibronectin forms theprovisional matrix. This provisional matrix helps in the recruitment ofinflammatory cells and then the fibroblasts and other accessory cells. During thistime, the epidermal cells begin to proliferate and migrate across the wound.

The next step in the wound healing process is the recruitment of inflammatorycells to the injured site. Neutrophils from the circulating blood reach the site earlyand they degranulate and die. Subsequently the number of macrophages at thesite increases thus helps in phagocytosis of cellular debris and othermicroorganisms. These macrophages are the source of chemoattractants andmitogens, which induce the migration of endothelial cells and fibroblasts to theinjured site followed by their proliferation. Effective wound healing processrequires the invasion of macrophages to the wounded site since it clears thecellular debris and pathogenic microorganisms. In the epidermal layer, the basallayer is reformed.

The invasion of fibroblasts results in the formation of granulation tissue in thedermis. The granulation tissue mainly consists of dense population of fibroblasts,macrophages and developing vasculature consisting of ECM components likefibronectin, collagen and hyaluronic acid. The fibroblasts present in thegranulation tissue produce collagen mainly type I and type III, which helps inincreasing the tensile strength of the wound. Myofibroblasts begins to contractand due to the shrinking of the wound the fluid oozes out. During this time, theepidermal layer gets stratified.

Then the matrix undergoes remodeling which involves synthesis and degradationof ECM components. During remodeling, the composition of the matrix changes.Previously, collagen III, which is dominant is converted to collagen I. Theremodeling process actually determines the scar formation. Although, the woundis healed at this point of time, lot of chemical and structural changes takes placewithin the wound site and may continue for many months till the composition ofmatrix returns to the original state.

**Engineered Wound Healing**

Tissue engineering strategies are being employed to enhance the wound healing process. For example, people have used a porous template made up of collagen I and chondroitin-6-sulphate to mimic the extracellular matrix of the dermis of skin. This

template was fabricated with a range of pore sites and degradationrate. First, the time required for the 50% reduction in the wound area was measured and the pore size in range of 20 and 120 micrometer was found to be optimum to cause delay in wound contracture thereby improving the wound healing due to the migration of fibroblasts. Usually wound contraction occurs after the migration of fibroblasts in the provisional matrix and subsequentconversion into myofibroblast. Hence, when the fibroblast migration is delayed,automatically wound contraction and scar formation is also delayed thus allowingsufficient time for the wound healing process.

Pore size and degradation rate plays an important role in determining the celladhesion and infiltration in the scaffold. At very small pore sizes, fibroblastscannot infiltrate while at the large pore sizes, the surface area available foradhesion is very low. Moreover, the degradation time should be fine tuned sothat the degradation time matches with the wound healing time. The normalwound healing rate for skin is three weeks and for peripheral nerves is six weeks.

Another alternative for the polymeric scaffolds are the cell-contractedextracellular matrix gels. It exploits the cell-based contraction for the formation oftissue like constructs. It is found that with increase in cell number, thecontraction increases and with increase in extracellular matrix contraction, thecontraction decreases. This cell-contracted extracellular matrix gels form dermalequivalents and are commercialized for tissue engineered skin product.

***Fetal wound healing***

Fetal wound healing occurs very rapidly without scar formation while adult woundhealing is a very slow process with the formation of fibrosis and scar at theinjured site. Moreover in adult wound healing there is poor reconstitution ofepidermal and dermal tissue at the healed wound site. However, the process ofre-epithelization and connective tissue contraction is almost same in adults andembryo but the mechanism by which they occur is different. In embryo, the gapin the epidermis is closed by the contraction of actin purse-string, but in the caseof adult, the epidermal cells migrate and fill the gap. Myofibroblasts in the adultwound helps in the contraction of the exposed connective tissue thereby bringthe two edges of the wound closer. On the other hand, in fetal wound healing,embryonic cells help in the contraction of the connective tissue by exertingsimilar traction forces.

Another major difference is that in adult wound healing, there is extensiveinflammatory response while in embryo there is no inflammatory response. Fetalwound contains “basket weave” collagen matrix and collagen III as the majorECM component, while adult wound contains bundles of collagen and fibronectinas the major ECM component. Fetal wounds exhibit low tension while adultwound exhibit high tension.Since there is no inflammatory response and minimal scar formation in fetalwound healing, it would be interesting to understand the mechanism so that theycan mimic them in the tissue engineered medical products (TEMPs) for effectivewound healing.

**Tissue dynamics as interacting cellular-fateprocesses**

The dynamic states of tissue function are a complex process, which involves theinterplay of different cell types. The cells communicate and co-ordinate via thecellular fate processes and it determines the dynamic states of tissue function.

The following are the five important cellular fate processes:

- Cell replication – increase in cell number
- Cell differentiation – change in gene expression and acquisition ofparticular function that is specific for the particular cell type
- Cell motility – movement of cell into a particular niche
- Cell apoptosis – programmed cell death
- Cell adhesion – binding of the cell to the neighboring cell or theextracellular matrix components

**Angiogenesis**

It is the process of new blood vessel formation. This process occurs both inhealthy individuals for wound healing applications and also for restoring bloodflow to tissue after injury as well as in pathological conditions like cancer. One ofthe critical components in the process is the successful host integration of mostcell based tissue engineered implants.

This process possess “on” and “off” switch mechanisms, which normallyregulates angiogenesis in healthy body. Stimulating factor such as angiogenin,angiopoietin, FGF, PDGF, VEGF are the major “on” switch. Substances such asangioarrestin, angiostatin, canstatin, endostatin are the potent inhibitors for theangiogenetic process called as

“off” switch. When the body loss control overangiogenesis, it will lead to either excessive new blood vessel formation leadingto cancer, rheumatoid arthritis, blinding disease, psoriasis or insufficient bloodvessel formation leading to coronary artery disease, stroke, and chronic wounds.Hence, the process of angiogenesis plays a vital role in diseases.

There are various steps involved in the new blood vessel formation. They are,

1. Immediately after injury, the injured tissues produce and releaseangiogenic growth factors, which diffuses into the nearby tissuesimmediately
2. The released signals (growth factor) can then bind with specificreceptors located on nearby endothelial cells
3. The binding of the growth factor on to the receptor activates theendothelial cells
4. Activated endothelial cells begin to produce new molecules mainlyenzymes used to create tiny holes in the basement membranesurrounding all existing blood vessels
5. These endothelial cells also start to proliferate and migrate via thedissolved tiny holes of the existing vessel towards the tumor
6. Integrins such as αvβ3 and αvβ5 helps to pull the sprouting new bloodvessel forward by serving as grappling hooks
7. Enzymes such as matrix metalloproteinase (MMP) are produced todissolve the adjacent tissue in order to accommodate the new bloodvessel
8. Once the vessel extends, again the surrounding tissue remoldedaround the vessel
9. Sprouted endothelial cells rolled up and develop a blood vessel tube
10. These individual blood vessel tubes connected together to form bloodvessel loops, which circulates the blood
11. At the end, newly formed vessels are stabilized by specialized musclecells, which could provide the structural support providing blood flow.

**How Cell-Extra cellular matrix interactions can co- ordinate cellfates?**

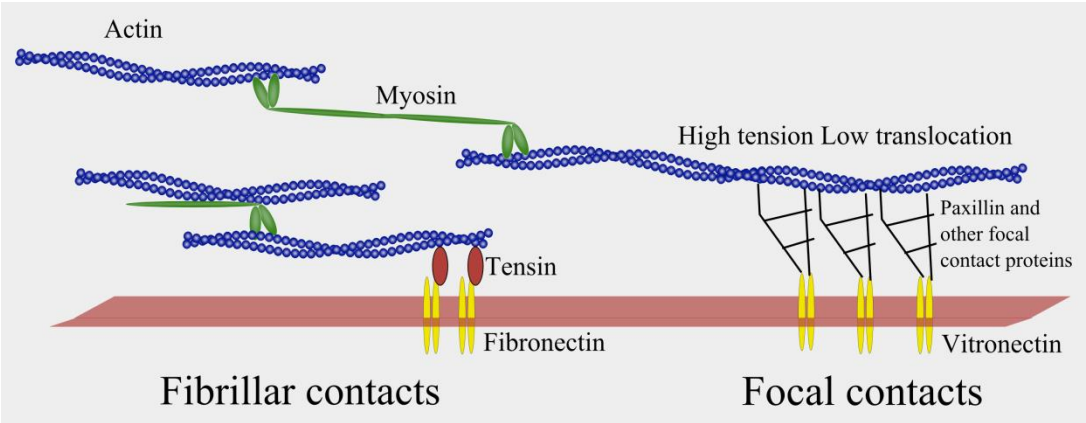
As we all know cell adhesion, migration, proliferation, differentiation and even cell death form a part of the cell fate processes. Now let us take one simple example i.e cells adhesion. Cell adhesion is the primary factor for controlling most of the cell fate. For example; cell migration where speed is mainly regulated by the cell-ECM interactions. This we have seen in the factors regulating the cell migration speed. Similarly let us take apoptosis. This is programmed cell death where the cells attain

round or spherical morphology due to the fragmentation of cytoskeleton proteins. The major role of cytoskeleton in migration and also adhesion has well understood. i.e cell surface receptor like integrins communicate the cell with the ECM thereby providing the link to the actin cytoskeleton. This is why the cell loses its specific phenotype during apoptosis and become round morphology via the fragmentation of cytoskeleton proteins. These examples make the readers understand the role of cell-ECM interaction in coordination of cell fate process.

The extracellular matrix comprising of different number of proteins like collagen, elastin and polysaccharides like glycosaminoglycans, proteoglycans, interconnects all of the cells in a tissue and their cytoskeleton elements. The ECM molecules are synthesized, secreted, oriented and modified by the cells present in the tissues. It is a multifunctional matrix. It can provide a structural as well as mechanical support to the tissues. The ECM can serve as a substrate for the cells to migrate and place to locate the signals for communication. Hence, the ECM dynamic and is being modified constantly. There are number of receptors on the cell surface facilitating the cell-ECM interactions. This signal may be signal for migration, replication, differentiation, and apoptosis. Hence, it is clear that the cell fate process has been regulated by the ECM components. ECM signals are very stable and even very specific and very strong as compared to diffusible growth factor signal.

Mainly, proteoglycans, special glycoprotein, collagen, soluble multiadhesive proteins like fibronectin are the ECM components. The binding of cell to the ECM is mainly through the integrins, which is class of heterodimeric proteins. There are 18 human α and 8 β subunits.

There are three different classes of integrin-based junctions called hemidesmosomes, focal contacts and fibrillar contacts. The integrins are the cell adhesion receptors and they bind to the small peptide sequence within the larger ECM molecules. Among the different integrin based junctions, focal and fibrillar contacts are attached through the specific proteins with actin cytoskeleton proteins in the cell. Thereby it is subjected to the contraction force generated by the actin-myosin interactions. In case of focal contacts, (oval structures) the cell is mainly attached to the matrix protein called vitronectin via αvβ3 integrins. This is because the vitronectin serves as a rigid substrate, thus αvβ3 integrins cannot move due to contractile forces. This in turn develops the high tension leading to the association of the specific proteins like paxillin, vinculin. However, for fibrillar contacts (elongated or dot like structure), matrix protein fibronectin attach with the cells through α5β1 integrins. Here there is no recruitment of vinculin and paxillin. Fibrillar contact contains tensin instead of vinculin and paxillin. Hemidesmosomes are the intracellular protein plaques found in the epithelial tissues, which can connect the basement membrane to cytoskeleton actin filaments via integrins.



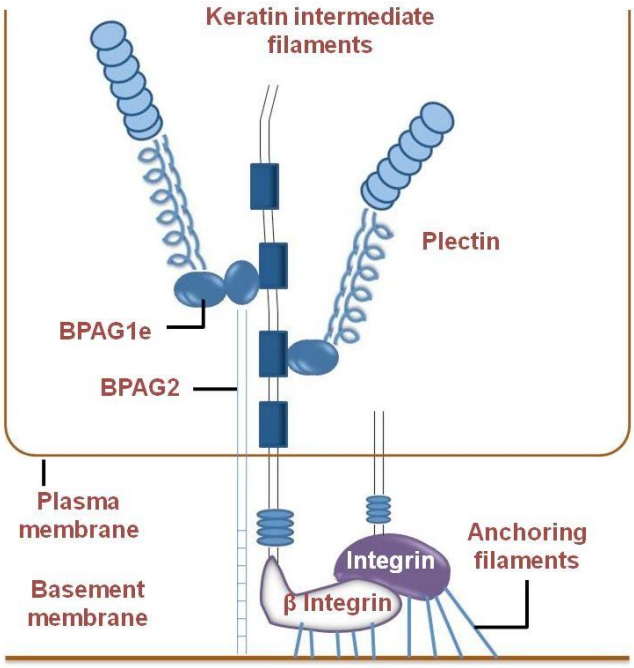



Fig1: Integrin-based adhesion junctions (A) Focal and Fibrillar contacts; (B) Hemidesmosome

Let us take an example of RGD. This peptide sequence has arginine-glycine- aspartic acid. This sequence can mimic some features of larger ECM components like fibronectin. Ligand binding to integrins requires the simultaneous binding of divalent cations. How many numbers of binding sites are required to generate a cell response? One research group, has immobilized the tripeptide binding sequence on a cell growth surface at varying densities. Then as function of surface density of RGD binding sites especially for fibroblast, cell attachment, spreading and growth were examined. From this experiment, they have identified that an average receptor spacing of 440 nm was sufficient for cell attachment and spreading and 160 nm for focal point adhesionformation.

Let us take another example of cell adhesion in the circulatory system. This is because cell adhesion is the primary component in response of immune cells to the specific infections. The cell has to attach to the endothelial surface first and then migrate to the target site. The balance between the bond length, dissociation rates and affinity strength can determine the process of adhesion.

## 1. Modifying theECM

Cells produce as well as degrade the ECM in their own environment. One good example for this is chondrocytes and surrounding ECM. Cartilage is a tissue chiefly composed of ECM, mainly collagen. It has been reported that the production of collagen occurs on the order of days with diffusion into the ECM about 100 μm per day. ECM in the cartilage tissue is constantly synthesised, degraded and also remodelled in response mechanical deformations. The deposition of proteoglycan in the ECM surrounding the chondrocyte was found in a direction perpendicular to that of the compression. Cells produce newly formed ECM molecules that may (i) migrate to the matrix where they may continue to; (ii) diffuse away from the cell; (iii) become immobilized within the existing matrix; (iv) proteolytic enzyme can cause degradation of the matrix leading to the release and transport of the degraded matrix molecules.

### *Malfunctions in ECMsignalling*

It is clear that the ECM regulates the cell behaviour and coordinates the cell function and homeostasis. In addition, the three-dimensional architecture, composition and remodelling of ECM contributes the micro-environmental signalling that direct the cell shape, migration, viability, growth and differentiation. Hence any malfunction of cell-ECM signalling leads to various pathological states such as degenerative, malignant,

developmental, immune and hemostatic disorders. Especially mutations in genes encoding ECM protein, ECM remodelling protein, ECM receptors will lead to diseases. This mutation can alter both structural properties ECM molecules as well as function of the protein that degrade the ECM mainly in the matrix metallo- protease (MMPs) family. MMPs are responsible for the interactions between the cell and the surrounding ECM.

***Malfunctioning morphoregulatory controlloop***

Tumour-suppressor genes, *Rb* and p53 and ECM interactions form a regulatory feedback loop. ECM as well as integrins controls the phosphorylation of *Rb* and transcription, translocation and degradation of p53. This in turn can regulate the expression of MMPs, thereby degrading the ECM. P53 regulates the cell-ECM interactions through the transcription control of ECM components such as collagen, laminin, fibronectin etc. This type of feed-back loop promotes cell fate process such as apoptosis and migration.

**Direct cell-cell contact**

Cells exhibit special proteins on their surface leading to direct cell-cell contact. This type of interaction is highly specific and direct communication between the two cells. However, both soluble signalling and ECM signalling are non-specific since there is no direct knowledge between the signalling cell and target cell. The cell-cell signalling molecules are in two different forms. In the first form, the signal allows mechanical contact between the two cells,

whereas in the second, the molecule that form junctions between the cells through which the molecules can be transported from one cell to the other.

There are four different junctions facilitating the direct cell-cell interaction.

1. Tight junction: This can form the impenetrable barrier by sealing the neighbouring cell membrane together. Thereby, it can prevent the leakage of molecules between the cells.

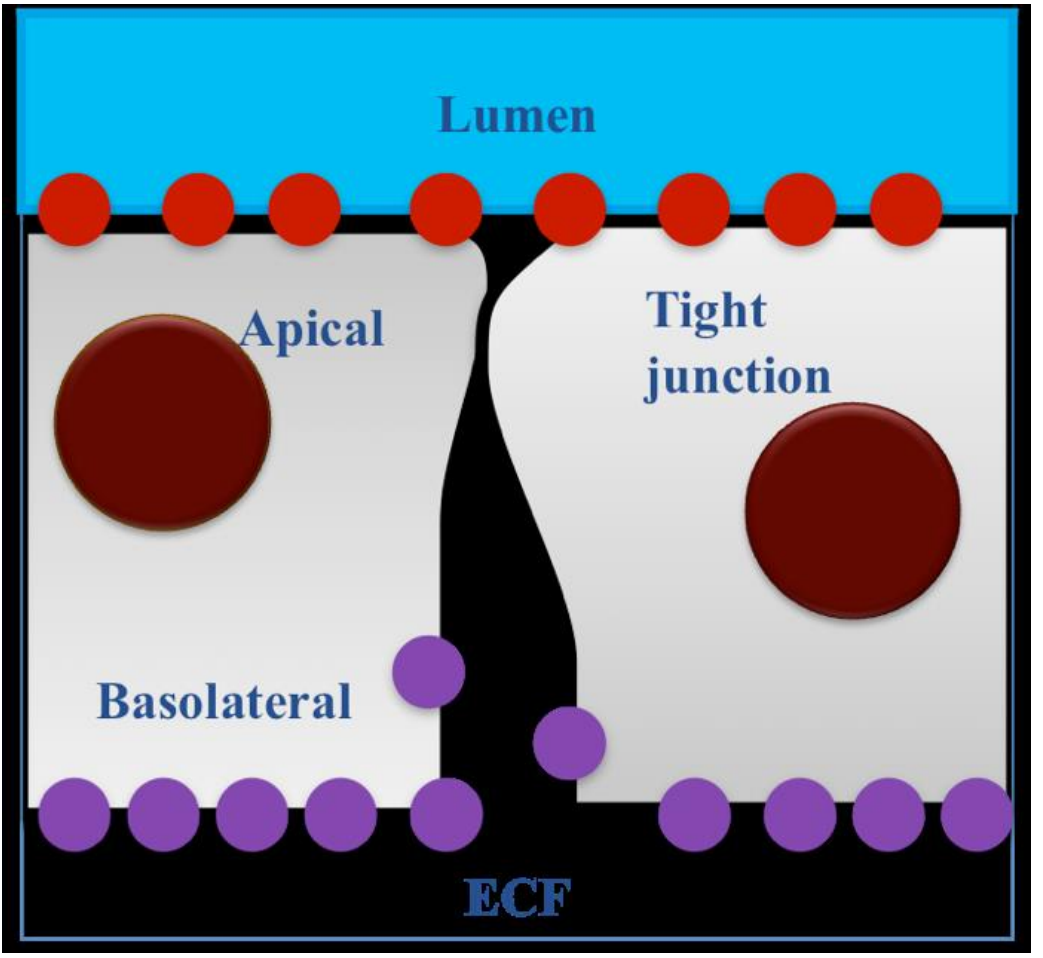
2. Belt desmosomes: this is also called as adheren junctions. This can join the actin cytoskeleton bundle of one cell to the similar bundle of the adjacent cell. This can form a circumferential belt of adhesion.

3. Spot desmosomes: Also called as spot welds. This attaches the tough intermediate filaments in one cell to the same in the neighbouring cell.

4. Gap junction: This can form a hollow channel between the adjacent cells thereby allow the passage of small cytoplasmic molecules across the membrane.

Tight junctions are the sheets of cells that can provide the interface between the cell mass and a cavity or a space (lumen). A cell exposed to the lumen is the apical surface and rest of the cell is called as a basolateral surface. This junction seals the neighbouring epithelial cells in a narrow band just beneath their apical surface. This will prevent the entry of molecules and ions across the space between the cells.

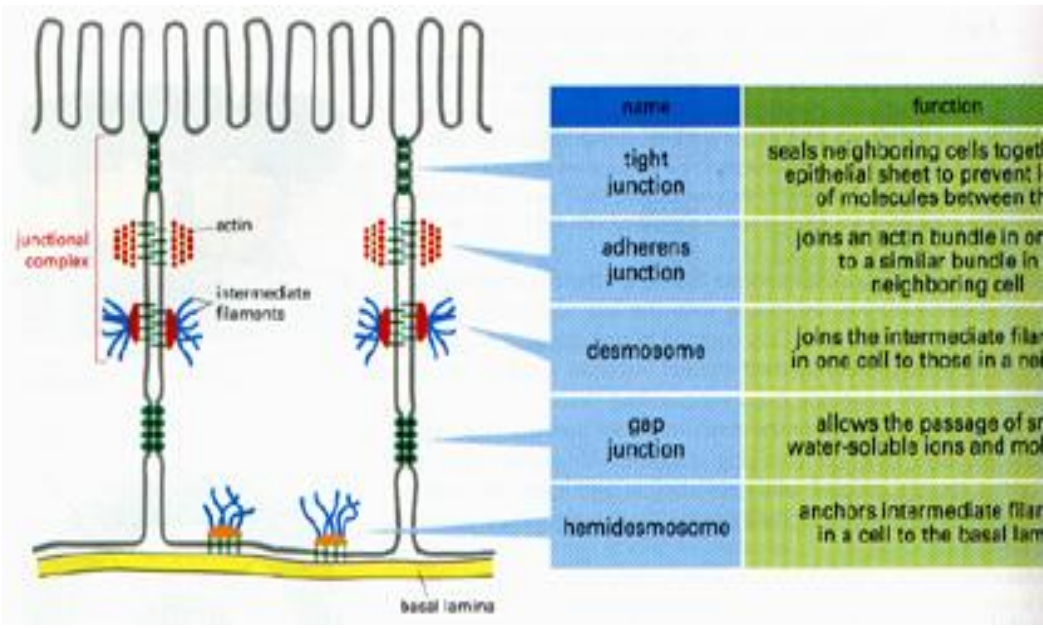
Materials can enter the cell only through diffusion or active transport. This junction also blocks the movement of integral membrane proteins.




Schematic of a tight junction

Adheren junctions provide strong mechanical attachments between the adjacent cells. This holds cardiac muscle cells tightly as the hearth expands and contracts and is also responsible for contact inhibition. Some of these junctions are present in narrow bands connecting the neighbouring cells, while others are present in discrete patches that hold the cells together. This junction is

built from the cadherins, which is a transmembrane protein. The extracellular segment binds to each other, while the intracellular segments bind to catenins and this in turn bind to actin filaments.

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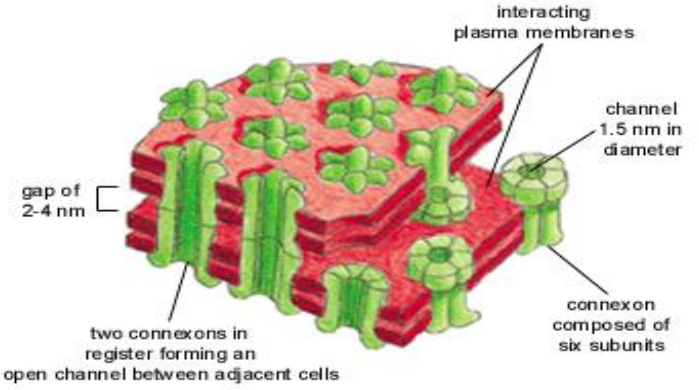
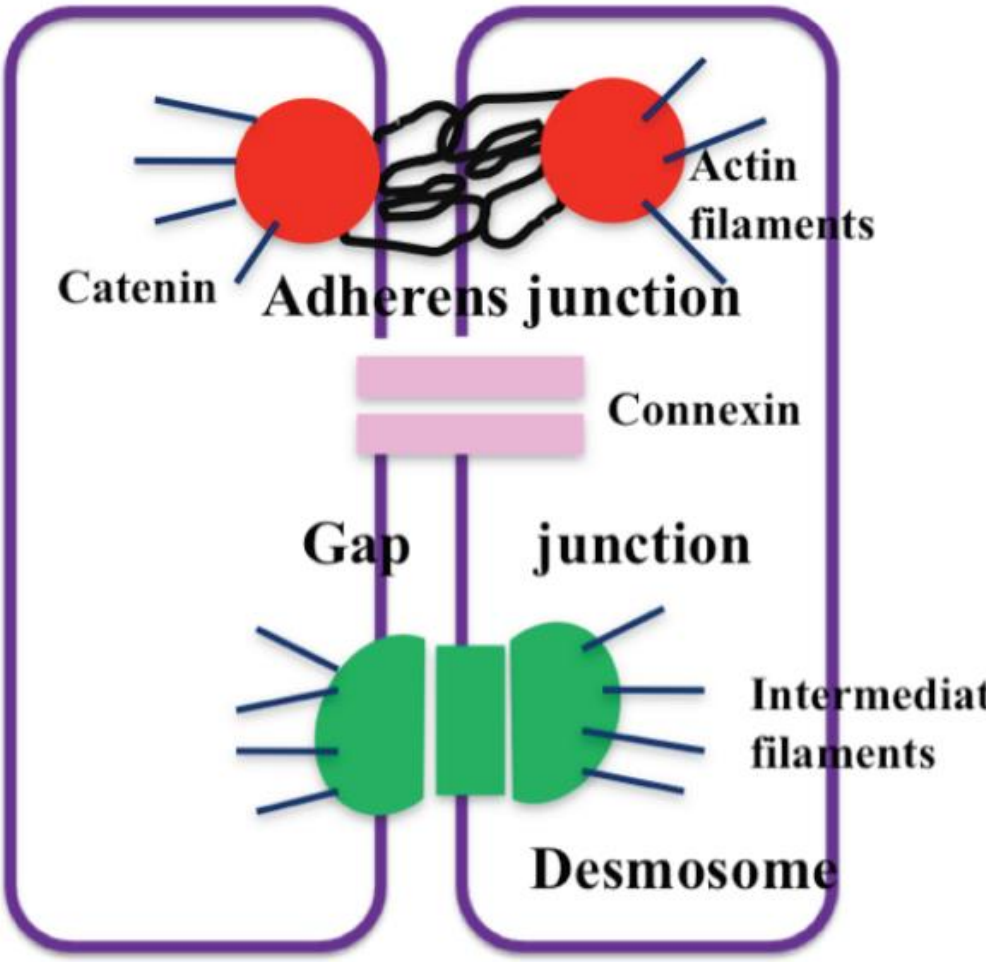
Gap junctions provide hollow intracellular channels of about 1.5-2 nm in diameter to permit the passage of ions and small molecules between the adjacent cells. The gap junction does not seal the membrane together as tight junction nor do they restrict the entry of molecules between the membranes. This junction is composed of array of channels, which permit the small water-soluble molecules to shuttle from one cell to another. In addition, this will allow the electrical and metabolic coupling among cells. Electrical coupling is abundant in cardiac and smooth muscle cells.




Depolarization of one group of muscle cell rapidly spreads to the adjacent cells, leading to the well co-ordinated contractions of those muscles. In case of metabolic coupling, many hormones act by elevating the intracellular concentration of secondary messengers such as cyclic AMP. This cAMP can readily pass through the gap junctions. Hexagonal tubes called connexons connect the two membranes of the adjacent cells. This connexons is constructed from 4 to 6 copies of transmembrane proteins called connexins. Orientation of connexins can form a pore across the gap. However, elevated intracellular calcium and low intracellular pH established the specific stimuli for closing the connexions rapidly.

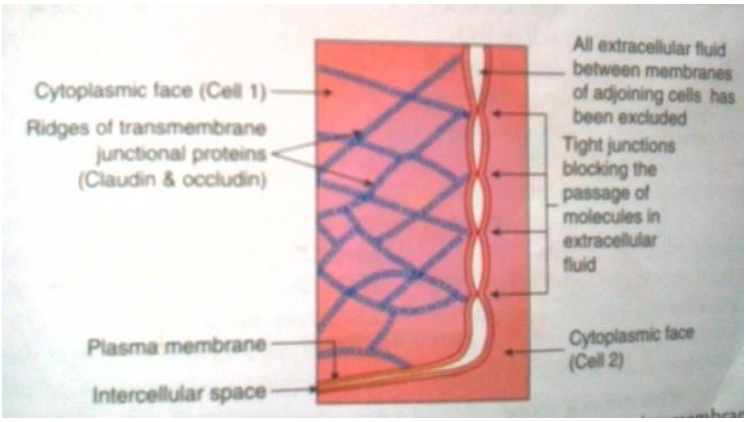



Types of cell-cell junctions

Desmosomes provide strong structures involved in the intercellular adhesion. This exists as a disc shaped structure on either side of the opposing cytoplasmic membrane. This disc can connect to inside of the own cell via intermediate filaments. This desmosome connect the cytoplasm of two cells and convey the stresses on one cell to its neighbours.

Molecular components in cell junctions mainly are membrane bound receptors involved in cell adhesion. They are cadherins, Ig

super family, cell adhesion molecule (CAM), selectins and integrins. Cadherins facilitate the adhesion via homophilic binding to other cadherins in a calcium dependent manner. This appears to be critical in segregating the embryonic cells into the tissues. This can anchor the cells through cytoplasmic actin in case of adheres junctions, whereas intermediate filament in case of desmosomes.




Immunoglobulin like adhesion molecule can function by both homo and heterophilic binding. Integrins is a group of heterodimeric glycoprotein, both alpha and beta subunits participate in the adhesion. This will facilitate the cell-ECM interaction. This exists in both active and inactive states. Selectins expressed only on the leucocytes and endothelial cells. Like integrins, they are so much important in many host defence mechanisms. This will bind to the carbohydrate ligands on the cells. Hence the binding forces are relatively weak.

Molecules involved in direct cell-cell contact are known as cell junction molecules. This junction may on the order of 1.5 nm diameter and allow the molecules less than 1000 Da to pass

through. Assume that the molecules being exchanged never leave the cells and their size is limited by the diameter of pore.

Consider the flux through a hole on a flat wall. The maximum flux *J* is calculated as follows:

$$J = \frac{4D}{\pi d}([C]_1 - [C]_2)$$

Where D is the diffusion coefficient; d is the diameter of the hole; $[C]_1$ is the soluteconcentration in the signalling cell and $[C]_2$ is the concentration in the receiving cell.

When we substitute in typical numerical values such as d= 4 nm; D = $10^{-5}$ $cm^2$/sec;$[C]_1$-$[C]_2$ = 100 µM, we obtain a flux of 2.4 x $10^5$ molecules/pore/sec. Therefore for100 pores, we obtain a flux of 2.4 x $10^7$ molecules/ sec.

**Cell Migration**

Cell migration plays an important role in all physiological as well as some pathological processes. Cell migration is very important during organogenesis and embryonic development. It plays a role in tissue-repair response during both wound healing and angiogenesis. On one hand, the immune system relies on cell migration, while on the other cancer metastasis is characterized by the cell motility. The above-mentioned are the ones that normally happen in the body. But there are cases where migration of cells is induced by infusing chemotactic growth factors. For example, bone

marrow stem cells can be induced to migrate out of the marrow into the blood circulation by infusing chemotactic growth factors into the patient.

Based on the stimuli, there are five different modes of cell migration

1. Chemotaxis - soluble concentration gradient
2. Haptotaxis - insoluble concentration gradient
3. Galvanotaxis - electrical currents
4. Contact guidance - surface topology
5. Contact inhibition - lamellipodium inhibited by neighbor adjacency

Cell migration is a five-step process

Step 1: Protrusion of membrane lamellopodia

Step 2: Adhesion to the matrix via integrin receptors

Step 3: Contraction of the cytoplasm by myosin based motors

Step 4: Rear release and cell displacement

Step 5: Recycling of remaining integrins

**Step 1**: Protrusion of the membrane lamellopodia occurs by the polymerization of actin. In the actin polymerization process, there is increase in the number of sites for actin polymerization followed by the addition of actin monomers on the filament growth sites near the membrane. The new sites for actin polymerization may arise either by the uncapping of existing filaments or the formation of new nucleation sites.

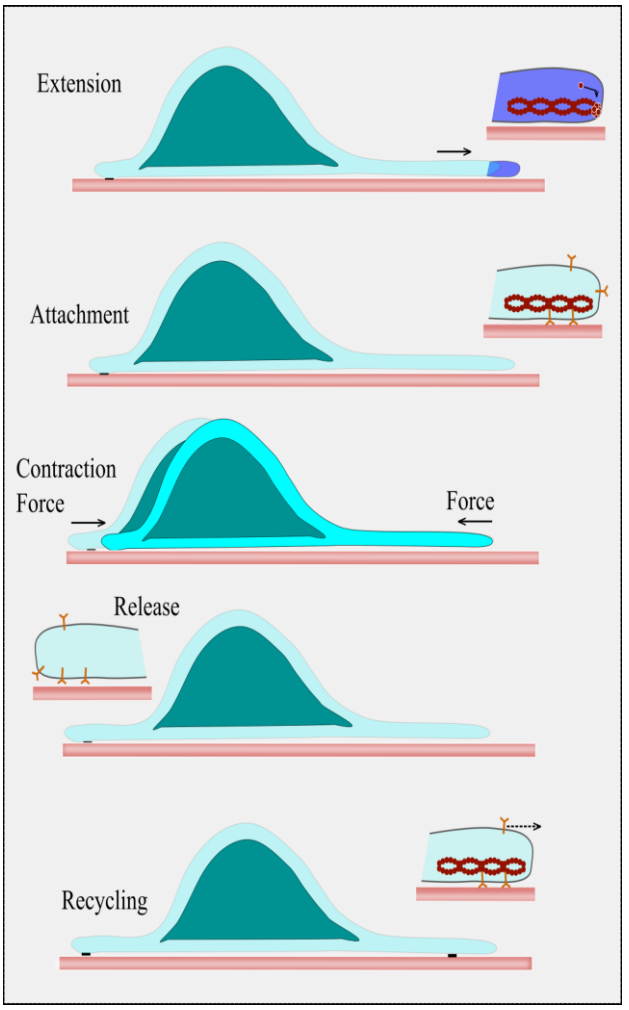
**Step 2**: The actin cytoskeleton is linked to the extracellular matrix via integrins, which are the cell surface receptors thus forming specialized complexes. Integrin receptors have the affinity towards

certain motifs like RGD that is present in the extracellular matrix. Upon binding to the ligands (RGD), integrin cross-linking occurs and matrix stiffness is increased. These specialized complexes are regulated by the *rho* subfamily of the *ras* family of GTP-binding proteins but however the mechanisms by which they exert the effects are not known.

**Step 3**: There are two forces involved - contractile force and the traction force. Contraction of cytoplasm occurs via myosin-based motors. However, for a cell to move, the cell must overcome the contraction force provided by the myosin-based motors. Fibroblast cells can generate a traction force of approximately 2X104 μdynes against the substrate.

**Step 4**: Rear side release of the integrins and the cell displacement involves both physical and enzymatic processes. Either by the transmission ofphysical forces or by regulating the local Ca2+ concentration, asymmetry in theadhesion of cell to the substrate can be brought out thus breaking the rearside contacts and the cell displacement.

**Step 5**: The integrins that are released from the rear side is endocytosed intovesicles which is then followed by intracellular diffusion and is directedtowards the cell surface of the leading edge.




Different steps involved in the migration of a cell

***Factors affecting cell migration speed***

There are two factors, which affect the cell migration speed is substratumadhesive strength and intracellular contraction force.Assume when the adhesion is very weak, then obviously intracellularcontraction force dominates, thereby preventing the traction force against thesubstratum. Therefore, the movement could be very negligible.

Suppose the intracellular contraction force is comparable with the substratumadhesive strength, cells can impart the traction force against substratepromoting the rear release by the detachment of rear cell-substrateattachment, thereby promoting cell speed significantly.However, if there is a strong adhesion between cell and substrate, the rearrelease could not be promoted and hence cell migration is very negligible.From these it is very clear that the ratio of intracellular contraction force andthe substratum adhesive strength will determine the cell migration speed.

SOLUBLE GROWTH FACTORS

Cells undergo various fate processes such as adhesion, replication, migration,differentiation and death in presence of different environmental signals. Inorder to coordinate these activities, cell should communicate with each other.This cell-cell communication can be achieved by biochemical, physical ormechanical stimuli.

- **Biochemical stimuli:** Cells secrete some soluble signals such ascytokines and chemokines and insoluble signals, which alter thephysical and chemical microenvironment through modification to theECM
- **Physical stimuli:** Cells can touch each other via direct cell-cell contacteg. gap junctions
- **Mechanical stimuli:** Cells respond to mechanical stimuli in theirmicroenvironment as similar to biochemical stimuli.

**Soluble growth factors**

Growth factors are small proteins of about 15,000 to 20,000Dalton in size. They are reasonably stable with long half-life. These

proteins are produced by the signalling cell and secreted to target cell through different routes:

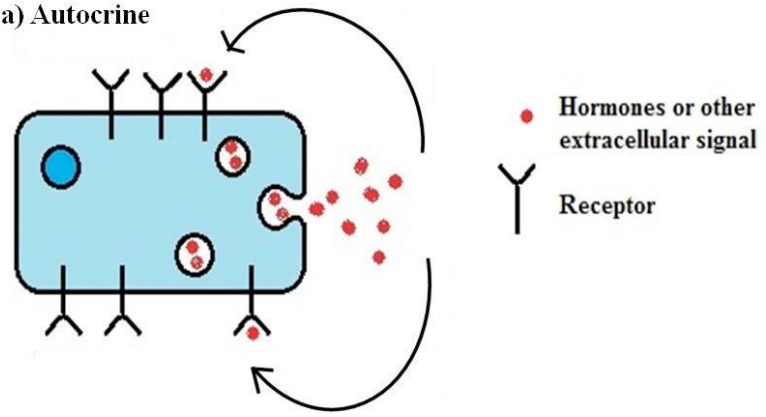
1. Autocrine - cell secrete growth factor and signal itself
2. Paracrine - cell secretes growth factor and signals neighbouringcells by diffusion
3. Endocrine - cell secretes growth factor into blood stream thatcarries growth factor to target cell

Autocrine signalling: This type of signalling mainly confined to pathologicalconditions like cancer. For example certain type of tumour cells synthesisethe growth factors, which are required for normal cell growth. However, thegrowth factors released can be used by the same cells, thereby promotingupregulated growth tumour cells.

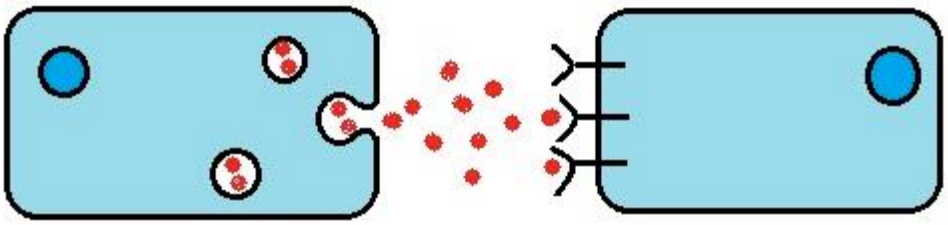
Paracrine signalling: Receiving cell is in the close proximity of the signaling cell. These signals will affect only the group of receiving cells adjacent to it.Examples of paracrine signalling are neurotransmitters, neurohormones.

Endocrine signalling: Signals from the signalling cell acts only on the distantset of receiving or target cells. Usually endocrine hormones are carried awayfrom the site of release to the target site.

a) Autocrine



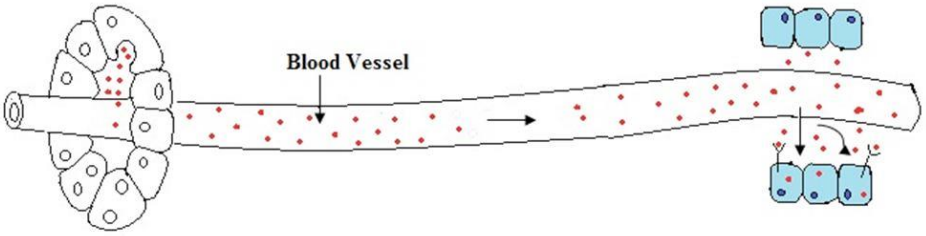

(b) Paracrine



Secretory Cell

Adjacent target cell

c) Endocrine




Hormone secretion into blood by endocrine gland

Distant target cells

Three general schemes for cell-cell signalling by extracellular chemicals

**What are the different types of signals available?**

• Cytokines are a class of regulatory proteins that are secreted by cells and classically cause proliferation or differentiation. eg. Hepatocytegrowth factor: Cytokine stimulates division in hepatocytes, epidermalkeratinocytes, renal tubular epithelial cells and melanocytes. Interleukin–
2: cytokine stimulates growth of T lymphocytes• Chemokines are a group of chemotactic factors that provide directionalcues for cell migration. eg. CCL27: chemokine attracts T cells to theskin. CX3CL1: leukocyte recruitment

• Autocrine: relating to, promoted by, or being a substance secreted by acell and acting on surface receptors of the same cell

• Paracrine: relating to, promoted by, or being a substance secreted by acell and acting on adjacent cells

• Endocrine: relating to, promoted by or being and substance secreted bya cell that is carried by the blood stream to a target cell

During the cell signalling process, signalling cell secretes a signaling molecule, which can diffuse away from the cell through molecular diffusion,thereby creating concentration gradient. The job of the target cell is to sensethe signal coming and the signalling molecule binds to the receptors on thetarget cell surface, thereby inducing the signal transduction process. Thissignal transduction from one cell to another depends upon the distancebetween the signalling and target cell and sensitivity of target cell to signaling molecule.

***How far can soluble signals propagate?***

The maximum secretion rate is mainly depends upon the following formulae:

At steady state, $$\frac{[C]}{K_d} = a \frac{R}{r}$$

$$a = \frac{\tau_{diffusion}}{\tau_{secretion}} = \frac{R^2/D}{K_d R/F}$$

and

where,

[C] is the concentration of a signal

Kd is the dissociation constant of a signal to receptors on the receivingcell

R is the radius of the cell

r is the distance between the cells

a is the ratio of two time constants

$\tau_{diffusion}$ is the diffusion time constant

$\tau_{secretion}$ is the secretion time constant

F is the secretion rate

D is the diffusion coefficient of the signalling molecule

When [C]=Kd, then,

$$a = \frac{r}{R}$$

Hence the maximal secretion rate is given by the ratio of two time constants -the time constant for diffusion away from the producing

cell and the secretiontime constant. Assume endpoint where signal strength is half of themaximum, hence leading to a time constant estimate for signalling process ofmaximal distance of about 200μm over 20 minutes. This distance is alteredproportionally to the secretion rate (F) and inversely with dissociation constant(Kd).

Popular growth factors in tissue regeneration.

- Ang, angiopoietin;
- bFGF, basic fibroblast growth factor;
- BMP, bonemorphogenetic protein;
- EGF, epidermal growth factor;
- FGF, fibroblast growth factor;
- HGF, hepatocyte growth factor;
- IGF,insulin-like growth factor;
- NGF, nerve growth factor,
- PDGF, platelet-derived growth factor;
- TFG, transforming growth factor;
- VEGF, vascular endothelial growth factor.

| abbreviation | tissues treated | representative function |
|---|---|---|
| Ang-1 | blood vessel, heart, muscle | blood vessel maturation and stability |
| Ang-2 | blood vessel | destabilize, regress and disassociate endothel surrounding tissues |
| FGF-2 | blood vessel, bone, skin, nerve, spine, muscle | migration, proliferation and survival of endo inhibition of differentiation of embryonic s |
| BMP-2 | bone, cartilage | differentiation and migration of osteoblasts |
| BMP-7 | bone, cartilage, kidney | differentiation and migration of osteoblasts, |
| EGF | skin, nerve | regulation of epithelial cell growth, proliferat differentiation |
| EPO | nerve, spine, wound healing | promoting the survival of red blood cells and precursors to red blood cells. |
| HGF | bone, liver, muscle | proliferation, migration and differentiation o cells |
| IGF-1 | muscle, bone, cartilage, bone liver, lung, kidney, nerve, skin | cell proliferation and inhibition of cell apopt |
| NGF | nerve, spine, brain | survival and proliferation of neural cells |
| PDGF-AB (or -BB) | blood vessel, muscle, bone, cartilage, skin | embryonic development, proliferation, migra endothelial cells |
| TGF-α | brain, skin | proliferation of basal cells or neural cells |
| TGF-β | bone, cartilage | proliferation and differentiation of bone-form proliferative factor for epithelial cells |
| VEGF | blood vessel | migration, proliferation and survival of endo |

## UNIT III

## BIOMATERIALS

### Biomaterials in tissue engineering

A Biomaterial is defined a material used in medical device intended to interact with the biological systems without provoking any harmful effects. A good example is calcium sulphate (Plaster of Paris), which has been used as a bone graft substitute. Similarly hip-joint prostheses have been fabricated from stainless steel, or titanium, special alloys, ceramics and ultrahigh molecular weight polyethylene. Expanded polytetrafluoroethylene (ePTFE) has been used as non-stick coating in the cooking vessel and has also been exploited as small artery grafts. Most of the skate shoes use polyurethane to provide maximum grip on the skateboard. The same material can be used to fabricate the hemodialysis access graft. Another good example is the use of biomaterials in the fabrication of intraocular lens. About 50% of elder population suffers from the cataracts where the natural lens loses its transparency and become cloudy. Hence biomaterials such as poly (methyl methacrylate), silicone elastomer, soft acrylic polymers has been used to fabricate the intraocular lens, substitute for the natural lens.

Although most of the identified biomaterials have improved the quality of patient's life, each manmade construct has its own limitations due to the variations in genes, body chemistries, sex, life style and degree of physical exercise from individual to individual. Hence choice of the biomaterial for the intended application plays a vital role for the success rate. For example use of calcium sulphate as a bone substitute cannot support the patient weight completely. Moreover, this will degrade faster than the healing rate of bone. Stress shielding, implant loosening and corrosion are the major drawbacks of using metal alloys in hip

prostheses. Use of ePTFE graft in small arteries can provoke foreign body response and exhibit complaint mismatch. Polyethylene terephthalate (PET) is highly thrombogenic promoting thrombosis and catastrophic complications in prosthetic vascular graft implantation. Additionally use of polyurethane as a graft can leach some harmful products. Hence a biomaterial should not be toxic to the system and provide mechanical support to the cells.

Mostly polymers have gained more attention for the use of biomaterials in biomedical applications such as orthopaedics, dental applications, soft tissue and cardiovascular regeneration. Polymer is a macromolecule composed of repeating units of same or different monomers. The polymers are mainly classified as natural (those which are derived from the living organism) and synthetic (those which are synthesised chemically). Molecular weight is the term used to describe the average length of the polymer chains, which are expressed as Daltons or atomic mass units. Number average molecular weight (Mn) and weight average molecular weight (Mw) are the major types of molecular weights. Number average is defined as the distribution of molecular weight over the number of molecules whereas weight average is the distribution of molecular weight over the weight of each chain. The ratio of Mw to Mn is known as polydispersity index (PDI), measures the breadth of the distribution. Usually PDI is around 1.5 – 2.0 for most of the commercial polymers and polymers, whose PDI is is said to be monodisperse.

**The biomaterial properties such as bulk, surface, mechanical and biological have found to influence dynamic interactions at the tissue – implant interface.**

In the **bulk**, solid materials differ from other state of the materials such as liquid and gas due to the strength of interatomic forces, which brings

the atoms together. There are three primary or strong inter atomic bonds (ionic, covalent, and metallic) and two secondary or weak inter atomic bonds (van der Waals and hydrogen bonding).

**Surface property** differs from that of the bulk since the atoms at the surface have high reactivity as well as special organization. Such surface can be modified for the intended function that can drive the biological functions such as protein adsorption, cell adhesion, cell growth, and blood compatibility in response to biomaterial. Hence it is very essential to characterise the surface using different methods of characterization.

**Mechanical properties**

The success of graft uptake by the host mainly depends upon the mechanocompatibility of the tissues. Or in other words, the mechanical properties of the biomaterials should closely match the properties of the tissues. Robert Hooke first discovered Hooke's law or law of elasticity in 1678 and it states that when a material is subjected to distraction force, it would lengthen in the direction of force, which is proportional to the applied load. This extension for a specific applied load may vary with the geometry and composition of the specimen. Hence stress ($\sigma$) is the normalized load or is the force divided by area. Whereas deformations may be converted in to strain ($\varepsilon$), which is the change in length or displacement to original length. Stress-strain curves are obtained by plotting stress versus strain, and the curves provide information of material's mechanical properties such as elastic limit, elasticity, plasticity, yield stress, yield point, ultimate tensile strength and fracture stress.

The elastic limit is the limit above, which the material will not go back to its original shape when the load is removed. Yield point is defined as the

point at which the material has substantial elongation without further increase in the load. Yield stress is defined as the stress at which the material starts to deform plastically. The slope of stress-strain curve provides the information of material elastic modulus. The ultimate tensile strength is the point at which maximum load that the material can withhold before failure (begins to lose the ability to sustain load). Fracture stress is the point at which the material ruptures. Area under the stress-strain curve provides the toughness of the material. Based on different mechanical properties, materials can be categorized as elastic, plastic, brittle and ductile. The ability of the material to retain its original shape when the force is removed is known as elasticity whereas the ability of the material to attain a new shape when the force is removed is called as plasticity. Brittle materials are the materials, which fail at very lower strain. However, ductile materials are the materials, which tend to withstand large strain before fracture.

**Biological properties**

Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application. Ideally, biomaterials should not provoke any harmful effects to the host system. The biocompatibility of the material is mainly influenced by the surface and bulk properties. Biocompatibility of a biomaterial depends on the roughness, smoothness, ridges, grooves, and pores, implant size, wettable property (hydrophobic / hydrophilic nature), degradation rate, degradation products and mechanical properties. Immunogenicity is defined as the ability to stimulate the immune response. The response may be generalized, nonspecific or specific. There are different methods to evaluate the biocompatibility both in vitro (using cell lines) and in vivo (using animal models).

Tissue engineering is an interdisciplinary field, which applies both engineering and life science principles for the development of biological substitutes to maintain or restore the tissue function.

Tissue scaffolds are temporary framework to lodge the cells and possess several cues to control the cell fate as native ECM. The ideal properties of scaffolds are

1. Biocompatibility: The scaffold should not elicit any toxic reactions inside the body

2. High porosity with desired pore size: High porosity is required for the infiltration of cells and diffusion of nutrients, oxygen and also by-products. The pore size should freely allow the cells to migrate throughout the scaffold i.e. cell infiltration

3. Adequate mechanical properties: The scaffold mechanical property should match the tissue properties. For example, based on the mechanical property, the tissue is categorized as hard tissues (eg. bone) and soft tissues (eg. nerve, skin). Hard tissue like bone is very tough in nature whereas the soft tissues are more elastic in nature. Hence, the mechanical property of the material should be designed according to the tissue of interest that should mimic the native ECM.

4. High surface area to volume ratio to improve the cell adhesion capacity of the scaffold

5. Biodegradation: This property should be required to avoid the longterm complication of the material inside the body. Additionally, the degradation products should be non-toxic to the system. Degradation rate of the scaffold plays a vital role in retaining the scaffold integrity in

terms of structure and mechanical integrity. That is the degradation rate should match the rate of neo-tissue formation at the site of injury.

6. Mimics native ECM: It should possess specific cues to regulate the cell fate process like adhesion, proliferation, and differentiation as native ECM.

2. Biodegradable polymers

Polymers, both natural and synthetic, have been extensively used as biomaterials. In addition, researchers have used proteins, polysaccharides and inorganic materials. Polymers are classified as synthetic and natural polymers based on the origin. Examples of synthetic polymers include poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lacticco- glycolic acid) (PLGA) and poly (ε-caprolactone) (PCL). Poly(hydroxyalkanoates) such as poly(hydroxybutyrate) and poly(hydroxybutyrate-co-valerate) are examples of natural polymers. Each has its own merits and demerits.

For example synthetic polymers are tailor made, and can be made biodegradable, but lack biological recognition. However, immunogenicity, poor processibility of natural polymers limits its use though it possess biological motif for cell adhesion. Poly(glycolic acid) is relatively hydrophilic and it degrades rapidly in aqueous medium thereby loses its mechanical integrity between two to four weeks. On the other hand, poly(lactic acid) is more hydrophobic having slower degradation rate, hence better mechanical stability. Copolymer of both polymers, PLGA has an intermediate degradation rate based on the ratio of each polymer and PLGA has been approved by FDA. PCL is a semi crystalline polymer and degrades very slowly when compared to other polymers like PLA, PGA, and PLGA.

Poly(hydroxybutyrate) (PHB) is natural polymer derived from the microorganism via fermentation. PHB degrades very slowly due to its hydrophobic nature. Other examples of natural polymers are collagen, gelatin, collagen-GAG copolymers, which are fibrous proteins. The major disadvantages of using collagen are the pathogen transmission, antigenicity and poor mechanical property with no control on the degradation rate. Silk fibroin is also a natural fibrous protein, used as a biomaterial due to its good tensile property, biocompatibility, and non-toxicity with slow degradation rate.

Similarly, polysaccharides such as alginate, chitosan, hyaluronate have been used as biomaterials. Porous bioactive glasses, calcium phosphates like α- tricalcium phosphate, hydroxyapatite, and polymer-ceramic composites have been used as inorganic materials due to its osteoconductive property. However, the brittle nature and poor processability limits its potential use towards biomedical applications.

**Application of Biomaterials**

The main applications of biomaterials can be classified into the categories below and described later:

- Cardiovascular medical devices (stents, grafts and etc.)
- Orthopedic and dental applications (implants, tissue engineered scaffolds and etc.)
- Ophthalmologic applications (contact lenses, retinal prostheses and etc.)
- Bioelectrodes and biosensors
- Burn dressings and skin substitutes
- Sutures
- Drug delivery systems

***Cardiovascular medical devices***. Heart valves, endovascular stents, vascular grafts, stent grafts and other cardiovascular grafts are common medical devices in cardiovascular applications. There are several major forms of valvular heart disease, most involving the aortic and/or the mitral valve. The most common type of valve disease and most frequent indication for valve replacement overall is calcific aortic stenosis obstruction at the aortic valve secondary to age-related calcification of the cusps of a valve that was previously anatomically normal. In case of vascular patholo- gies, stents and vascular graft is used. Different polymers and metals with or without coating can be applied in this category (titanium, polytetrafluoroethylene and etc.) . ***Tissue engineering scaffolds***. Tissue engineering is one of the most important ways to achieve tissues for repair or replacement applications. Its goal is to design and fabricate reproducible, bioactive and bioresorbable 3D scaffolds with tailored properties that are able to maintain their structure and integrity for predictable times, even under load-bearing conditions. Scaffolds can be applied in different tissues. It is only important to note that it only in designing the scaffold, type of fabrication and biomaterial selection depending on the target organ and its cells that can be affected on final application. Chemistry, architecture, porosity and rate of degradation should provide a sufficient mechanical environment and should facilitate cell attachment, proliferation and migration, waste nutrient exchange, vascularization and tissue ingrowth. Also there should be a proper ratio between
degradation of the scaffold and tissue ingrowth .

There are various types of scaffold fabrication methods. At first, only polymeric scaffolds were used but gradually composite scaffolds

and especially ceramic/pol- ymer scaffolds have been used. The main important scaffold fabrication methods are: fiber bonding, solvent casting and particulate leaching, compression molding, extrusion, freeze-drying, phase emulsion, solid free form fabrication and electro-spinning. Differences between these methods are temperature, pressure, solvent type, porogen (which is responsible for making pores) and etc.

Recently researchers used mesostructured materials in scaffolds to supply drug and biological agents in situ during degradation of scaffold and growing new tissue.

***Ophthalmologic applications***. Vision impairment/low vision, blindness, refrac- tive error (Myopia and Hyperopia), astigmatism, presbyopia, cataracts, primary open-angle glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy are common ophthalmologic diseases. To improve the life of these patients, many implants have been applied. The main biomaterials which are used in this category are summarized in Table 1.

***Bioelectrodes and biosensors***. Bioelectrodes are sensors used to transmit infor- mation into or out of the body. Surface or transcutaneous electrodes used to moni- tor or measure electrical events that occur in the body are considered monitoring or recording electrodes. Typical applications for recording electrodes include elec-trocardiography, electroencephalography, and electromyography information into or out of the body. Various parameters influence the material selection of elec- trodes (Table 2).

These bioelectrodes are mainly applied in cardiology and neurology applica- tions. A biosensor is a sensor that uses biological molecules, tissues, organisms or principles to measure chemical or biochemical concentrations. Biosensors can

**Table 1** Ophthalmic implant materials commonly used

| Implant | Materials which used |
|---|---|
| Contact lenses | Poly(methyl methacrylate) (PMMA), 2-hydroxy- ethyl |
| Inlays or onlays | Hydrogels, collagen, permeable |
| Intraocular lenses | Optic: PMMA, hydrophobic acrylic, silicone, hydro- philic acrylic |
| Ophthalmic viscosurgical device (OVD) | Chrondroitin sulfate, sodium hyaluronate, hyalu- ronic acid, |
| Glaucoma shunts | Plates: silicone (impregnated with barium), polypropylene |
| Vitreous replacements | Silicone oil, gases |

**Table 2** Parameters influencing the material selection of electrodes

| | |
|---|---|
| Electrode | Surface area, geometry, and surface condition |
| Electrical | Potential, current, and quantity of charge |
| Environmental | Mass-transfer variables and solution variables |
| Engineering | Availability, cost, strength, and fabricability |

be used in many medical and non-medical applications. Biomedical sensors are sensors that detect medically relevant parameters; these could range from simple physical parameters like blood pressure or temperature, to analyses for which biosensors are appropriate (e.g. blood glucose). Biosensors can work by changes in pH, ions, blood gases ($O_2$, $CO_2$ and etc.), drugs, hormones, proteins, viruses, bacteria, tumors and etc.

***Burn dressing and skin substitutes***. Skin is the largest organ that protects body from microorganisms and external forces, integrates complex sensory nervous and immune systems, controls fluid loss, and serves important aesthetic function. Deep skin injuries due to deep cuts, burns or degloving injuries can cause significant physiological derangement, expose the body to a risk of systemic infection, and become a life threatening problem. So the need of skin substitutes depending on wound depth is felt. An ideal skin substitute must be inexpensive, long lasting, a bacterial barrier, semipermeable to water, elastic, easy to apply, painless to the patient, non-antigenic and non-toxic and has durable shelf-time. Today a lot of commercial skin substitutes are applied.

***Sutures***. Suture is any strand of material that is used to ligate blood vessels or approximate tissue. Ligatures are used to achieve hemostasis or to close a structure to prevent leakage. The suture device is comprised of: the suture strand; the surgi- cal needle; and the

packaging material used to protect the suture and needle during storage. The ideal suture must be biocompatible, sterile, compliant, adequate knot/ straight strength, secure and stable knot, strength and mass loss profiles adequate for proposed usage, low friction, adequate needle attachment strength, atraumatic needle design, non-electrolytic, non-capillary, non-allergenic, non-carcinogenic, minimally reactive, uniform and predictable performance. Silk, nylon, polyester, cotton, polypropylene, ultra-high molecular weight polyethylene (UHMWPE), stainless steel and synthetic absorbable polymers such as poly glicolic acid (PGA), p-dioxanone (PDO) and etc. are the main materials that are used as sutures yet. ***Drug delivery systems*** (***DDS***). Drug delivery systems introduced as formula- tions or instruments which enable to control the release rate of a biological agent (especially a drug) in the target site. Drug delivery systems are an interface between patient and drug. Drugs can be introduced to the organ by different anatomical routes due to disease and drug type: Digestive system (oral, anal), oral, rectal, parenteral (subcutaneous, intramuscular, intravenous, arterial), mucous membranes, respiratory tract by inhalation, subcutaneous or intraosseous are man anatomical routes.

By increasing the size the dosage in single dose administration, side effects would appear so in order to reduce these side effects, coatings with varying thickness, are

**Table 3** DDS systems

Macroscale DDS ("zero order" constant delivery rate DDS)

- Implants (e.g. subcutaneous or intramuscular)
- Inserts (e.g. vaginal, ophthalmic)
- Ingested DDS (e.g. osmotic pumps, hydrogels)
- Topical DDS (e.g. skin patches)

Macroscale and microscale DDS (site-specific, sustained delivery rate DDS)

- Surface-coated DDS (e.g. oral tablets, catheters, drug-eluting stents)
- Injected DD depots (e.g. degradable microparticles and phase separated masses) Nanoscale DDS (targeted DDS)
- Injected nanocarrier DDS (e.g. PEGylated drugs, polymer-drug conjugates, PEGylated liposomes, PEGylated polymeric micelles, and drug nanoparticles, sometimes targeted by monoclonal antibodies or cell membrane receptor ligands)

applied. Such formulations are now known as "sustained release" or "prolonged release" products. However, the pharmacokinetics of such products depended greatly on the local in vivo patient environment and as such, vary from patient to patient. These systems are called "zero order" systems because they release drug during time in a constant rate. These reasons were among the most important driving forces that led to the birth of the field of "controlled drug delivery" (CDD) in the mid to late 1960s that became known as "macro-scale devices" that exhibit constant or zero order drug delivery rates, leading to constant plasma drug concentrations over long time durations of drug delivery. By the rapid growth of nanoscale materials, inject- able targeting drug delivery systems appear (see Table 3).

***Dental materials***. Restorative materials have been used as tooth crowns and root replacements. Four groups of materials which are used in dentistry today are metals, ceramics polymers and composites. Despite recent advances in material science and dentistry, there still is not a proper material for restorative dentistry. Characteristics of an ideal restorative material are listed below:

- Be biocompatible
- Bond permanently to tooth structure or bone
- Match the natural appearance of tooth structure and other visible tissues

- Exhibit properties similar to those of tooth enamel, dentin and other tissues
- Be capable of initiating tissue repair or regeneration of missing or damaged tissue

Dental materials can be classified in two categories: preventive materials, restorative material. Preventive dental materials include pit and fissure sealants, sealing agents that prevent leakage, materials that are used primarily for the antibacterial effects, liners, bases, cements and restorative materials that are used primarily because the release fluoride, chlorhexidine or other therapeutic agents used to prevent or inhibit the progression of tooth decay. This type of materials used for short-term application. Restorative dental materials consist of all synthetic components that can be used to repair or replace tooth structure, including primes, bonding agents, liners, cement bases, amalgams, resin based composites, compomers, metal-ceramics, hybrid iono- mers, cast metals and denture polymers. Restorative materials can be used for both short and long-term applications. Restorative materials can be classified as *direct restorative materials* and *indirect restorative materials* dependent on whether they are used. Direct fabricated intraorally and indirect fabricated extraorally.

Because of importance of restorative dental materials, explain more about this part. Dental amalgam has been used traditionally for filling dental cavities. Amalgam is a mixture of copper, tin, zinc, mercury, silver and other trace metals. Later cement dental restorative materials were used as restorative materials. To achieve adhesive bonding in the general case of two rigid solids, such as a tooth enamel surface and an orthodontic bracket, it is necessary to apply a fluid adhesive between them.

Moreover, the fluid must be of appropriate chemical formulation to initially wet both surfaces, exhibiting a low contact angle. One or both surfaces may have been subjected to some form of pre-treatment or conditioning with an etchant or primer that, inter alia, may have modified surface porosity. In this case, the adhesive fluid may be drawn into the solid surface layers by capillary action. The presence of a suitable fluid between two solids greatly enhances the potential for intermolecular force interactions at each solid–fluid boundary.

Dental cements can be classified to:

- Conventional acid-base cements
- Poly-electrolyte cements: Zinc poly carboxylates and glass ionomers
- Resin-modified glass-ionomer cements
- Dual-setting resin-based cements

**Conventional acid-base cements**

Dental cements are, traditionally, fast-setting pastes obtained by mixing solid and liquid components. Most of these materials set by an acid-base reaction, and sub- sequently developed resin cements harden by polymerization. They have various compositions. This

material is composed primarily of zinc oxide powder and a 50 % phosphoric acid solution containing aluminum and zinc. The mixed material sets to a hard, rigid cement by formation of an amorphous zinc phosphate binder. The bonding arises entirely from penetration into mechanically produced irregu- larities on the surface of the prepared tooth and the fabricated restorative material. Classifications of dental cements are summarized in Table 4.

**Poly-electrolyte cements: Zinc poly carboxylates and glass ionomers**

Poly (carboxylic acid) cements were developed in 1967 to provide materials with properties comparable to those of phosphate cements, but with adhesive properties of calcified tissues. This type of cement is composed of zinc oxide and aqueous poly (acrylic acid) solution. The metal ion cross-links the polymer structure via carboxyl groups, and other carboxyl group's complex to Ca ions in the surface of the tissue. Adequate physical properties, excellent biocompatibility in the tooth, and adhesion to enamel and dentin are main advantages of these cements being opaque is the main problem with these cements. The need for a translucent mate- rial led to the development of the glass-ionomer cements (GIC). GICs are also based on poly (acrylic acid) or its copolymers with itaconic or maleic acids, but utilize a calcium aluminosilicate glass powder instead of zinc oxide. GICs set by cross-linking of the polyacid with calcium and aluminum ions from the glass, together with formations of a silicate gel structure.

**Resin-modified glass-ionomer cements**

Polyacid molecules contain both ionic carboxylate and polymerizable meth- acrylate groups. It is induced to set by both an acid-base reaction and visible light polymerization. Adhesive bonding but not complete sealing is obtained, because of the imperfect adaptation to the bonded surfaces under practical conditions.

**Dual-setting resin-based cements**

Dual-setting resin-based cements are fluid or paste-like monomer systems based on aromatic or urethane dimethacrylates. They are normally consisting of two-component materials that are mixed to induce setting. They may also be light-cured. These set materials are strong, hard, rigid, insoluble and cross-linked polymers.

**Table 4** Classification of dental cements

| Dental cements | Components | Setting mechanism |
|---|---|---|
| Zinc phosphate | Zinc oxide powder, phos- phoric acid liquid | Acid–base reactions; Zn complexation |
| Zinc polycarboxylate | Zinc oxide powder, aqueous poly(acrylic | Acid–base reactions; Zn complexation |
| Glass ionomer (polyalkenoate ) | Ca, Sr, Al silicate glass powder aqueous poly(acrylic acid-itaconic | Acid–base reactions; Metal ion complexation |

| | | |
|---|---|---|
| Resin modified glass ionomer | Dimethacrylate monomers. Aqueous poly(acrylic acid methacrylate) co- | Peroxide-amine or photo- initiated polymerization |
| Resin-based | Aromatic or urethane dimeth- acrylates, HEMA | Photoinitiated addition polymerization |
| Dentin adhesive | Etchant: Phosphoric acid (aq.)<br>Primer: HEMA in ethanol or acetone<br>Bond resin: Dimethacrylate | Photoinitiated addition polymerization |

## Applications of Nanotechnology in Tissue Engineering

The applications of nanotechnology in tissue engineering in three aspects: biomaterials scaffolding, cellular engineering, and biomolecular manipulation.

### *Applications of Nanotechnology in Biomaterial Scaffolds*

A biomaterial scaffold creates a milieu within which cells are instructed to form a tissue or organ in a highly controlled way. The principal function of a scaffold is to direct cell behaviors such as migration, proliferation, differentiation, maintenance of phenotype, and apoptosis, by facilitating sensing and responding to the environment via cell–matrix commu- nications and cell–cell communications. Therefore, the desirable physical characteristics of biomaterial scaffolds for tissue engineering applications include high porosity, large surface area, large pore size, and uniformly distributed interconnected porous structures throughout the matrix . In addition, the scaffold has to provide spatial signals to modulate the organization of the cells as well as that of the extracellular matrix derived from them. Using nanotechnology, biomaterial scaffold can be manipulated at atomic, molecular, and macromolecular levels and constructed into specific geometrical and topological structures at 1–100 nm scales. Creating tissue engineering scaffolds in nanoscale may bring unpredictable new properties to the material, such as mechanical (stronger), physical (lighter and more porous), optical (tunable optical emission), color, chemical reactivity (more active or less cor- rosive), electronic properties (more electrically conductive), or magnetic properties (super- paramagnetic) and may come up with new functionalities as well, which are unavailable at micro- or macroscales. Other advantages of using

nanotechnology for scaffold fabri- cation may include enhancing biocompatibility, improving contact guidance, reducing friction and therefore wear for joint applications, reducing the need for revision surgery, altering physical or chemical characteristics of the scaffold, and promoting tissue growth around the implant. For example, it has been found that nanosculpting the surface of such scaffolds may stimulate cell growth; the cells rapidly follow the nanoscopic etched tracks, resulting in a faster filling of the matrix with the required cells or tissues. More importantly, some complex specific tissue functions cannot be easily mimicked with macroresolution scaffolds. Nanoscale precision scaffolds can be built in three ways, i.e., atom-by-atom, molecule-by- molecule (also called top-down), or self-assembly (also called self-organization or bottom- up). Top-down means fabricating nanodevices from the microlevel to the nanolevel, for example, stripping a virus particle down form a viral cage; while bottom-up means obtain- ing nanodevices from the atoms and small molecules at a level smaller than the nanolevel, for example, building supermolecular architectures from single small molecules, even single atoms.

*Three-Dimensional Nanofibrous Scaffold*

Collagen is a natural extracellular matrix (ECM) molecule found in many tissues such as bone, skin, tendons, ligaments, and other connective tissues. It has a fibrillar structure with a fiber diameter ranging from 50 to 500 nm. Collagen provides a substrate for cellular recognition and promotes cell attachment, proliferation, and differentiated function. Cellu- lar recognition is an advantage for promoting cell adhesion, migration, and proliferation. However, sometimes advantages can be disadvantages. For example, cellular recognition can cause immunogenicity, which is not desirable and a common problem with natural poly- mers. Other problems with natural polymers are inconsistencies in their mechanical prop- erties, their degradability, and their reproducibility between samples. To mimic the size and morphology of natural extracellular matrix for engineering tissues, such as fibrous colla- gen matrix, Ma et al. have developed a three-dimensional nanofibrous scaffold using a phase-separation technique from biodegradable synthetic polymers, which avoids the con- cerns of pathogen transmission and immunorejection associated with collagen from animal and cadaver sources. To further improve the mass transport and new tissue

organization, they have built three-dimensional macroporous architectures into the nanofibrous matrices by incorporating water-soluble sugar fibers with special arrangement. Briefly, polymer was dis- solved in a solvent to form a homogenous solution. Polymer solutions are cast over the sugar fibers assemblies in a mold and are then thermally phase-separated to form nanofibrous matrices. The sugar fibers are leached out with water to finally form the synthetic nano-fibrous scaffold with predesigned macroporous architectures. The overall macroporous architectural design provides channels for improved mass transport and neovascularization

and allows uniform seeding of cells throughout the whole 3D scaffold. The nanofibrous matrices provide a large area for cell attachment, growth, and differentiation.

*Electrospinning*

Electrospinning is another technique for nanofibrous scaffold fabrication. Polymers are dis- solved into a proper solvent or melt before being subjected to a voltage to overcome the surface tension and viscoelastic forces and form different size fibers (50 nm to 30 $\mu$m in diameter), which features a morphologic similarity to the extracellular matrix (ECM) of nat- ural tissue, high porosity, and effective mechanical properties. Such a structure meets the essential design criteria of an ideal engineered scaffold; therefore, the sizes of electro- spun fibers represents an attractive size range for tissue engineering, wound healing, and related applications. Electrospun nanofibers have been shown to support cell attachment and proliferation of smooth muscle cells and fibroblasts . Cells seeded on this structure tend to maintain phenotypic shape and guided growth according to nanofiber orientation. Matt hews et al. have demonstrated that it is possible to tailor mechanical properties of electrospun nanofiber substrates by controlling fiber orientation.

Adjusting process parameters and polymer solution characteristics can vary fiber sizes and properties. Many polymers are adaptable for an electrospinning process, such as polyethylene oxide, poly(ethylene-*co*-vinyl alcohol), DNA, polyaramids, polycaprolactone, PLA, PGA, polyaniline, and polypeptides. Electrospinning can be used to prepare bioac- tive nanofibers, which may be used for carrying active biomolecules. For example, enzymes can be immobilized via material engineering and improved stability and activity of the enzymes were observed [26]. The electrospun structure, composed of fibers ranging from 50 to 1000 nm in diameter, features a morphologic similarity to the extracellular matrix (ECM) of natural tissue. A wide range of pore diameter distribution, high porosity, and effective mechanical properties could be obtained through the well-developed electrospinning tech- nique. Adjustable structural and mechanical properties meet the essential design criteria of scaffolds

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*Self-Assembly*

Self-assembly, or self-organization, refers to the reversible and cooperative assembly of pre- defined components into an ordered superstructure. Self-assembly is a key process for life, for example, nucleic acid synthesis, protein synthesis, and energy transduction are associ- ated to self-assembly processes. From a tissue engineering standpoint, self-assembly could be used to engineer different types of surface topography on the nano- and microscale to influence cell adhesion, migration, function, and tissue integration, to establish a controlled local microenvironment (protein and other macromolecular patterning) via surface function- lization of biomaterials to generate micro- and nanoscale mechanical stresses affecting cell– biomaterials interactions, and to position cells precisely on scaffold surface to control cell interactions. However, these structures are not very stable, due to noncovalent interactions, such as hydrogen bonds, ionic bonds, hydrophobic interactions, and van der Waals interac- tions, among the assembled molecules. Self-assembly can be used to produce a variety of structures, such as films, bilayer, membranes, nanoparticles, fibers, micelles, capsule, tubes, coils, mesophases, or unilamellar and multilamellar vesicles. These structures can fur- ther self-organize into the superstructures, such as lamellar, hexagonal, and cubic structures, which are especially attractive for fabrication of zeolite-like nanoporous materials that are superb for scaffold uses. Peptides and proteins are mostly used for fabricating scaffolds by the self-assembly approach, owing to their versatile structures for building suprastructures. For example, natural scaffolds in human tissue are superstructures self-assembled from protein and peptide blocks; these superstructures include collagen, elastin, keratin, etc. The possible applications include the preparation of nanoparticles, the exploitation of biomineralization in bone tissue engineering, the design of nanomotors, the development of functionalized delivery vectors, and templating of nanostructures on scaffold surfaces.

***a.*** *Templating* Templating is an interesting phenomena during the normal hard tissue development, organic phase, in which collagen fibers work as a template to guide inor- ganic phase formation in bone and teeth. Bones and teeth are biocomposites that require controlled mineral deposition during their

self-assembly to form tissues with unique mechanical properties. He et al. used dentin matrix protein 1 (DMP1), an acidic protein, to nucleate the formation of hydroxyapatite *in vitro* in a multistep process. The nucleated amorphous calcium phosphate precipitates ripen and nanocrystals form . In summary, the templating process involves a number of distinct steps. the first step is design, synthesis, and modification of the self-assembling molecules. The second step is examination of the self-assembly behaviors and characterization of the assem- bled microstructure. If desired microstructures are obtained, the structures will be used as a template for ruggedization. The templated structures will be characterized and ready for application.

***b.*** *Ionic Self-Complementary Peptide* The peptides can form stable *β*-strand and *β*-sheet conformations, with side chains with one polar side and one nonpolar side, and then undergo self-assembly to form nanofibers. These nanofibers can form interwoven mats that form three-dimensional hydrogels, with high water contents (>99.5%), which may by suitable for tissue engineering. The other feature of this peptide is that if the charge orientation is changed, entirely different molecules can be obtained.

***c.*** *Bionanotubes/Lipid Tubules* Using this concept, lipid tubules can be coated using sol–gels to fabricate ceramic rods or hollow cylinders with diameters down to 0.5 $\mu$m, coated with silanes to change the chemical nature of the tubule's surface or coated with metals . Modified tubules as microvials can be used for controlled release applications as well. The use of small hollow cylinders may offer several advantages over a sphere. Because the length and diameter are fixed and not a function of osmotic pressure, they will not change as the contents are released, and these parameters can be easily optimized for the desired release profile.

***d.*** *Nanometer-Thick Coating* Using self-assembly, chemical, physical, and biological properties (e.g., surface texture, functional groups, integrins, and adhesive motifs) of a scaf- fold surface can be modified to promote specific cell adhesion, differentiation, migration, and orientation .

***e.*** *Switchable Surface* As reported recently in *Chemical Engineering News* , the properties of a surface can be controlled by changing the conformation of molecules formed by self-assembly. The molecules in the mono- layer have

large globular end groups with a free carboxylate end group on each molecule. When an electrical potential is applied to the underlying gold substrate, the carboxylate is attracted to the surface, bending the alkane chains and exposing them to the surroundings. Such surfaces have potential as drug delivery controllers.

***f.*** *Three-Dimensional Scaffold* In order to engineer functional tissues and organs, three-dimensional scaffolds are very necessary. The major difficulty with the self-assembly approach is to fabricate a three-dimensional scaffold with reproducible gross and microstruc- ture and satisfied mechanical properties. Most three-dimensional scaffolds formed by self- assembly are the hydrogel type. By exposing self-assembling peptides to a salt solution or a physiological media, small amounts of macroscopic structures, mostly membranes, may be obtained for studying the cell behavior on self-assembled peptides. Macroscopic structures are formed with self-assembled nanofibers with diameters ~10 nm and pores ~5–200 nm. To improve mechanical properties of the scaffolds, artificial amphiphilic protein scaffolds with over 200 amino acids are synthesized. These scaffolds can resist high temperatures, over 90 $^{\circ}$C, as well . One major direction in this area is fabricating scaffolds with controllable/reproducible macroscopic and microscopic structures and mechanical properties.

*Molecular Biomimetics*

One promising approach to fabricate tissue engineering scaffolds is to mimic the ability of specific cells to synthesize and organize hierarchical materials with very fine features or res- olution at the nanometer level. However, based on the knowledge we have currently on natural molecule synthesis and tissue remodeling/organization, it is very difficult to achieve this objective. A hybrid approach called molecular biomimetics can be used to synthesize scaffolds with required mechanical properties and functional characteristics for engineering specific tissues from synthetic materials. Highly functionalized scaffolds with bioactive prop- erties or some natural polymers, such as proteins, polysaccharides, and lips, can be obtained. Molecular biomimetics uses microstructures and functional domains of organismal design principles to synthesize new materials

or structures. Nanotechnology offers great opportu- nities to combine physical and biological knowledge together to generate hybrid materials with potentials of assembling scaffolds at the molecular level. One good example is using recognition properties of proteins to control the organization and specific functions of scaf- folding materials. To ensure precise control over the molecular structure and organization, inorganic surface-specific polypeptides/proteins can be designed through genetics. Inorganic- binding peptides/proteins may be designed using a theoretical molecular approach; however, it is currently impractical because it is extremely expensive and time consuming. One interesting approach is using extracted biomineralizing proteins from hard tissue to mimic the binding between minerals and proteins in normal tissue. There are limitations with this approach as well. One is that there are multiple proteins involved in inorganic and pro- tein binding; the other is that tissue extracted proteins are very specific as to the types of inorganics the bind and are of limited practical use . Due to lack of knowledge on natural protein folding prediction and surface-bonding chemistry, customized peptides and proteins, obtained from massive libraries of synthetic polypeptides, would be a preferred route to find a specific sequence for binding specific inorganics. Surface-specific proteins are used as linkers to bind synthetic nanostructures, such as functional polymers, onto the templates. This type of structure can be used to specifically promote a certain wanted cell type to attach and proliferation on the scaffold surface but ward off the unwanted cell types. One typical example is for neuronal regeneration, to engineer the guidance bridge surface with matrix and cell surface adhesion molecules/ligands that are able to activate signaling pathways within the growth cone and have selectivity to allow the attachment of axons and growth cones yet ward off other cell types of inhibitory nature (Fig. A). This also called immobilization. Inorganic-binding proteins could potentially be used as linkers to bind two inorganic particles together as well (Fig. B).

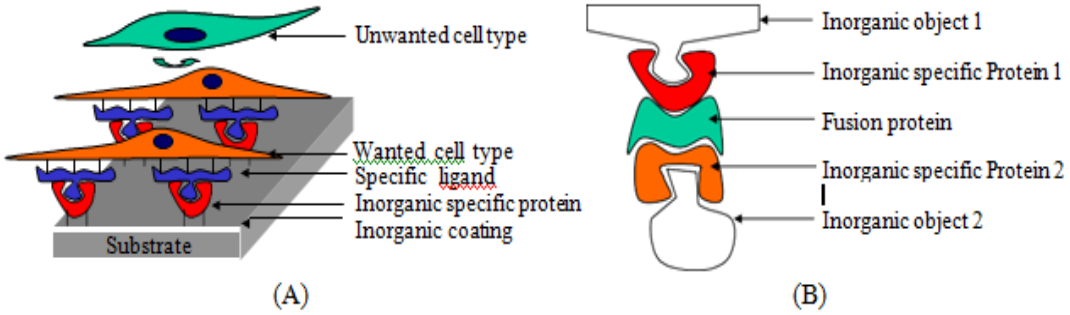



Figure. (A) Specific ligands could be immobilized on inorganic substrate to selectively promote wanted cell type attach and ward off unwanted cell types. (B) Inorganic-binding proteins could potentially be used as linker to bind two inorganic particles together.

*Patterning*

***a.*** *Microfabrication* Cells not only can respond to microscale topography but also may respond to nanoscale topography. Highly reproducible nanotopography with several nano- meters resolution can be obtained by advanced techniques such as electron beam lithography (EBL). Using such a method, very uniform surface textures can be obtained, although the fabrication process is very costly and time consuming, especially if large areas are needed. Such surfaces can be used as very important scientific tools to systematically study the cell respond to different size topography from several nanometers to several hundred nanometers. The behaviors of different cell types on different nanoscale textures provide fundamental information about sensitivity of cells to topography. Microfabrication technologies are a sophisticated way to obtain reproducible delicate structures. Depending on the applications, nanostructure can be fabricated either through bulk micromachining (build- ing texture out of bulk materials) or surface micromachining (building the structure on a surface). Photolithography, etching, and deposition are mostly utilized. Etching is one popular way to generate an organized texture on a scaffold surface. Etching can be wet or dry, depending on the chemical used. If a liquid chemical is used, it is called wet etching. If a gas-phase chemistry is applied, it is called dry etching. For a thin film, sil- icon, and glass substrates, both wet and dry etching will work very well. Plastics are usually sputter etched. Some materials, such as photoresist and parylene C can be etched in oxygen plasma.

Using nanofabrication methods, our group is developing aligned channels on scaffolds for tissue engineering application to mimic the bands of bungner structure in peripheral nerves and honeycomb architecture fashioned by bees. Scaffolds with highly aligned channels may provide excellent guidance, which is especially important for nervous tissue regeneration, for the formation of three-dimensional architecture. Wen and Zhang are collaborating on the design and fabrication of honeycomb thin sheets for the regeneration of retina as well. Paralleled multichannels, schematically shown in Figure 3A, are fabricated from biodegradable polymers with average channel size around 5–10 $\mu$m; the rod photoreceptor cells (95%) and cone photoreceptor cells (5%) cells are seeded into the "honeycomb" channels (Fig. 3B). By use of a similar approach, bipolar cells are seeded onto the honeycomb sheet to form an aligned monolayer; retina ganglion cells are seeded onto the sheet to form the third layer. By stacking three layers together, a tissue-engineered retina structure is expected: retina ganglion cells form the outmost layer, photoreceptor cells constitute the innermost layer, and the biopolar cell layer is sandwiched in the middle.

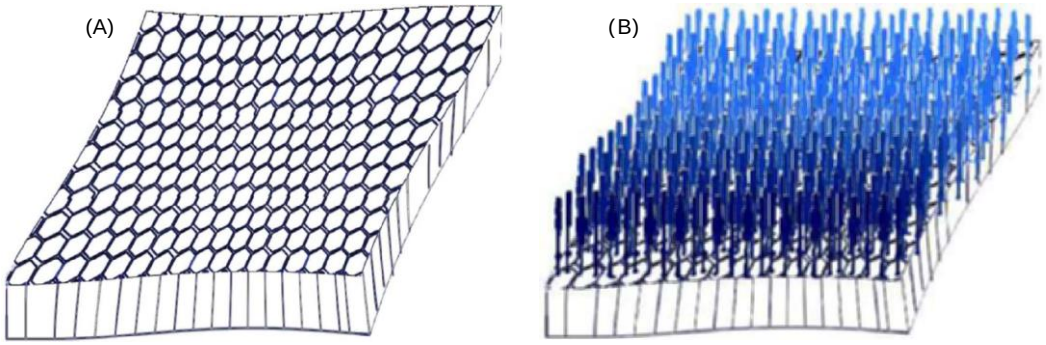



Figure 3. (A) Hone-cone like scaffold fabricated with nanotechnology; (B) photoreceptor cells are seeded into the channels to form aligned monolayer sheet for tissue-engineered retina.

Scanning probe microscopes (SPM), atomic force microscopy, and scanning tunneling microscopy are useful tools for the construction of nanosystems with 3D precision at up to 0.01-nm resolution. However, these tools require manual manipulations, which are time- consuming and barely reproducible. Nanoplanning system is an automating 2D assembly tool for fabrication of nanoobjects. One advantage is that it possesses the possibility of using artificial intelligence to control molecular level manufacturing .

***b.*** *Soft Lithography* (*Nonphotolithographic Techniques*) Deposition and patterning biomolecules and cells are an important application. There are three types of methods to accomplish this job. One is ink-jet printing, or may be called "protein printing", "cell printing", "tissue printing", or even "organ printing". However, the patterns generated by ink-printing lack resolution. A second method is based on protein adsorption. Since only physical adsorption of a protein from a solution to a substrate occurs, patterns created lack the resolution in handling living cells and biomolecules. A third type of patterning using stamps and channels to transfer patterns on polymers has been developed, including microcontact print, microfluid channels, and laminar fluid patterning. Microcontact print, which is excellent for patterning cells and fragile molecules, relies on an elastomeric stamp which is cast from a textured surface made by photolithography, tooling, or molding. The stamps can be used to print cell and biomolecular favorable zones on scaffold surfaces. The shapes and degrees of spreading of cells can be controlled: for example, well- spread cells can be placed next to rounded cells, and square cells can be placed next to circular cells . The advantages of microcontact print include that it utilizes cell and biomolecule friendly conditions (e.g., no exposure to harsh chemical or physical treatments, printing occurs under ambient conditions, and postprinting steps occur in aqueous solu- tions that do not denature proteins) and it is an operation friendly process (e.g., allows patterning large areas, is easy to operate, and has low cost)

***c.*** *Polymer Demixing* In order to produce large areas of nanotopography at low cost and high efficiency, Muller-Buschbaum et al. developed a polymer demixing

method to produce organized topographic features such as pits, islands, or ribbons of varying height or depth. Briefly, polystyrene and poly(4-bromostyrene) blends spontaneously undergo phase separation during spin casting onto silicon wafers. The scale of the textures can be adjusted by controlling polymer ratio and polymer concentration. By varying the ratio of the polymers, topography shape can be controlled; by varying the concentration of the polymer, topo- graphic features can be changed. They studied endothelial cells and fibroblasts response to this type of topography and found that 13-nm-high islands increase cell spreading and proliferation when compared with 95-nm-high islands for both cell types. Cytoskeletal conformation, filopodia formation, lamellapodia formation, proliferative gene expression, G-protein receptors, G-protein regulators, and other G-protein related genes are influenced by nano- scaletopography.

***d.*** *Selective Cell Attachment* In order to achieve desired cell adhesion but repel unwanted attachment, scaffold surfaces have been made nonadherent with hyaluronan (HA), poly(ethylene oxide) (Pluronics) , PEG, Glycocalyx, antibacterial with silver or N-alkylated poly(vinylpyridine) coatings, and bioadhesive with RGD peptide insertion, growth factor attachment, other bioactive groups decoration, plasma etching, or other chem- ical modifications . With designed patterns, the spatial organization of the cells in two and three dimensions may be obtained. Cell morphology and cellular activities can be managed by patterning as well. By creating specific patterns of surface chemistry and/or texture, cell behaviors can be confined with physical or chemical ultrastructures, which can be used to control cellular activity.

*Composite Materials*

Many natural tissues possess a composite microstructure, such as bone and teeth. These complex composite structures play critical roles for the physical and biological properties of the tissue. To mimic the natural tissue structure, biomedical polymers, bioceramics, and other inorganic materials are to be combined for superior properties. Polymer–inorganic nanoparticle composites can be used as scaffolds for tissue-engineered bone. When com- pared with conventional polymer composites, nanoparticle ftlled polymers have many superior properties,

such as less weight, better mechanical properties, improved durability, and bioactive interface, which is a key factor to a successful and long-term use of prostheses. Nanostructured polylactic/glycolic acid (PLGA) and titania composites, cre- ated by chemically treating micrometer-structured PLGA with NaOH and mixing with nanometer grain size titania, were investigated for bone engineering using osteoblasts (bone-forming cell) and chondrocytes (cartilage-synthesizing cell). Increased osteoblast and chondrocyte adhesion were observed on polymer surfaces of nanometer scale roughness when compared with that of micrometer scale roughness.

*Nanogels*

Nanogels refer to hydrogels in nanoscale; micrometer scale hydrogels are called "micro- gels", in order to tell the difference. Nanogels are formed by collapse of swollen hydrophilic polymer gels. Drugs can then be attached to the nanogels through weak bonds (such as, elec- trostatic interactions, hydrogen bond, and hydrophobic interactions) and cleavable covalent bonds between the drug moiety and the gel surface .

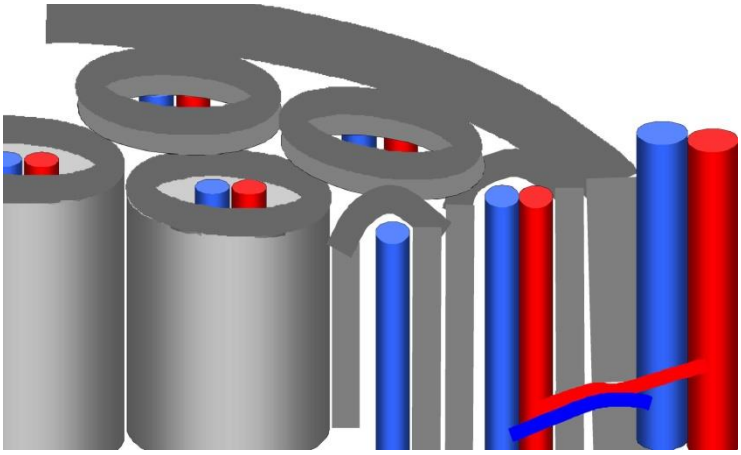


Figure 4. Different diameter nanoHA-degradable polyurethane hollow fiber membranes are used to construct a macro-structure highly mimic the natural bone structure with pores to promote vascular formation throughout the whole graft.

The chemical composition, size, polydispersity, stability, and swelling behavior of

the nanogels were investigated by NMR, light scattering, TEM, and AFM. The cell viability, uptake, and physical stability of nanogel–DNA complexes were evaluated under physiological conditions. Cationic nanogels formed monodisperse com- plexes with oligonucleotides and showed enhanced oligonucleotide uptake in cell culture. Vinogradov et al. developed nanosized cationic nanogels of cross-linked poly(ethylene oxide) (PEO) and polyethyleneimine (PEI). Nanogel materials could be formed by the interaction of anionic amphiphilic molecules or oligonucleotides with PEO–PEI nanogels to form distinct hydrophobic polyion-complex regions and hydrophilic PEO chains. This sys- tem allows for immobilization of negatively charged biologically active compounds such as retinoic acid, indomethacin, and oligonucleotides (bound to polycation chains) or hydrophobic molecules (incorporated into nonpolar regions of polyion–surfactant complexes) on the nanogels. To allow for the targeted delivery of nanogels in the body, the nanogel surface can be modified with various biospecific ligands. Various coupling strategies can be employed for this purpose, including covalent attachment of the ligand moiety to the free amino groups of PEI segments.

*DNA Nanotechnology*

The use of DNA nanotechnology can be used for tissue engineering for two purposes, one is to construct a scaffold from specific geometrical and topological DNA structure, for exam- ple, the use of DNA structure to assemble a tissue engineering scaffold using synthetic or natural biomaterials; the other is to drive nanomechanical devices through DNA transi- tions . Nanomechanical devices may be used for drug delivery, growth factor release, and cellular engineering. Although most studies related to DNA nanotechnology are for electronics applications, such as memory-device construction, there are possibilities of using this technology for tissue engineering scaffold development. Many of the DNA nano- technologies are based on branched DNA motifs, which permit guidance of the formation of long lines, circles, knots, catenanes, networks, or close mimicking natural ECM struc- tures through nanotechnology. The advantages of using DNA nanotechnology for tissue engineering scaffold fabrication include readily programmed and reliably predicted DNA structures, availability of arbitrary sequences because of convenient solid support synthesis, stable structures, and easy

manipulation and modification of DNA structures. DNA nanotechnology has been explored for a long time; however, its application is mainly at the stage of ideas and relatively far from reality. Tissue engineering applications may bring DNA technology into reality sooner.

*Angiogenesis*

One vital requirement for tissue engineered grafts to survive is the sufficient supply of oxy- gen and nutrients and removal of carbon dioxide and waste. Both the supply and removal process involve the transport of substances in the tissue possibly in all forms, gas, liquid, and solid. Transport in tissue engineering has two main issues: to design tissue with a well- perfused transport network and to create tissues that would have the function of transport, such as blood vessels. To properly perfuse large organs, such as kidney and liver, diffusion and convection alone cannot meet the requirements. A well-established vascular network is essential. However, techniques to grow a blood vessel network throughout the tissue have not been developed. Using nanofabrication, different size scale textures and structures can be obtained from a few nanometers to tens of micrometers. These structures are good sub- strates for learning the endothelial cell behaviors on different structures. Moldovan and Ferrari developed a silicon-based "angiogenesis assistant device", which is a nanofilter-based, drug delivery silicon capsules. The nanofilter with controlled size ranging from 10 to 200 nm for angiogenic growth fac- tor delivery is jointed with a millimeter-scale silicon frame with an endothelial cell coating for blood compatibility and vascularization. The nanofilter is created with micromachin- ing and sacrificial layer techniques. This device would possess several advantages in both research and clinical applications, such as a controllable delivery if there is a combined sensor and remote control device in the design, a supply of endothelial cells for better blood compatibility, a delivery of angiogenic factors for improved vascularization, and ease of implantation.

*Nanosensor*

To fully mimick organ functions, for example, secretagogue-triggered hormone release, minia- turization of sensors to nanodimensions may decrease the typical time constant down to the milliseconds time scale , which is very close to the trigger

system in a normal human body. With huge advantages in ultrasmall size, nanosensors can be easily embedded into the scaffolds, even the cell membrane, to establish smart/responsible implantable tissues grafts.

*Sweeping Vehicle*

Some researchers have proposed that nanorobots can be used as sweeping vehicles in the human body, such as teeth cleaning robots that collect harmful bacteria from the mouth, blood vessel cleaning robots that remove extra fat from the circulation system, and lung cleaning cleaning robots that can be used in the lungs to clean dust, which mimic the natural macrophages in alveoli. However, natural macrophages are unable to metabolize foreign particles like fibers of asbestos and reverse the toxic effects of smoking in the lungs. With robots, these particles may be completely cleaned out of the lung. A similar idea may be used in biomaterials and tissue engineering. For example, ultratiny robots may be used to remove biomaterial debris, which may come from degradation or wear at joint surfaces, from the transplanted grafts or implanted artificial implants for better integration and function.

# UNIT IV

# BASIC BIOLOGY OF STEM CELLS

**I. What are stem cells, and why are they important?**

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. In addition, in many tissues they serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell.

Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions.

Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. The functions and characteristics of these cells will be explained in this document. Scientists discovered ways to derive embryonic stem cells from early mouse embryos more than 30 years ago, in 1981. The detailed study of the biology of mouse stem cells led to the discovery, in 1998, of a method to derive stem cells from human embryos and grow the cells in the laboratory. These cells are called human embryonic stem cells. The embryos used in these studies were created for reproductive purposes through in vitro fertilization procedures. When they were no longer needed for that purpose, they were donated for research with the informed consent of the donor. In 2006, researchers made another breakthrough by identifying conditions that would allow some specialized adult cells to be "reprogrammed" genetically to assume a stem cell-like state. This new type of stem cell, calledinduced pluripotent stem cells (iPSCs), will be discussed in a later section of this document.

Stem cells are important for living organisms for many reasons. In the 3- to 5-day-old embryo, called a blastocyst, the inner cells give rise to the entire body of the organism, including all of the many specialized cell types and organs such as the heart, lungs, skin, sperm, eggs and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

Given their unique regenerative abilities, stem cells offer new potentials for treating diseases such as diabetes, and heart disease. However, much work remains to be done in the laboratory and the clinic to understand how to use these cells for cell-based therapies to treat disease, which is also referred to as regenerative or reparative medicine.

Laboratory studies of stem cells enable scientists to learn about the cells' essential properties and what makes them different from specialized cell types. Scientists are already using stem cells in the laboratory to screen new drugs and to develop model systems to study normal growth and identify the causes of birth defects.

Research on stem cells continues to advance knowledge about how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. Stem cell research is one of the most fascinating areas of contemporary biology, but, as with many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

**II. What are the unique properties of all stem cells?**

Stem cells differ from other kinds of cells in the body. All stem cells—regardless of their source—have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types.

Stem cells are capable of dividing and renewing themselves for long periods. Unlike muscle cells, blood cells, or nerve cells—which do not normally replicate themselves—stem cells may replicate many times, or proliferate. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

Scientists are trying to understand two fundamental properties of stem cells that relate to their long-term self-renewal:

1. Why can embryonic stem cells proliferate for a year or more in the laboratory without differentiating, but most adult stem cells cannot; and

2. What are the factors in living organisms that normally regulate stem cellproliferation and self-renewal?

Discovering the answers to these questions may make it possible to understand how cell proliferation is regulated during normal embryonic development or during the abnormal cell division that leads to cancer. Such information would also enable scientists to grow embryonic and non-embryonic stem cells more efficiently in the laboratory.

The specific factors and conditions that allow stem cells to remain unspecialized are of great interest to scientists. It has taken scientists many years of trial and error to learn to derive and maintain stem cells in the laboratory without them spontaneously differentiating into specific cell types. For example, it took two decades to learn how to grow human embryonic stem cells in the laboratory following the development of conditions for growing mouse stem cells. Likewise, scientists must first understand the signals that enable a non-embryonic (adult) stem cell population to proliferate and remain unspecialized before they will be able to grow large numbers of unspecialized adult stem cells in the laboratory.

Stem cells are unspecialized. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. For example, a stem cell cannot work with its neighbors to pump blood through the body (like a heart muscle cell), and it cannot carry oxygen molecules through the bloodstream (like a red blood cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

Stem cells can give rise to specialized cells. When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are just beginning to understand the signals inside and outside cells that trigger each step of the differentiation process. The internal signals are controlled by a cell's genes, which are interspersed across long strands of DNA and carry coded

instructions for all cellular structures and functions. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that restrict DNA expression in the cell and can be passed on through cell division.

Many questions about stem cell differentiation remain. For example, are the internal and external signals for cell differentiation similar for all kinds of stem cells? Can specific sets of signals be identified that promote differentiation into specific cell types? Addressing these questions may lead scientists to find new ways to control stem cell differentiation in the laboratory, thereby growing cells or tissues that can be used for specific purposes such as cell-based therapies or drug screening.

Adult stem cells typically generate the cell types of the tissue in which they reside. For example, a blood-forming adult stem cell in the bone marrow normally gives rise to the many types of blood cells. It is generally accepted that a blood-forming cell in the bone marrow—which is called a hematopoietic stem cell—cannot give rise to the cells of a very different tissue, such as nerve cells in the brain. Experiments over the last several years have purported to show that stem cells from one tissue may give rise to cell types of a completely different tissue. This remains an area of great debate within the research community. This controversy demonstrates the challenges of studying adult stem cells and suggests that additional research using adult stem cells is necessary to understand their full potential as future therapies.

**III. What are embryonic stem cells?**

**A. What stages of early embryonic development are important for generating embryonic stem cells?**

Embryonic stem cells, as their name suggests, are derived from embryos. Most embryonic stem cells are derived from embryos that develop from eggs that have been fertilized in vitro—in an in vitro fertilization clinic—and then donated for research purposes with informed consent of the donors. They are not derived from eggs fertilized in a woman's body.

**B. How are embryonic stem cells grown in the laboratory?**

Growing cells in the laboratory is known as cell culture. Human embryonic stem cells (hESCs) are generated by transferring cells from a preimplantation-stage embryo into a plastic laboratory culture dish that contains a nutrient broth known asculture medium. The cells divide and spread over the surface of the dish. In the original protocol, the inner surface of the culture dish was coated with mouse embryonic skin cells specially treated so they will not divide. This coating layer of cells is called a feeder layer. The mouse cells in the bottom of the culture dish provide the cells a sticky surface to which they can attach. Also, the feeder cells release nutrients into the culture medium. Researchers have now devised ways to grow embryonic stem cells without mouse feeder cells. This is a significant scientific advance because of the risk that viruses or other macromolecules in the mouse cells may be transmitted to the human cells.

The process of generating an embryonic stem cell line is somewhat inefficient, so lines are not produced each time cells from the preimplantation-stage embryo are placed into a culture dish. However, if the plated cells survive, divide and multiply enough to crowd the dish, they are removed gently and plated into several fresh culture dishes. The process of re-plating or subculturing the cells is repeated many times and for many months. Each cycle of subculturing the cells is referred to as

apassage. Once the cell line is established, the original cells yield millions of embryonic stem cells. Embryonic stem cells that have proliferated in cell culture for six or more months without differentiating, are pluripotent, and appear genetically normal are referred to as an embryonic stem cell line. At any stage in the process, batches of cells can be frozen and shipped to other laboratories for further culture and experimentation.

**C. What laboratory tests are used to identify embryonic stem cells?**

At various points during the process of generating embryonic stem cell lines, scientists test the cells to see whether they exhibit the fundamental properties that make them embryonic stem cells. This process is called characterization.

Scientists who study human embryonic stem cells have not yet agreed on a standard battery of tests that measure the cells' fundamental properties. However, laboratories that grow human embryonic stem cell lines use several kinds of tests, including:

- Growing and subculturing the stem cells for many months. This ensures that the cells are capable of long-term growth and self-renewal. Scientists inspect the cultures through a microscope to see that the cells look healthy and remainundifferentiated.

- Using specific techniques to determine the presence of transcription factors that are typically produced by undifferentiated cells. Two of the most important transcription factors are Nanog and Oct4. Transcription factors help turn geneson and off at the right time, which is an important part of the processes of celldifferentiation and embryonic development. In this case, both Oct 4 and Nanog are associated with maintaining the stem cells in an undifferentiated state, capable of self-renewal.

- Using specific techniques to determine the presence of particular cell surface markers that are typically produced by undifferentiated cells.

- Examining the chromosomes under a microscope. This is a method to assess whether the chromosomes are damaged or if the number of chromosomes has changed. It does not detect genetic mutations in the cells.

- Determining whether the cells can be re-grown, or subcultured, after freezing, thawing, and re-plating.

- Testing whether the human embryonic stem cells are pluripotent by 1) allowing the cells to differentiate spontaneously in cell culture; 2) manipulating the cells so they will differentiate to form cells characteristic of the three germ layers; or 3) injecting the cells into a mouse with a suppressed immune system to test for the formation of a benign tumor called a teratoma. Since the mouse's immune system is suppressed, the injected human stem cells are not rejected by the mouse immune system and scientists can observe growth and differentiation of the human stem cells. Teratomas typically contain a mixture of many differentiated or partly differentiated cell types—an indication that the embryonic stem cells are capable of differentiating into multiple cell types.

**D. How are embryonic stem cells stimulated to differentiate?**

As long as the embryonic stem cells in culture are grown under appropriate conditions, they can remain undifferentiated (unspecialized). But if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve cells, and many other cell

types. Although spontaneous differentiation is a good indication that a culture of embryonic stem cells is healthy, the process is uncontrolled and therefore an inefficient strategy to produce cultures of specific cell types.

So, to generate cultures of specific types of differentiated cells—heart muscle cells, blood cells, or nerve cells, for example—scientists try to control the differentiation of embryonic stem cells. They change the chemical composition of the culture medium, alter the surface of the culture dish, or modify the cells by inserting specific genes. Through years of experimentation, scientists have established some basic protocols or "recipes" for the directed differentiation of embryonic stem cells into some specific cell types (Figure 1).

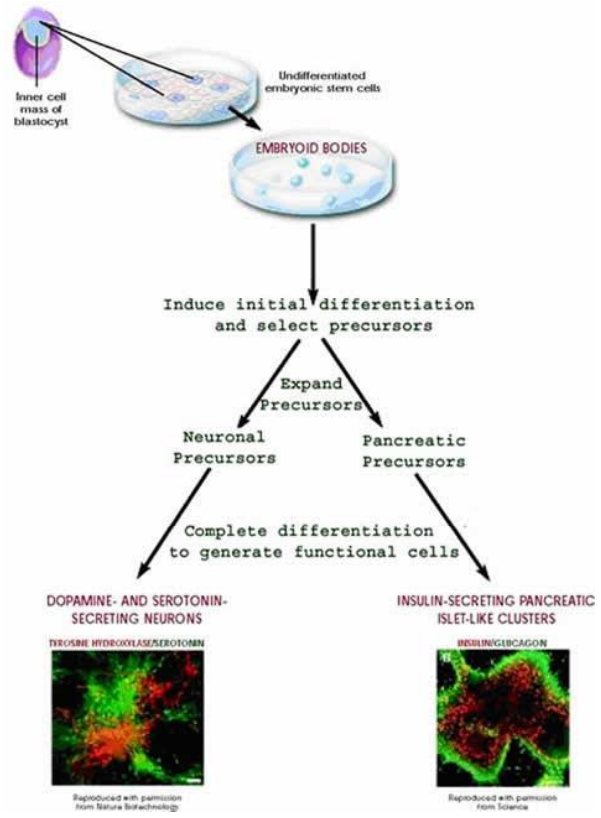



Figure 1. Directed differentiation of mouse embryonic stem cells.

**IV. What are adult stem cells?**

An adult stem cell is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ. The adult stem cell can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Scientists also use the term somatic stem cell instead of adult stem cell, where somatic refers to cells of the body (not the germ cells, sperm or eggs). Unlike embryonic stem cells, which are defined by their origin (cells from the preimplantation-stage embryo), the origin of adult stem cells in some mature tissues is still under investigation.

Research on adult stem cells has generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led researchers and clinicians to ask whether adult stem cells could be used for transplants. In fact, adult hematopoietic, or blood-forming, stem cells from bone marrow have been used in transplants for more than 40 years. Scientists now have evidence that stem cells exist in the brain and the heart, two locations where adult

stem cells were not at first expected to reside. If the differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies.

The history of research on adult stem cells began more than 60 years ago. In the 1950s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal stem cells (also called mesenchymal stem cells, or skeletal stem cells by some), were discovered a few years later. These non-hematopoietic stem cells make up a small proportion of thestromal cell population in the bone marrow and can generate bone, cartilage, and fat cells that support the formation of blood and fibrous connective tissue.

In the 1960s, scientists who were studying rats discovered two regions of the brain that contained dividing cells that ultimately become nerve cells. Despite these reports, most scientists believed that the adult brain could not generate new nerve cells. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types—astrocytes andoligodendrocytes, which are non-neuronal cells, and neurons, or nerve cells.

**A. Where are adult stem cells found, and what do they normally do?**

Adult stem cells have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. They are thought to reside in a specific area of each tissue (called a "stem cell niche"). In many tissues, current evidence suggests that some types of stem cells are pericytes, cells that compose the outermost layer of small blood vessels. Stem cells may remain quiescent (non-dividing) for long periods of time until they are activated by a normal need for more cells to maintain tissues, or by disease or tissue injury.

Typically, there is a very small number of stem cells in each tissue and, once removed from the body, their capacity to divide is limited, making generation of large quantities of stem cells difficult. Scientists in many laboratories are trying to find better ways to grow large quantities of adult stem cells in cell culture and to manipulate them to generate specific cell types so they can be used to treat injury or disease. Some examples of potential treatments include regenerating bone using cells derived from bone marrow stroma, developing insulin-producing cells for type 1 diabetes, and repairing damaged heart muscle following a heart attack with cardiac muscle cells.

**B. What tests are used to identify adult stem cells?**

Scientists often use one or more of the following methods to identify adult stem cells: (1) label the cells in a living tissue with molecular markers and then determine the specialized cell types they generate; (2) remove the cells from a living animal, label them in cell culture, and transplant them back into another animal to determine whether the cells replace (or "repopulate") their tissue of origin.

Importantly, scientists must demonstrate that a single adult stem cell can generate a line of genetically identical cells that then gives rise to all the appropriate differentiated cell types of the tissue. To confirm experimentally that a putative adult stem cell is indeed a stem cell, scientists tend to show either that the cell can give rise to these genetically identical cells in culture, and/or that a purified population of these candidate stem cells can repopulate or reform the tissue after transplant into an animal.

**C. What is known about adult stem cell differentiation?**

As indicated above, scientists have reported that adult stem cells occur in many tissues and that they enter normal differentiation pathways to form the specialized cell types of the tissue in which they reside.

Normal differentiation pathways of adult stem cells. In a living animal, adult stem cells are available to divide for a long period, when needed, and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue. The following are examples of differentiation pathways of adult stem cells (Figure 2) that have been demonstrated in vitro or in vivo.

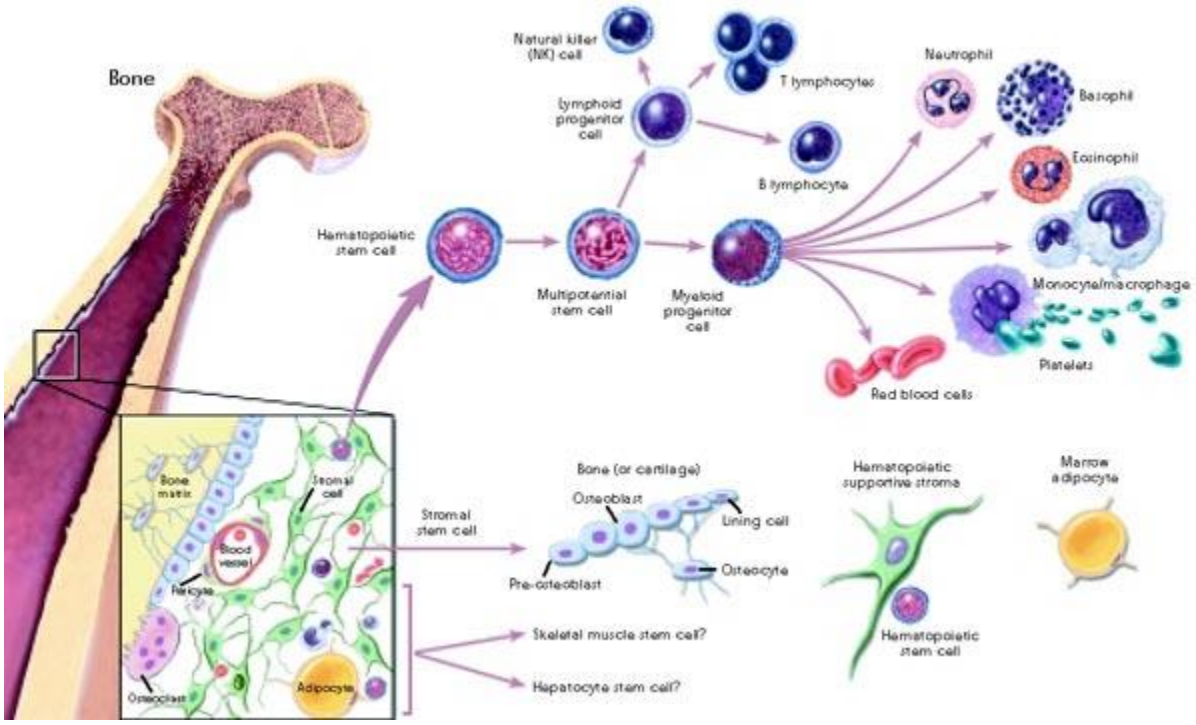



Figure 2. Hematopoietic and stromal stem cell differentiation.

- Hematopoietic stem cells give rise to all the types of blood cells: red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, and macrophages.

- Mesenchymal stem cells have been reported to be present in many tissues. Those from bone marrow (bone marrow stromal stem cells, skeletal stem cells) give rise to a variety of cell types: bone cells (osteoblasts and osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and stromal cells that support blood formation. However, it is not yet clear how similar or dissimilar mesenchymal cells derived from non-bone marrow sources are to those from bone marrow stroma.

- Neural stem cells in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of non-neuronal cells—astrocytes and oligodendrocytes.

- Epithelial stem cells in the lining of the digestive tract occur in deep crypts and give rise to several cell types: absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells.

- Skin stem cells occur in the basal layer of the epidermis and at the base of hair follicles. The epidermal stem cells give rise to keratinocytes, which migrate to the surface of the skin and form a protective layer. The follicular stem cells can give rise to both the hair follicle and to the epidermis.

Transdifferentiation. A number of experiments have reported that certain adult stem cell types can differentiate into cell types seen in organs or tissues other than those expected from the cells' predicted lineage (i.e., brain stem cells that differentiate into blood cells or blood-forming cells that differentiate into cardiac muscle cells, and so forth). This reported phenomenon is called transdifferentiation.

Although isolated instances of transdifferentiation have been observed in some vertebrate species, whether this phenomenon actually occurs in humans is under debate by the scientific community. Instead of transdifferentiation, the observed instances may involve fusion of a donor cell with a recipient cell. Another possibility is that transplanted stem cells are secreting factors that encourage the recipient's own stem cells to begin the repair process. Even when transdifferentiation has been detected, only a very small percentage of cells undergo the process.

In a variation of transdifferentiation experiments, scientists have recently demonstrated that certain adult cell types can be "reprogrammed" into other cell types in vivo using a well-controlled process of genetic modification (see Section VI for a discussion of the principles of reprogramming). This strategy may offer a way to reprogram available cells into other cell types that have been lost or damaged due to disease. For example, one recent experiment shows how pancreatic beta cells, the insulin-producing cells that are lost or damaged in diabetes, could possibly be created by reprogramming other pancreatic cells. By "re-starting" expression of three critical beta cell genes in differentiated adult pancreatic exocrine cells, researchers were able to create beta cell-like cells that can secrete insulin. The reprogrammed cells were similar to beta cells in appearance, size, and shape; expressed genes characteristic of beta cells; and were able to partially restore blood sugar regulation in mice whose own beta cells had been chemically destroyed. While not transdifferentiation by definition, this method for reprogramming adult cells may be used as a model for directly reprogramming other adult cell types.

In addition to reprogramming cells to become a specific cell type, it is now possible to reprogram adult somatic cells to become like embryonic stem cells (induced pluripotent stem cells, iPSCs) through the introduction of embryonic genes. Thus, a source of cells can be generated that are specific to the donor, thereby increasing the chance of compatibility if such cells were to be used for tissue regeneration. However, like embryonic stem cells, determination of the methods by which iPSCs can be completely and reproducibly committed to appropriate cell lineages is still under investigation.

D. What are the key questions about adult stem cells?

Many important questions about adult stem cells remain to be answered. They include:

- How many kinds of adult stem cells exist, and in which tissues do they exist?

- How do adult stem cells evolve during development and how are they maintained in the adult? Are they "leftover" embryonic stem cells, or do they arise in some other way?

- Why do stem cells remain in an undifferentiated state when all the cells around them have differentiated? What are the characteristics of their “niche” that controls their behavior?

- Do adult stem cells have the capacity to transdifferentiate, and is it possible to control this process to improve its reliability and efficiency?

- If the beneficial effect of adult stem cell transplantation is a trophic effect, what are the mechanisms? Is donor cell-recipient cell contact required, secretion of factors by the donor cell, or both?

- What are the factors that control adult stem cell proliferation and differentiation?

- What are the factors that stimulate stem cells to relocate to sites of injury or damage, and how can this process be enhanced for better healing?

**V. What are the similarities and differences between embryonic and adult stem cells?**

Human embryonic and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. One major difference between adult and embryonic stem cells is their different abilities in the number and type of differentiated cell types they can become. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin.

Embryonic stem cells can be grown relatively easily in culture. Adult stem cells are rare in mature tissues, so isolating these cells from an adult tissue is challenging, and methods to expand their numbers in cell culture have not yet been worked out. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies.

Scientists believe that tissues derived from embryonic and adult stem cells may differ in the likelihood of being rejected after transplantation. We don't yet know for certain whether tissues derived from embryonic stem cells would cause transplant rejection, since relatively few clinical trials have tested the safety of transplanted cells derived from hESCS.

Adult stem cells, and tissues derived from them, are currently believed less likely to initiate rejection after transplantation. This is because a patient's own cells could be expanded in culture, coaxed into assuming a specific cell type (differentiation), and then reintroduced into the patient. The use of adult stem cells and tissues derived from the patient's own adult stem cells would mean that the cells are less likely to be rejected by the immune system. This represents a significant advantage, as immune rejection can be circumvented only by continuous administration of immunosuppressive drugs, and the drugs themselves may cause deleterious side effects.

**VI. What are induced pluripotent stem cells?**

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell–like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells. Although these cells meet the defining criteria for pluripotent stem cells, it is not known if iPSCs and embryonic stem cells differ in clinically significant ways. Mouse iPSCs were first reported in 2006, and human iPSCs were first reported in late 2007. Mouse iPSCs demonstrate important characteristics of pluripotent stem cells, including expressing stem cell markers, forming tumors containing cells from all three germ layers, and being able to contribute to many different tissues when injected into mouse embryos at a very early stage in development. Human iPSCs also express stem cell markers and are capable of generating cells characteristic of all three germ layers.

Although additional research is needed, iPSCs are already useful tools for drug development and modeling of diseases, and scientists hope to use them in transplantation medicine. Viruses are currently

used to introduce the reprogramming factors into adult cells, and this process must be carefully controlled and tested before the technique can lead to useful treatment for humans. In animal studies, the virus used to introduce the stem cell factors sometimes causes cancers. Researchers are currently investigating non-viral delivery strategies. In any case, this breakthrough discovery has created a powerful new way to "de-differentiate" cells whose developmental fates had been previously assumed to be determined. In addition, tissues derived from iPSCs will be a nearly identical match to the cell donor and thus probably avoid rejection by the immune system. The iPSC strategy creates pluripotent stem cells that, together with studies of other types of pluripotent stem cells, will help researchers learn how to reprogram cells to repair damaged tissues in the human body.

**VII. What are the potential uses of human stem cells and the obstacles that must be overcome before these potential uses will be realized?**

There are many ways in which human stem cells can be used in research and the clinic. Studies of human embryonic stem cells will yield information about the complex events that occur during human development. A primary goal of this work is to identify how undifferentiated stem cells become the differentiated cells that form the tissues and organs. Scientists know that turning genes on and off is central to this process. Some of the most serious medical conditions, such as cancer and birth defects, are due to abnormal cell division and differentiation. A more complete understanding of the genetic and molecular controls of these processes may yield information about how such diseases arise and suggest new strategies for therapy. Predictably controlling cell proliferation and differentiation requires additional basic research on the molecular and genetic signals that regulate cell division and specialization. While recent developments with iPS cells suggest some of the specific factors that may be involved, techniques must be devised to introduce these factors safely into the cells and control the processes that are induced by these factors.

Human stem cells are currently being used to test new drugs. New medications are tested for safety on differentiated cells generated from human pluripotent cell lines. Other kinds of cell lines have a long history of being used in this way. Cancer cell lines, for example, are used to screen potential anti-tumor drugs. The availability of pluripotent stem cells would allow drug testing in a wider range of cell types. However, to screen drugs effectively, the conditions must be identical when comparing different drugs. Therefore, scientists must be able to precisely control the differentiation of stem cells into the specific cell type on which drugs will be tested. For some cell types and tissues, current knowledge of the signals controlling differentiation falls short of being able to mimic these conditions precisely to generate pure populations of differentiated cells for each drug being tested.

Perhaps the most important potential application of human stem cells is the generation of cells and tissues that could be used for cell-based therapies. Today, donated organs and tissues are often used to replace ailing or destroyed tissue, but the need for transplantable tissues and organs far outweighs the available supply. Stem cells, directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat diseases including macular degeneration, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis.

## Hemapoetic stem cells

Blood cells are responsible for constant maintenance and immune protection of every cell type of the body. This relentless and brutal work requires that blood cells, along with skin cells, have the greatest powers of self-renewal of any adult tissue.

The stem cells that form blood and immune cells are known as hematopoietic stem cells (HSCs). They are ultimately responsible for the constant renewal of blood—the production of billions of new blood cells each day. Physicians and basic researchers have known and capitalized on this fact for more than 50 years in treating many diseases. The first evidence and definition of blood-forming stem cells came from studies of people exposed to lethal doses of radiation in 1945.

Basic research soon followed. After duplicating radiation sickness in mice, scientists found they could rescue the mice from death with bone marrow transplants from healthy donor animals. In the early 1960s, Till and McCulloch began analyzing the bone marrow to find out which components were responsible for regenerating blood . They defined what remain the two hallmarks of an HSC: it can renew itself and it can produce cells that give rise to all the different types of blood cells

**What Is a Hematopoietic Stem Cell?**

A hematopoietic stem cell is a cell isolated from the blood or bone marrow that can renew itself, can differentiate to a variety of specialized cells, can mobilize out of the bone marrow into circulating blood, and can undergo programmed cell death, called apoptosis—a process by which cells that are detrimental or unneeded self-destruct.

A major thrust of basic HSC research since the 1960s has been identifying and characterizing these stem cells. Because HSCs look and behave in culture like ordinary white blood cells, this has been a difficult challenge and this makes them difficult to identify by morphology (size and shape). Even today, scientists must rely on cell surface proteins, which serve, only roughly, as markers of white blood cells.

Identifying and characterizing properties of HSCs began with studies in mice, which laid the groundwork for human studies. The challenge is formidable as about 1 in every 10,000 to 15,000 bone marrow cells is thought to be a stem cell. In the blood stream the proportion falls to 1 in 100,000 blood cells. To this end, scientists began to develop tests for proving the self-renewal and the plasticity of HSCs.

The "gold standard" for proving that a cell derived from mouse bone marrow is indeed an HSC is still based on the same proof described above and used in mice many years ago. That is, the cells are injected into a mouse that has received a dose of irradiation sufficient to kill its own blood-producing cells. If the mouse recovers and all types of blood cells reappear (bearing a genetic marker from the donor animal), the transplanted cells are deemed to have included stem cells.

These studies have revealed that there appear to be two kinds of HSCs. If bone marrow cells from the transplanted mouse can, in turn, be transplanted to another lethally irradiated mouse and restore its hematopoietic system over some months, they are considered to be long-term stem cells that are capable of self-renewal. Other cells from bone marrow can immediately regenerate all the different types of blood cells, but under normal circumstances cannot renew themselves over the long term, and these are referred to as short-term progenitor or precursor cells. Progenitor or precursor cells are relatively immature cells that are precursors to a fully differentiated cell of the same tissue type. They are capable of proliferating, but they have a limited capacity to differentiate into more than one cell type as HSCs do. For example, a blood progenitor cell may only be able to make a red blood cell (see Figure 1. Hematopoietic and Stromal Stem CellDifferentiation).

Harrison et al. write that short-term blood-progenitor cells in a mouse may restore hematopoiesis for three to four months. The longevity of short-term stem cells for humans is not firmly established. A true stem cell, capable of self-renewal, must be able to renew itself for the entire lifespan of an organism. It is these long-term replicating HSCs that are most important for developing HSC-based cell therapies. Unfortunately, to date, researchers cannot distinguish the long-term from the short-term cells when they are removed from the bloodstream or bone marrow.

The central problem of the assays used to identify long-term stem cells and short-term progenitor cells is that they are difficult, expensive, and time-consuming and cannot be done in humans. A few assays are now available that test cells in culture for their ability to form primitive and long-lasting colonies of cells, but these tests are not accepted as proof that a cell is a long-term stem cell. Some genetically altered mice can receive transplanted human HSCs to test the cells' self-renewal and hematopoietic capabilities during the life of a mouse, but the relevance of this test for the cells in humans—who may live for decades—is open to question.

The difficulty of HSC assays has contributed to two mutually confounding research problems: definitively identifying the HSC and getting it to proliferate, or increase its numbers, in a culture dish. More rapid research progress on characterizing and using HSCs would be possible if they could be readily grown in the laboratory. Conversely, progress in identifying growth conditions suitable for HSCs and getting the cells to multiply would move more quickly if scientists could reliably and readily identify true HSCs.

**Can Cell Markers Be Used to Identify Hematopoietic Stem Cells?**

HSCs have an identity problem. First, the ones with long-term replicating ability are rare. Second, there are multiple types of stem cells. And, third, the stem cells look like many other blood or bone marrow cells. So how do researchers find the desired cell populations? The most common approach is through markers that appear on the surface of cells.

Listed here are cell surface markers found on mouse and human hematopoietic stem cells as they exist in their undifferentiated state *in vivo* and *in vitro.* As these cells begin to develop as distinct cell lineages the cell surface markers are no longer identified.

| Mouse | Human |
|---|---|
| $CD34^{low/-}$ | $CD\ 34^{+}$ |
| $SCA\text{-}1^{+}$ | $CD59^{+}$* |
| $Thy1^{+/low}$ | $Thy1^{+}$ |
| $CD38^{+}$ | $CD38^{low/-}$ |
| $C\text{-}kit^{+}$ | $C\text{-}kit^{-/low}$ |
| $lin^{-}$* | $lin^{-}$** |

** Only one of a family of CD59 markers has thus far been evaluated.*
*** $Lin^{-}$ cells lack 13 to 14 different mature blood-lineage markers.*

**Table 5.1. Proposed cell-surface markers of undifferentiated hematopoietic stem cells.**

Table 1. Proposed cell-surface markers of undifferentiated hematopoietic stem cells.

Such cell markers can be tagged with monoclonal antibodies bearing a fluorescent label and culled out of bone marrow with fluorescence-activated cell sorting (FACS).

The groups of cells thus sorted by surface markers are heterogeneous and include some cells that are true, long-term self-renewing stem cells, some shorter-term progenitors, and some non-stem cells. Weissman's group showed that as few as five genetically tagged cells, injected along with larger doses of stem cells into lethally irradiated mice, could establish themselves and produce marked donor cells

in all blood cell lineages for the lifetime of the mouse. A single tagged cell could produce all lineages for as many as seven weeks, and 30 purified cells were sufficient to rescue mice and fully repopulate the bone marrow without extra doses of backup cells to rescue the mice  Despite these efforts, researchers remain divided on the most consistently expressed set of HSC markers. Connie Eaves of the University of British Columbia says none of the markers are tied to unique stem cell functions or truly define the stem cell. "Almost every marker I am aware of has been shown to be fickle," she says.

More recently, Diane Krause and her colleagues at Yale University, New York University, and Johns Hopkins University, used a new technique to home in on a single cell capable of reconstituting all blood cell lineages of an irradiated mouse. After marking bone marrow cells from donor male mice with a nontoxic dye, they injected the cells into female recipient mice that had been given a lethal dose of radiation. Over the next two days, some of the injected cells migrated, or homed, to the bone marrow of the recipients and did not divide; when transplanted into a second set of irradiated female mice, they eventually proved to be a concentrated pool of self-renewing stem cells. The cells also reconstituted blood production. The scientists estimate that their technique concentrated the long-term stem cells 500 to 1,000- fold compared with bone marrow.

**What Are the Sources of Hematopoietic Stem Cells?**

**Bone Marrow**

The classic source of hematopoietic stem cells (HSCs) is bone marrow. For more than 40 years, doctors performed bone marrow transplants by anesthetizing the stem cell donor, puncturing a bone—typically a hipbone—and drawing out the bone marrow cells with a syringe. About 1 in every 100,000 cells in the marrow is a long-term, blood-forming stem cell; other cells present include stromal cells, stromal stem cells, blood progenitor cells, and mature and maturing white and red blood cells.

Peripheral Blood

As a source of HSCs for medical treatments, bone marrow retrieval directly from bone is quickly fading into history. For clinical transplantation of human HSCs, doctors now prefer to harvest donor cells from peripheral, circulating blood. It has been known for decades that a small number of stem and progenitor cells circulate in the bloodstream, but in the past 10 years, researchers have found that they can coax the cells to migrate from marrow to blood in greater numbers by injecting the donor with a cytokine, such as granulocyte-colony stimulating factor (GCSF). The donor is injected with GCSF a few days before the cell harvest. To collect the cells, doctors insert an intravenous tube into the donor's vein and pass his blood through a filtering system that pulls out CD34+ white blood cells and returns the red blood cells to the donor. Of the cells collected, just 5 to 20 percent will be true HSCs. Thus, when medical researchers commonly refer to peripherally harvested "stem cells," this is something of a misnomer. As is true for bone marrow, the CD34+ cells are a mixture of stem cells, progenitors, and white blood cells of various degrees of maturity.

In the past three years, the majority of autologous (where the donor and recipient are the same person) and allogeneic (where the donor and recipient are different individuals) "bone marrow" transplants have actually been white blood cells drawn from peripheral circulation, not bone marrow.

**Umbilical Cord Blood**

In the late 1980s and early 1990s, physicians began to recognize that blood from the human umbilical cord and placenta was a rich source of HSCs. This tissue supports the developing fetus during pregnancy, is delivered along with the baby, and, is usually discarded. Since the first successful umbilical cord blood transplants in children with Fanconi anemia, the collection and therapeutic use of these cells has grown quickly. The New York Blood Center's Placental Blood Program, supported by NIH, is the largest U.S. public umbilical cord blood bank and now has 13,000 donations available for transplantation into small patients who need HSCs. Since it began collecting umbilical cord blood in 1992, the center has provided thousands of cord blood units to patients. Umbilical cord blood recipients—typically children—have now lived in excess of eight years, relying on the HSCs from an umbilical cord blood transplant.

There is a substantial amount of research being conducted on umbilical cord blood to search for ways to expand the number of HSCs and compare and contrast the biological properties of cord blood with adult bone marrow stem cells. There have been suggestions that umbilical cord blood contains stem cells that have the capability of developing cells of multiple germ layers (multipotent) or even all germ layers, e.g., endoderm, ectoderm, and mesoderm (pluripotent). To date, there is no published scientific evidence to support this claim. While umbilical cord blood represents a valuable resource for HSCs, research data have not conclusively shown qualitative differences in the differentiated cells produced between this source of HSCs and peripheral blood and bone marrow.

**Fetal Hematopoietic System**

An important source of HSCs in research, but not in clinical use, is the developing blood-producing tissues of fetal animals. Hematopoietic cells appear early in the development of all vertebrates. Most extensively studied in the mouse, HSC production sweeps through the developing embryo and fetus in waves. Beginning at about day 7 in the life of the mouse embryo, the earliest hematopoietic activity is indicated by the appearance of blood islands in the yolk sac. The point is disputed, but some scientists contend that yolk sac blood production is transient and will generate some blood cells for the embryo, but probably not the bulk of the HSCs for the adult animal. According to this proposed scenario, most stem cells that will be found in the adult bone marrow and circulation are derived from cells that appear slightly later and in a different location. This other wave of hematopoietic stem cell production occurs in the AGM—the region where the aorta, gonads, and fetal kidney (mesonephros) begin to develop. The cells that give rise to the HSCs in the AGM may also give rise to endothelial cells that line blood vessels. These HSCs arise at around days 10 to 11 in the mouse embryo (weeks 4 to 6 in human gestation), divide, and within a couple of days, migrate to the liver. The HSCs in the liver continue to divide and migrate, spreading to the spleen, thymus, and—near the time of birth—to the bone marrow.

**Embryonic Stem Cells and Embryonic Germ Cells**

In 1985, it was shown that it is possible to obtain precursors to many different blood cells from mouse embryonic stem cells. Perkins was able to obtain all the major lineages of progenitor cells from mouse embryoid bodies, even without adding hematopoietic growth factors.

Mouse embryonic stem cells in culture, given the right growth factors, can generate most, if not all, the different blood cell types , but no one has yet achieved the "gold standard" of proof that they can produce long-term HSCs from these sources—namely by obtaining cells that can be transplanted into lethally irradiated mice to reconstitute long-term hematopoiesis .

**Self-renewal of Hematopoietic Stem Cells**

Scientists have had a tough time trying to grow—or even maintain—true stem cells in culture. This is an important goal because cultures of HSCs that could maintain their characteristic properties of self-renewal and lack of differentiation could provide an unlimited source of cells for therapeutic transplantation and study. When bone marrow or blood cells are observed in culture, one often observes large increases in the number of cells. This usually reflects an increase in differentiation of cells to progenitor cells that can give rise to different lineages of blood cells but cannot renew themselves. True stem cells divide and replace themselves slowly in adult bone marrow.

New tools for gene-expression analysis will now allow scientists to study developmental changes in telomerase activity and telomeres. Telomeres are regions of DNA found at the end of chromosomes that are extended by the enzyme telomerase. Telomeraseactivity is necessary for cells to proliferate and activity decreases with age leading to shortened telomeres. Scientists hypothesize that declines in stem cell renewal will be associated with declines in telomere length and telomerase activity. Telomerase activity in hematopoietic cells is associated with self-renewal potential.

Because self-renewal divisions are rare, hard to induce in culture, and difficult to prove, scientists do not have a definitive answer to the burning question: what puts—or perhaps keeps—HSCs in a self-renewal division mode? HSCs injected into an anemic patient or mouse—or one whose HSCs have otherwise been suppressed or killed—will home to the bone marrow and undergo active division to both replenish all the different types of blood cells and yield additional self-renewing HSCs. But exactly how this happens remains a mystery that scientists are struggling to solve by manipulating cultures of HSCs in the laboratory.

Two recent examples of progress in the culturing studies of mouse HSCs are by Ema and coworkers and Audet and colleagues [2, 15]. Ema et al. found that two cytokines—stem cell factor and thrombo-poietin—efficiently induced an unequal first cell division in which one daughter cell gave rise to repopulating cells with self-renewal potential. Audet et al. found that activation of the signaling molecule gp130 is critical to survival and proliferation of mouse HSCs in culture.

Work with specific cytokines and signaling molecules builds on several earlier studies demonstrating modest increases in the numbers of stem cells that could be induced briefly in culture. For example, Van Zant and colleagues used continuous-perfusion culture and bioreactors in an attempt to boost human HSC numbers in single cord blood samples incubated for one to two weeks [58]. They obtained a 20-fold increase in "long-term culture initiating cells."

**Differentiation of HSCs into Components of the Blood and Immune System**

Producing differentiated white and red blood cells is the real work of HSCs and progenitor cells. M.C. MacKey calculates that in the course of producing a mature, circulating blood cell, the original hematopoietic stem cell will undergo between 17 and 19.5 divisions, "giving a net amplification of between ~170,000 and ~720,000" .

Through a series of careful studies of cultured cells—often cells with mutations found in leukemia patients or cells that have been genetically altered—investigators have discovered many key growth factors and cytokines that induce progenitor cells to make different types of blood cells. These factors interact with one another in complex ways to create a system of exquisite genetic control and coordination of blood cell production.

**Migration of Hematopoietic Stem Cells Into and Out of Marrow and Tissues**

Scientists know that much of the time, HSCs live in intimate connection with the stroma of bone marrow in adults. But HSCs may also be found in the spleen, in peripheral blood circulation, and other tissues. Connection to the interstices of bone marrow is important to both the engraftment of transplanted cells and to the maintenance of stem cells as a self-renewing population. Connection to stroma is also important to the orderly proliferation, differentiation, and maturation of blood cells.

**Apoptosis and Regulation of Hematopoietic Stem Cell Populations**

The number of blood cells in the bone marrow and blood is regulated by genetic and molecular mechanisms. How do hematopoietic stem cells know when to stop proliferating? Apoptosis is the process of programmed cell death that leads cells to self-destruct when they are unneeded or detrimental. If there are too few HSCs in the body, more cells divide and boost the numbers. If excess stem cells were injected into an animal, they simply wouldn't divide or would undergo apoptosis and be eliminated. Excess numbers of stem cells in an HSC transplant actually seem to improve the likelihood and speed of engraftment, though there seems to be no rigorous identification of a mechanism for this empirical observation.

The particular signals that trigger apoptosis in HSCs are as yet unknown. One possible signal for apoptosis might be the absence of life-sustaining signals from bone marrow stroma. Michael Wang and others found that when they used antibodies to disrupt the adhesion of HSCs to the stroma via VLA-4/VCAM-1, the cells were predisposed to apoptosis.

**What Are the Clinical Uses of Hematopoietic Stem Cells?**

**Leukemia and Lymphoma**

Among the first clinical uses of HSCs were the treatment of cancers of the blood—leukemia and lymphoma, which result from the uncontrolled proliferation of white blood cells. In these applications, the patient's own cancerous hematopoietic cells were destroyed via radiation or chemotherapy, then replaced with a bone marrow transplant, or, as is done now, with a transplant of HSCs collected from the peripheral circulation of a matched donor. A matched donor is typically a sister or brother of the patient who has inherited similar human leukocyte antigens (HLAs) on the surface of their cells. Cancers of the blood include acute lymphoblastic leukemia, acute myeloblastic leukemia, chronic myelogenous leukemia (CML), Hodgkin's disease, multiple myeloma, and non-Hodgkin's lymphoma.

**Inherited Blood Disorders**

Another use of allogeneic bone marrow transplants is in the treatment of hereditary blood disorders, such as different types of inherited anemia (failure to produce blood cells), and inborn errors of metabolism (genetic disorders characterized by defects in key enzymes need to produce essential body components or degrade chemical byproducts). The blood disorders include aplastic anemia, beta-thalassemia, Blackfan-Diamond syndrome, globoid cell leukodystrophy, sickle-cell anemia, severe

combined immunodeficiency, X-linked lymphoproliferative syndrome, and Wiskott-Aldrich syndrome. Inborn errors of metabolism that are treated with bone marrow transplants include: Hunter's syndrome, Hurler's syndrome, Lesch Nyhan syndrome, and osteopetrosis. Because bone marrow transplantation has carried a significant risk of death, this is usually a treatment of last resort for otherwise fatal diseases.

**Hematopoietic Stem Cell Rescue in Cancer Chemotherapy**

Chemotherapy aimed at rapidly dividing cancer cells inevitably hits another target—rapidly dividing hematopoietic cells. Doctors may give cancer patients an autologous stem cell transplant to replace the cells destroyed by chemotherapy. They do this by mobilizing HSCs and collecting them from peripheral blood. The cells are stored while the patient undergoes intensive chemotherapy or radiotherapy to destroy the cancer cells. Once the drugs have washed out of a patient's body, the patient receives a transfusion of his or her stored HSCs. Because patients get their own cells back, there is no chance of immune mismatch or graft-versus-host disease. One problem with the use of autologous HSC transplants in cancer therapy has been that cancer cells are sometimes inadvertently collected and reinfused back into the patient along with the stem cells. One team of investigators finds that they can prevent reintroducing cancer cells by purifying the cells and preserving only the cells that are CD34+, Thy-1+.

**Graft-Versus-Tumor Treatment of Cancer**

One of the most exciting new uses of HSC transplantation puts the cells to work attacking otherwise untreatable tumors. A group of researchers in NIH's intramural research program recently described this approach to treating metastatic kidney cancer. Just under half of the 38 patients treated so far have had their tumors reduced. The research protocol is now expanding to treatment of other solid tumors that resist standard therapy, including cancer of the lung, prostate, ovary, colon, esophagus, liver, and pancreas.

**EMBRYONIC STEM CELLS**

Properties

- This is derived from the inner cell mass of blastocyst at age of the embryo
- Pluripotent
- Long term self renewal
- It exhibits stable, normal karyotype
- Capable of integrating fetal tissues during development; clonogenic
- Express transcription factor oct-4
- Lacks G1 checkpoint in cell cycle

Factors that affect the differentiation:

- Microenviroment
- Cell-cell interaction
- Growth factors like proteins and nutrients
- Extracellular matrix
- Activation and deactivation of genes

Surface markers that are present only in the undifferentiated cells like oct-4 determine the presence of the stem of cells.

Mainly two techniques called in vitro fertilization and somatic cell nuclear transfer are used to prepare embryonic stem cells. However, each technique has its own limitations. In in vitro fertilization, limited number of cell lines are available for federally funded research and risk of developing teratomas i.e tumours from the implantation of undifferentiated stem cells are the two main limitations of this

technique. Whereas somatic cell nuclear transfer has not yet achieved with human cells and here also there is potent chance for creating the teratomas.

**In vitro fertilization:** This technique involves the removal of cells from the microscopic inner cell mass of blastocyst and then cultures them to become a cell line.

**Somatic cell nuclear transfer:** This involves removal of nucleus from an egg cell and also from the adult somatic cell. Transfer the nucleus from the somatic cell to egg cell; provide the stimulation to the new cell to grow.

Major risk associated with the use of embryonic stem cells are tumour formation; immune rejection and also risk of infection.When these stem cells used for the person from whom it was removed will give 100% success rate. Depending upon the condition, there are 25% chances for perfect match for a sibling.

This is rich in haematopoietic stem cells and progenitor cells used to treat various nonmalignant and malignant haemoatopoietic diseases. The isolation of such stem cells is through the non-invasive collection, which does not harm the mother or infant. In addition, it is more immune as compared to other stem cells and these young cells possess long telomeres thereby having long self-renewing capacity.

**MESENCHYMAL STEM CELLS**

Mesenchymal stem cells are the adult stem cells derived from the bone marrow. This cell type has the capacity to differentiate into many lineages namely osteocyte for bone; chondrocyte for cartilage; myoblast for muscle; fibroblast for tendon or ligament; and other connective tissue cell types under specific culture conditions. In addition to this, these cells can be isolated from the patients since they avoid the immune complications. Generally, various dimensional scaffold structures with porous characteristics have been evaluated for the proliferation as well as the differentiation of the MSC. This is because porous characteristics are very essential for the vascularization, cell infiltration and also for the diffusion of nutrients to and waste from the scaffold.

Whatever the tissue may be to regenerate, design of proper ECM play a major role in the stem cells aided tissue engineering. This is because stem cells alone are not sufficient to construct the three-dimensional organization of tissues. In addition, stem cell delivery is mainly to replace the lost cells at that site of injury. However, delivering stem cells to the target tissue without any cell loss is really tough to achieve. Hence, targeted delivery of MSC is highly efficient to restore the function of lost tissues. This MSC delivery can be achieved using different carriers such as injectable gels, scaffold matrixes, films, or even immunoprotective microencapsulation. Though, the delivery is achieved by targeted carrier, the next important challenge is to provide the appropriate signal to achieve a desired differentiation. Number of polymeric candidates is widely explored for the tissue engineering applications such as natural materials like collagen, alginate, gelatin and synthetic polymers like polyethylene glycol, polycaprolactone, polyglycolic acid, polylactic acid, and even poly(lactide-co-glycolide).

Bone marrow derived mesenchymal stem cells has found to have different positive markers such as STRO-1, CD29, CD44, CD71, CD90, CD106 and so on, which are required to isolate the cells from the heterogeneous population. These isolated stem cells will then be expanded in vitro without affecting its differentiation potential. Under certain conditions, some exogenous growth factors are required to stimulate the osteogenic differentiation of BM-MSCs for bone regeneration. It was also found that the osteogenic induction with the growth promoting signals improved the formation of alkaline phophatase positive colonies with a cuboidal morphology and finally progressed to mineralized bone formation. Generally osteogenic induction medium comprises essential elements such

as β- glycerophosphate, ascorbic acid along with dexamethasone. Other exogenic factors such as recombinant bone morphogenic protein (rh-BMP) and parathyroid hormone related peptide will used for the osteogenic differentiation and proliferations. However, care should be taken always with the passage number of stem cells, which detemines the multilineage potential of the stem cells.

Though the mesenchymal stem cells alone are used as therapeutic agents for bone formation, there are a few reports proved the efficiency of 3-D scaffold seeded along with the BM-MSCs. For example, BM-MSCs were seeded within collagen sponge scaffold and also self-assembled peptide nanofiber scaffold has been used for the in vitro and in vivo osteogenic differentiation, showing significant improvement in the bone formation. There are various reports indicated the use of BM-MSCs-hydroxyapatite hybrid particles in bone formation. MSCs have also been used in the cartilage regeneration, which can promote the chondrogenesis in the presence of transforming growth factor-β. Poly(L-lactide-co- ε-caprolactone) (PLCL) has been modified with chitosan and evaluated the adhesion, phenotypic change as well as proliferation of human bone marrow derived MSCs. Chitosan modified PLCL scaffold has shown improved cell compatibility with the adequate mechanical strength towards chondrogenesis as compared to unmodified PLCL scaffold. Hence, many attempts have been made to evaluate the material property with proper modification on MSCs proliferation as well as differentiation.

The major elements present in the chondrogenic differentiation medium are TGF-β, insulin, dexamethasone along with BMP 6. The cell source that they have tested are human chondrocytes (HC), embryonic stem cells (ESC), embryonic stem cell derived mesenchymal stem cells (ESC-MSC) and also mesenchymal stem cells derived from bone marrow and adipose tissue (BM-MSC; ASC). This study has even made use of two different scaffolds namely chitosan and silk fibroin scaffolds with loaded BMP 6. The metabolic activity of the cells was found to be higher in the silk scaffolds as compared to chitosan scaffolds due to difference in pore size. This result confirms the role of scaffolds and its architecture on these cells metabolic activity. Among all cells, EMS-MSCs has shown better chondrogenic property in both the scaffolds and identified as a promising cell for chondrogenesis. This type of experiments has proven the role of cell source for regeneration.

**CANCER STEM CELLS**

Cancer stem cells (CSCs) are cancer cells (found within tumors or hematological cancers) that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. CSCs are therefore tumorigenic (tumor-forming), perhaps in contrast to other non-tumorigenic cancer cells. CSCs may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. Such cells are hypothesized to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. Therefore, development of specific therapies targeted at CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for patients with metastatic disease.

Existing cancer treatments have mostly been developed based on animal models, where therapies able to promote tumor shrinkage were deemed effective. However, animals do not provide a complete model of human disease. In particular, in mice, whose life spans do not exceed two years, tumor relapse is difficult to study.

The efficacy of cancer treatments is, in the initial stages of testing, often measured by the ablation fraction of tumor mass (fractional kill). As CSCs form a small proportion of the tumor, this may not necessarily select for drugs that act specifically on the stem cells. The theory suggests that conventional chemotherapies kill differentiated or differentiating cells, which form the bulk of the tumor but do not generate new cells. A population of CSCs, which gave rise to it, could remain untouched and cause relapse.

Cancer stem cells were first identified by John Dick in acute myeloid leukemia in the late 1990s. Since the early 2000s they have been an intense cancer research focus.

## INDUCED PLUORIPOTENT STEM CELLS

Induced pluripotent stem cell, or IPS cell, is a stem cell that has been created from an adult cell such as a skin, liver, stomach or other mature cell through the introduction of genes that reprogram the cell and transform it into a cell that has all the characteristics of an embryonic stem cell. The term pluripotent connotes the ability of a cell to give rise to multiple cell types, including all three embryonic lineages forming the body's or- gans, nervous system, skin, muscle and skeleton.

### Advantages of induced pluripotent stem cells

Bioethics: Induced stem cells have the obvious edge of not having to be derived from human embryos, a major ethical consideration. The ability to re- program an adult cell to behave like an embryonic stem cell may also enable scientists to sidestep other controversial methods, notably somatic cell nuclear transfer, also known as cloning, a technique that has additional ethical considerations and that is extremely difficult to do routinely, as unfertilized human eggs are required.

Genetically matched cell lines: Another critical advantage of induced pluripotent stem cell tech- nology is that, in theory, it allows for the creation of cell lines that are genetically customized to a patient. The issue of immune rejection, in which the body's immune system recognizes implanted cells or tissues as foreign and attacks them, is a barrier to the therapeutic application of cell-based therapies. If cells for therapy can be created using a patient's own cells, the issue can be potentially overcome.
Easier to create: Finally, the other key benefit of induced stem cells is that the technique can be performed in any moderately equipped molecular biology lab and does not require materials — human eggs or embryos — that can be more difficult to obtain.

How do we know induced pluripotent stem cells can match embryonic stem cells? So far, induced pluripotent stem cells appear to exhibit the same key features of embryonic stem cells: the ability to differentiate from a blank-slate state to any of the 220 types of cells in the human body, and the ability to reproduce indefinitely in culture. Because in- duced stem cells are relatively new, however, scientists must compare the cells to those obtained from embryos to assess their charac- teristics in detail and ensure that there are no significant differences.

Do induced pluripotent stem cells mean we no longer need embryonic stem cells? No. It remains to be seen whether repro- grammed cells differ in significant ways from embryonic stem cells. Comparative studies of embryonic and induced pluripotent stem cells are necessary to ensure that they are the same. It is essential for science to explore the full spectrum of research options to bring stem cell research to clinical fruition as soon as possible.

## HEPATIC STEMCELLS

The identification and elucidation of hepatic stem cell niche composition and function in the normal and regenerating liver are pivotal to developing therapeutic strategies for patients with impaired liver regeneration, which is usually seen in chronic liver diseases. Moreover, proper in vitro expansion of stem/progenitor cells without loss of their developmental potential, as well as establishment of cell differentiation protocols for the generation of functional hepatocytes, are important for therapeutic cell transplantation. Substantial progress has been made in recent years in all of these areas. However, additional fundamental research using rigorous test systems, such as those established in the hematopoietic stem cell field, is required to fully characterize liver stem cells and to understand the architecture of the stem cell niches in the liver. It is not clear yet whether the expansion of ducts by

proliferating hepatic progenitor cells during liver regeneration exactly mirrors the niche in the canals of Hering. Therefore, future studies should also investigate the stem cell niche in the normal liver as well. The selective inhibition of niche components by using knockout animals and specific inhibitors of receptors may provide new insights. Possible targets include niche elements such as cell-cell contacts (e.g., neuronal cadherin), signaling pathways (e.g., canonical/noncanonical WNT, Notch, TGF-β family, Hh), the CXCL12/CXCR4 axis, basement membrane proteins (e.g., laminin, collagen type IV), and integrins. Moreover, putative stem cells in the canals of Hering should be better defined by unique markers to enable their exact characterization and to clarify the presence of stem cells without hepatobiliary markers at this site. Homing of transplanted EpCAM+ hepatic stem cells in the canals of Hering has not yet been reported and would further support the concept of the stem cell niche. In contrast, homing of migrating hematopoietic stem cells can reoccur in the space of Disse of the adult liver under certain conditions. Additionally, transplanted stellate cells are capable of homing to the space of Disse (92). The first evidence is provided by in vitro experiments examining possible interactions of niche elements in the space of Disse, but these findings need to be confirmed in animal models as described above.

As general features of stem cell niches can be found in both the canals of Hering and the space of Disse, it is worth outlining the differences between these two sites. The canals of Hering appear to contain slow-cycling precursor cells in the normal liver, whereas the space of Disse normally contains quiescent stellate cells and hematopoietic stem/progenitor cells that only rarely divide. The direct exposure of hepatic stem cells to high concentrations of bile acids in the canals of Hering represents a prominent difference from the environment in the space of Disse. Bile acids act not only as emulgators of lipids but also as signaling molecules that can support liver regeneration. Another difference in the composition of these two niches is the presence of LSECs in the space of Disse and cholangiocytes in the canals of Hering, both of which can release CXCL12 to attract CXCR4-expressing cells. Additional differences and their consequences may become apparent in further analyses. Future research should also address how hepatic stem cell niches are affected by chronic diseases and aging. Are niches altered or lost, and are these changes responsible for impaired liver regeneration? Variation in the composition and abundance of basement membrane proteins can be observed in liver diseases, which may also adversely affect the maintenance of stem cell niches. An age-related decline in stem cell niche integrity is known and could lead to impaired self-renewal and inappropriate differentiation of stem cells in their niche. Indeed, further characterization of the liver stem cell niches as well as changes in liver diseases are challenges for future research that promise new insights into liver physiology.

## PRIMORDIAL GERM CELLS

Primordial germ cells (PGCs) are the precursors of sperm and eggs, which generate a new organism that is capable of creating endless new generations through germ cells. PGCs are specified during early mammalian postimplantation development, and are uniquely programmed for transmission of genetic and epigenetic information to subsequent generations.

Primordial germ cells are highly specialised cells that are precursors of gametes, which, following meiosis, develop as haploid sperm and eggs that generate a new organism upon fertilisation. They transmit genetic and epigenetic information between generations and ensure the survival of a species. Although germ cells are set aside during early development in almost all animals, the mechanism of germ cell specification is not conserved among animals. Typically, specification of germ cells occurs either through the inheritance of preformed germ plasm, or is induced among equipotent cells by instructive signals. For example, germ cell specification in Xenopus and C. elegans occurs via

the inheritance of germ plasm, whereas, in axolotls, germ cell specification occurs in animal cap cells in response to signals. In principle, both these mechanisms ensure suppression of somatic fate while promoting the onset of the germ cell programme. In mice, primordial germ cells (PGCs) originate from the early postimplantation epiblast cells, which also give rise to all somatic cells in response to signals from the extraembryonic tissues.

Mammalian sperm and eggs make an equal genetic contribution to a new organism, but their epigenetic contributions are unique and complementary. Both a male and a female genome are necessary for development to term because of the parent of origin-dependent 'imprinting' of the genome in the germ line. Investigations of mammalian germ cells provide a unique insight into how epigenetic information with respect to 'imprints' is first erased and then re-initiated, and becomes heritable from the germ line into adulthood. Imprinting results in the parent of origin-dependent monoallelic expression of imprinted genes during development. Faulty imprints can lead to developmental, physiological and behavioural anomalies in mice, and result in diseases in humans. The process of robust erasure and resetting of the epigenome in early PGCs also ensures that aberrant epigenetic information is not transmitted to the offspring. Nonetheless, there are recent reports suggesting that environmental factors can induce epigenetic changes that can be transmitted through the germline to subsequent generations with detrimental consequences. Studies on the germ cell lineage might test the validity of these claims or provide the mechanistic basis for them.

The resetting of the germline epigenome is also crucial for the establishment of the totipotent state following fertilisation. The underlying mechanisms involved may be applicable to the experimental reprogramming of the epigenome and to the manipulation of cell fates in vitro, and potentially to the reprogramming of the endogenous cells of diseased tissues. This has important implications for regenerative medicine and human diseases, including germ cell tumours. Germ cells also provide opportunities with which to uncover the mechanisms of chromatin modifications, as well as of DNA methylation/demethylation. How the factors involved induce significant epigenetic changes might be useful in other contexts during normal and aberrant development, and could lead to the development of novel therapeutic agents.

More directly, investigation of germ cells could lead to advances in reproductive medicine. For example, defective mitochondria transmitted by oocytes have major health implications; how they accumulate and are transmitted thereafter is of particular interest, as are the other causes of infertility. Advances in germ cell biology and genome editing, together with the ability to generate germ cells from pluripotent stem cells might lead to advances in assisted reproduction in some mammals.

# UNIT V

## GENE THERAPY: INTRODUCTION AND METHODS

### Introduction

Gene therapy is a novel treatment method which utilizes genes or short oligonucleotide sequences as therapeutic molecules, instead of conventional drug compounds. This technique is widely used to treat those defective genes which contribute to disease development. Gene therapy involves the introduction of one or more foreign genes into an organism to treat hereditary or acquired genetic defects. In gene therapy, DNA encoding a therapeutic protein is packaged within a "vector", which transports the DNA inside cells within the body. The disease is treated with minimal toxicity, by the expression of the inserted DNA by the cell machinery. In 1990 FDA for the first time approved a gene therapy experiment on ADA-SCID in the United States after the treatment of Ashanti DeSilva. After that, approximately 1700 clinical trials on patients have been performed with various techniques and genes for numerous diseases. Many diseases such as ADA-SCID, X-linked SCID, Leber's congenital amaurosis (a retinal disease), Parkinson's disease, multiple myeloma, chronic and acute lymphocytic leukemia, adrenoleukodystrophy have reported of successful clinical trials. But these are still not approved by FDA. Some other diseases on which gene therapy based research is going on are Haemophilia, Tyrosinemia, Hyperbilirubinemia (Criglar-NijjarSyndrom), Cystic Fibrosis and many other cancers. After 30 years of research and clinical trials, only one product called Glybera got approval in November 2012 which may be available in market in late 2013. It has the ability to cure lipoprotein lipase deficiency (LPLD) a very raredisease.

### Types of gene therapy

There are several approaches for correcting faulty genes; the most common being the insertion of a normal gene into a specific location within the genome to replace a non functional gene. Gene therapy is classified into the following two types:

1. Somatic genetherapy
2. Germ line genetherapy

#### Somatic Gene Therapy

In somatic gene therapy, the somatic cells of a patient are targeted for foreign gene transfer. In this case the effects caused by the foreign gene is restricted to the individual patient only, and not inherited by the patient's offspring or later generations.

#### Germ Line Gene Therapy

Here, the functional genes, which are to be integrated into the genomes, are inserted in the germ cells, i.e., sperm or eggs. Targeting of germ cells makes the therapy heritable.

***Gene Therapy Strategies***

**Gene Augmentation Therapy (GAT)**

In GAT, simple addition of functional alleles is used to treat inherited disorders caused by genetic deficiency of a gene product, e.g. GAT has been applied to autosomal recessive disorders. Dominantly inherited disorders are much less amenable to GAT. Figure 1 shows the GATstrategy

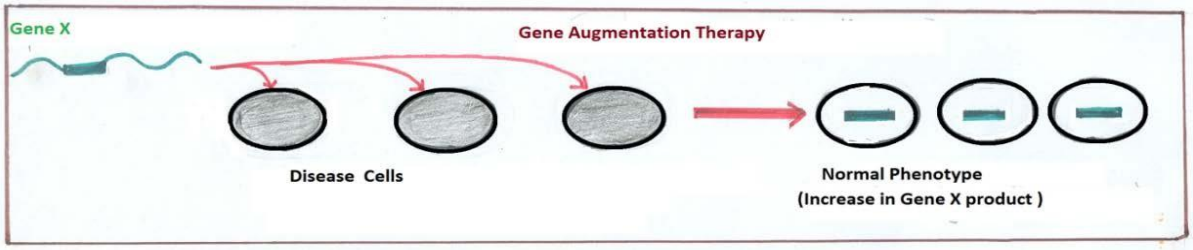



**Figure : A gene therapy vector has been designed to treat the diseased cells with a gene X. This vector was introduced inside the diseased cells by various gene transfer methods. After a successful homologous recombination the treated cells will show the presence of gene X product as well as normal phenotype.**

**Targeted Killing of Specific Cells**

It involves utilizing genes encoding toxic compounds (**suicide genes**), or **prodrugs**(reagents which confer sensitivity to subsequent treatment with a drug) to kill the transfected/ transformed cells. This general approach is popular in cancer gene therapies. This is shown in figure 2a &2b

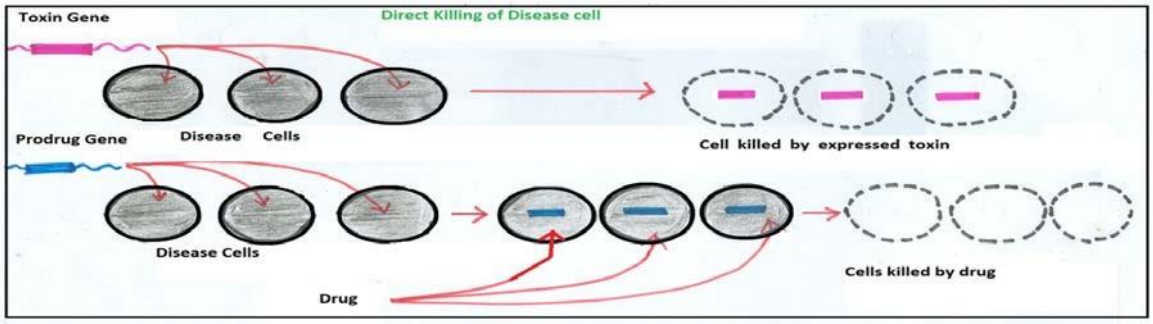



**Figure : a) Direct killing of diseased cells by two methods. The first method is the introduction of toxin gene into the diseased cell which when expresses toxin protein the cells die. The second method involves incorporation of a certain gene (e.g. TK) in the gene therapy vector which shows a suicidal property on introducing certain drug (e.g. ganciclovir).**

Thymidine kinase (TK) phosphorylates the introduced prodrugganciclovir which is further phosphorylated by endokinases to form ganciclovir triphosphate, an competitive inhibitor of deoxyguanosine triphosphate. Ganciclovir triphosphate causes chain termination when incorporated into DNA.

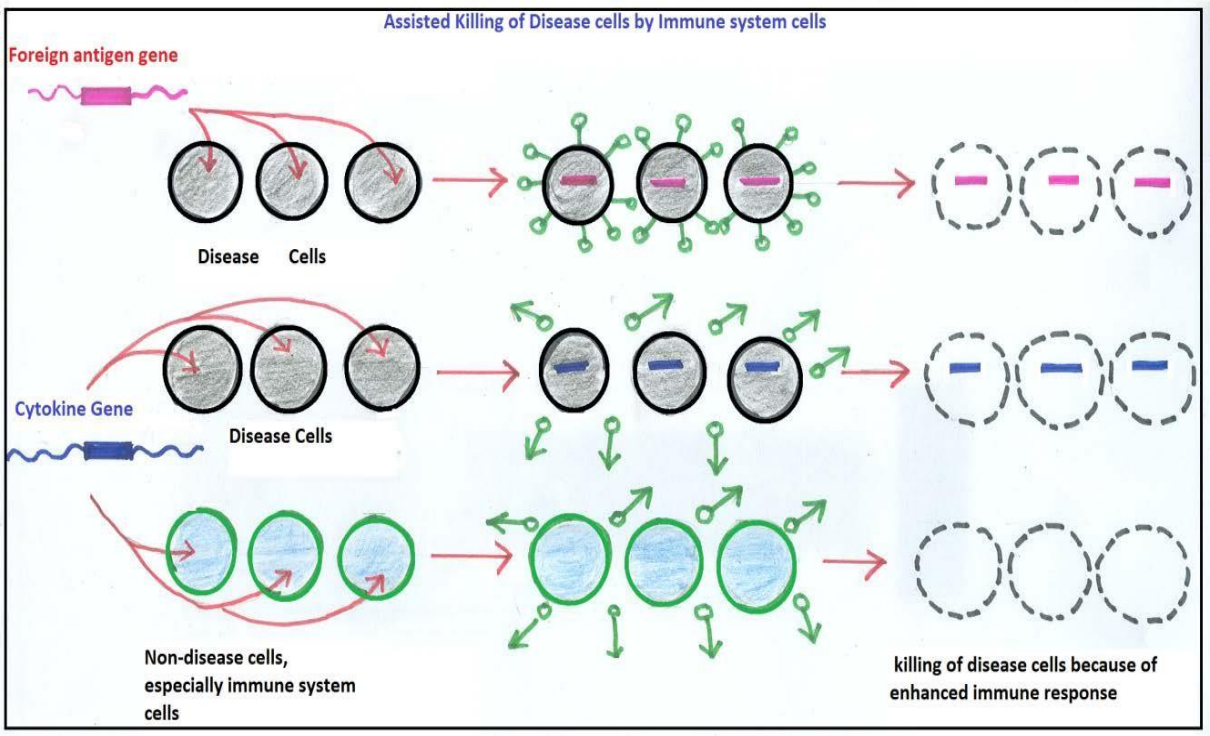



Figure : b) Assisted killing is another strategy of killing diseased cells. Here one method is to insert a well known foreign antigen coding gene which induces immune cells for the killing of the diseased cells. Few more methods are based on immune cells activation in which a certain cytokine encoding gene incorporated into gene therapy vector and inserted into either diseased cells or non-diseased cells. This will lead to enhanced immune response followed by killing of diseased cells.

**Targeted Inhibition of Gene Expression**

This is to block the expression of any diseased gene or a new gene expressing a protein which is harmful for a cell. This is particularly suitable for treating infectious diseases and somecancers.

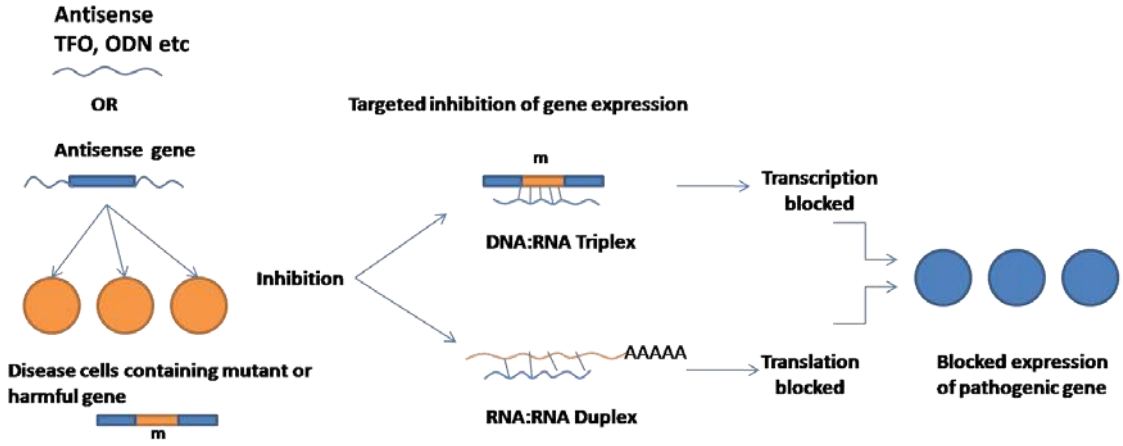



Figure To inhibit the target gene expression in diseased cell the antisense mRNA coding gene inserted vector or triplex-forming oligonucleotides (TFO) or antisense oligonucleotide (ODN) can be introduced which will inhibit the gene expression either by forming DNA:RNA triplex inside the nucleus or forming RNA:RNA duplex by forming complementary mRNA strand of disease protein coding mRNA. This may lead to blocking of disease causing protein expression.

**Targeted Gene Mutation Correction**

It is used to correct a defective gene to restore its function which can be done at genetic level by homologous recombination or at mRNA level by using therapeutic ribozymes or therapeutic RNA editing.

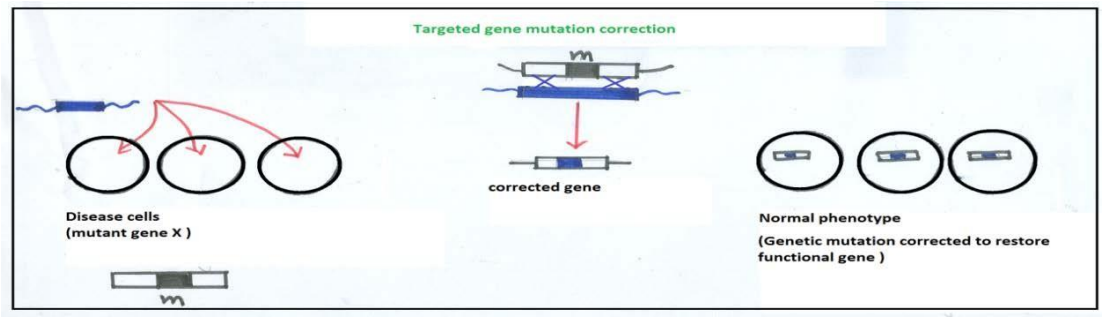



Figure This is used for disease caused by mutation. The corrected gene will be swapped by the mutant gene X (m).

**Then diseased cells will become normal after the correction of mutation by gene therapy.**

***Gene Therapy Approaches***

**Classical Gene Therapy**

It involves therapeutic gene delivery and their optimum expression once inside the target cell. The foreign genes carry out following functions.

- Produce a product (protein) that the patientlacks;
- Produces toxin so that diseased cell is killed.
- Activate cells of the immune system so as to help in killing of diseased cells.

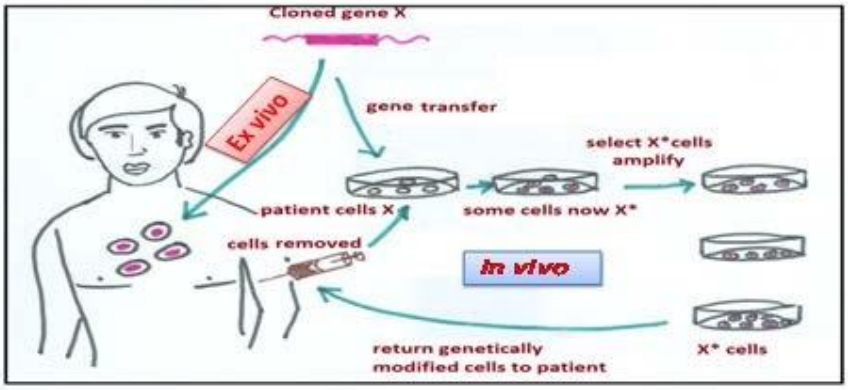
**Non-classical gene therapy**

It involves the inhibition of expression of genes associated with the pathogenesis, or to correct a genetic defect and restore the normal gene expression.

***Methods of gene therapy***

There are mainly two approaches for the transfer of genes in gene therapy:

1. Transfer of genes into patient cells outside the body (*ex vivo genetherapy*)
2. Transfer of genes directly to cells inside the body (*invivo*).




**Gene Therapy using autologous cells: Cells are used, i.e. cells are removed from the patient, cultured *in vitro,* before being returned to the patient's body. In this figure *in vivo* and *ex vivo* gene therapy is diagrammatically explained.**

*Ex vivo* gene therapy

- In this mode of gene therapy genes are transferred to the cells grown in culture, transformed cells are selected, multiplied and then introduced into thepatient.
- The use of autologous cells avoids immune system rejection of the introducedcells.
- The cells are sourced initially from the patient to be treated and grown in culture before being reintroduced into the sameindividual.
- This approach can be applied to the tissues like hematopoietic cells and skin cells which can be removed from the body, genetically corrected outside the body and reintroduced into the patient body where they become engrafted and survive for a long period oftime.
- Figureshows a self explanatory schematic diagram for e*x vivo* genetransfer.

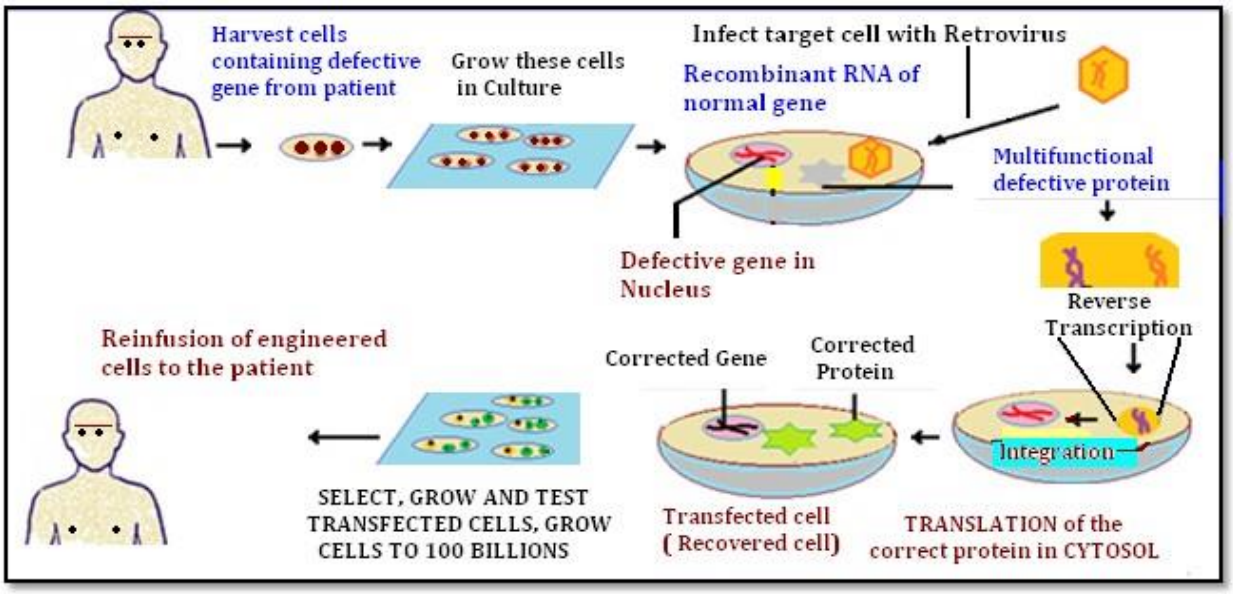



**Figure *Ex vivo* therapy involves tightly regulated cellular manipulation in harvested cells**

*In Vivo* Gene Therapy

- *In vivo* method of gene transfer involves the transfer of cloned genes directly into the tissues of thepatient.
- This is done in case of tissues whose individual cells cannot be cultured *in vitro* in sufficient numbers (like brain cells) and/or where re-implantation of the cultured cells in the patient is notefficient.
- Liposomes and certain viral vectors are employed for this purpose because of lack of any other mode ofselection.
- In case of viral vectors such type of cultured cells were often used which have been infected with the recombinant retrovirus *in vitro* to produce modified viral vectors regularly.These cultured cells will be called as vector-producing cells (VPCs)). The VPCs transfer the gene to surrounding diseasecells.
- The efficiency of gene transfer and expression determines the success of this approach, because of the lack of any way for selection and amplification of cells which take up and express the foreigngene.
- Figure shows various steps of *in vivo* genetransfer.

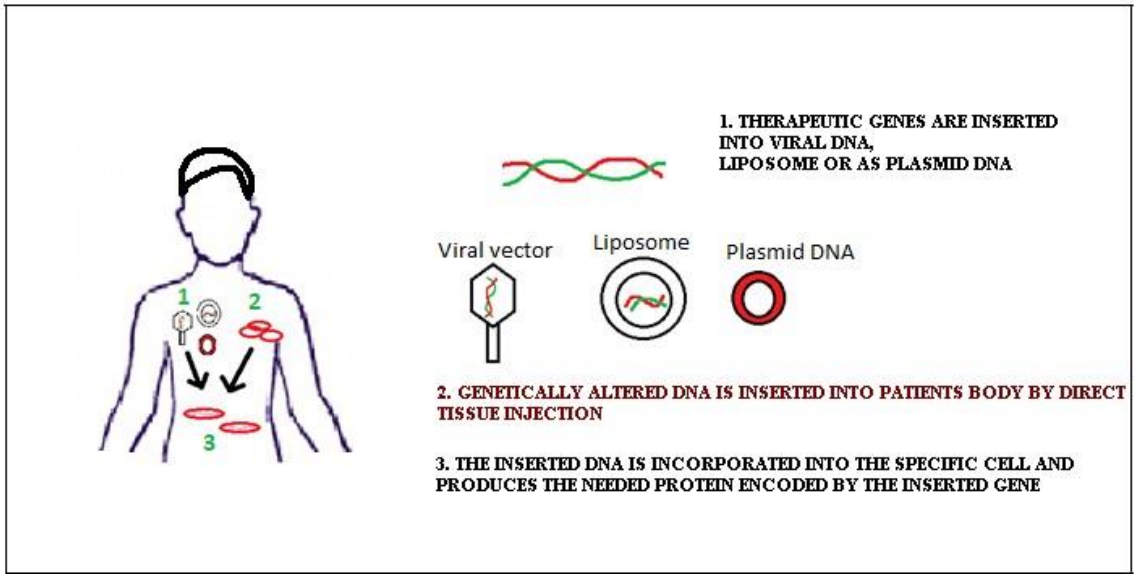



**Figure :various steps of *in vivo* gene transfer**

**Table 1: Differences between In Vivo and Ex Vivo gene therapy**

| **Difference Between *in vivo* and *ex vivo* Gene Delivery Systems** | |
|---|---|
| ***In vivo*** | ***Ex vivo*** |
| Less invasive | More invasive |
| Technically simple | Technically complex |
| Vectors introduced directly | No vectors introduced directly |
| Safety check not possible | Safety check possible |
| Decreased control over target cells | Close control possible |

***Target sites for Gene Therapy***

Therapeutic genes have to be delivered to specific target sites for a specific type of disease. This table describes the list of such disease and their target sites for gene therapy.

**Table 2: Target cells for gene transfer**

| **Target cells for gene transfer** | |
|---|---|
| **Disease** | **Target Cells** |
| Cancer | Tumor cells, antigen presenting cells (APCs), blood progenitor cells, T cells, fibroblasts, muscle cells |
| Inherited monogenic disease | Lung epithelial cells, macrophages, T cells, blood progenitor cells, hepatocytes, muscle cells |
| Infectious disease | T cells, blood progenitor cells, antigen presenting cells (APCs), muscle cells |
| Cardiovascular disease | Endothelial cells, muscle cells |
| Rheumatoid arthiritis | Sinovial lining cells |
| Cubital tunnel Syndrome | Nerve cells |

***Vectors for gene therapy***

Vectors for gene therapy can be classified into two types:

1. Viralvectors
2. Non-viral

**Table : Vectors used in gene therapy**

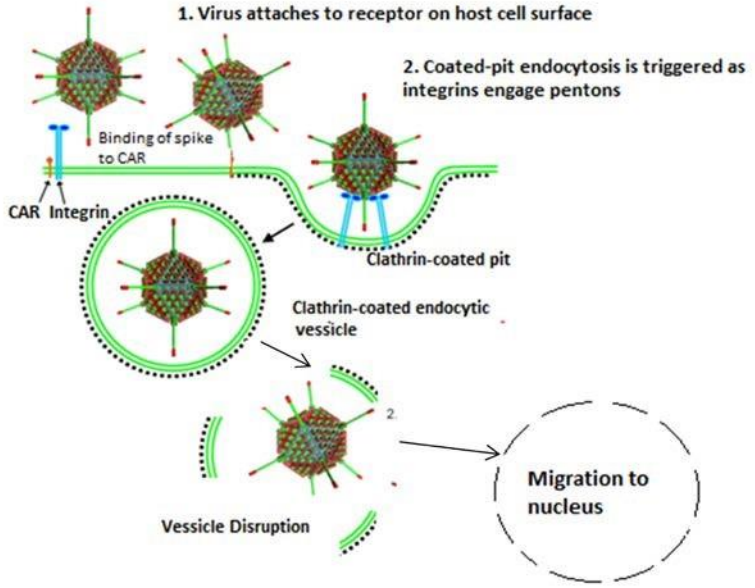
| **Vectors used in gene therapy** | |
|---|---|
| **Viral Vector** | **Non-viral Vectors** |
| Adenovirus | Lipid complex |
| Retrovirus | Liposomes |
| Adeno- Associated Virus | Peptide/protien |
| Lentivirus | Polymers |
| Vaccinia virus | |
| Herpes simplex virus | |
| | |
| Direct gene transfer methods like mechanical, electroporation, gene gun are also ued to transfer genes | |

**Viral vectors**

Retroviruses, adenoviruses and adeno-associated viruses (AAV) some commonly used viral vectors whereas some less commonly used viral vectors are derived from the Herpes simplex virus (HSV-1), the baculovirus etc.

- ***Adenoviral vectors***

Adenoviruses are large linear double-stranded DNA viruses that are commonly used for preparing gene transfer vectors. Adenovirus vectors are known to be the second most popular gene delivery vector for gene therapy of various diseases like cystic fibrosis and certain types of cancer. Figure 8-1.7.1.1 shows how the adenoviruses enter cells by receptor-mediated endocytosis. A primary cellular receptor binds to viral fiber then the virus interacts with secondary receptors which are responsible for its internalization. Coxsackie and Adenovirus Receptor (CAR), Heparansulphateglycosaminoglycans, sialic acid, CD46, CD80, CD86, alpha domain of MHC I are the primary receptors which are specific for specific strains of adenovirus. Integrins are the secondary receptors which helps in the internalization of viral particles. Some adenovirus directly interacts with integrins like in the case of fiber deficient Ad2virions.




The adenoviral DNA has inverted terminal repeats (ITRs) and a terminal protein (TP) is attached covalently to 5' termini. The adenoviral genome is classified as early and late regions based on the proteins they express. Proteins encoded by early region (E1, E2, E3, E4) genes are involved in viral DNA replication, cell cycle modulation and defense system. The late region genes (L1, L2, L3, L4, L5) encodes the viral structural proteins. Three classes of adenoviral vectors namely first, second and third generation viral vectors are developed for gene therapy purpose.

- ***Adeno- Associated Virus(AAV)***

Adeno-associated viruses (AAVs) are a group of small, single-stranded DNA viruses which cannot usually undergo productive infection without co-infection by a helper virus, such as an adenovirus.

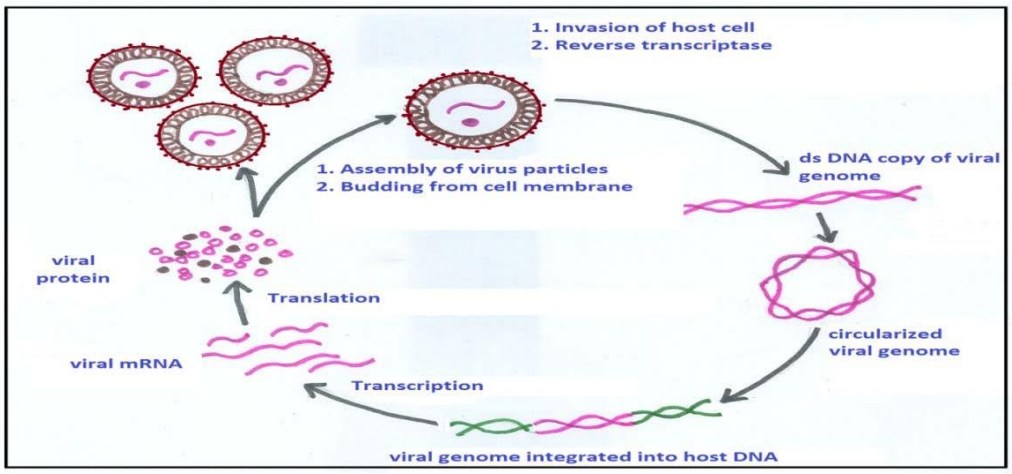
- The insert size for AAV is 4.5 kb, with the advantage of long-term gene expression as they integrate into chromosomalDNA.
- AAVs are highly safe as the recombinant adeno associated vectors contains only gene of interest and 96% viral genes aredeleted.

Adeno-associated viruses are explained in detail in Module 5-Lecture 1.

- ***RetroviralVectors***

Retroviruses are RNA viruses which possess a reverse transcriptase activity, enabling them to

synthesize a complementary DNA. Following infection (transduction), retroviruses deliver a nucleoprotein complex (pre-integration complex) into the cytoplasm of infected cells. The viral RNA genome is reverse transcribed first and then integrates into a single site of thechromosome.




**Figure**

- **Tumor retroviruses**, example Moloney's murine leukemia virus (MoMuLV), is widely used for the generation of recombinant vectors. these are produced at low titers as all the viral genes aredeleted.
- **Oncoretroviruses:** The cells that divide shortly after infection can only be transduced by oncoretrovirus. The preinitiation complex is excluded and their entry is restricted in to the nucleus as they can only enter when nuclear membrane dissolves during cell division the target cells for this viral vector is limited

- **Recombinant lentiviruses**are being developed that are non- pathogenic to humans and have the ability to transduce stationarycells.

➢ ***Other Viral Vectors:***

These include herpes simplex virus vectors and baculovirus.

**Herpes simplex virus vectors**: Herpes simplex virus-1 (HSV-1) is a 150 kb double- stranded DNA virus with a broad host range that can infect both dividing and non- dividing cells. the insert size is comparatively larger (>20kb) but have a disadvantage of short-term expression due to its inability to integrate into the host chromosome

**Baculovirus:** They can take up very large genes and express them highly efficiently. They help in recombinant protein expression in insect cell. They can infect hepatocytes as an only mammalian cell type and the gene expresses under the control of either mammalian or viral promoter.

**Simian Virus 40 Vectors (SV40):** SV40 are icosahedral papovavirus with a circular double stranded DNA of 5.2kb size as genetic material. The genome encodes for early proteins viz; large T antigen (Tag) and small t antigen (tag) and late protein viz; a regulatory protein agnoprotein and three structural proteins (VP1, VP2, VP3). The Tag gene is removed as it is responsible for

inducing immunogenicity in the recombinant SV40 vector. All the structural proteins except the major capsid protein VP1 is removed resulting in a genome of 0.5kb size which includes origin of replication (ori) and encapsidation sequence. Recombinant SV40 vectors allows expression of transduced gene

**Non- viral vectors**

It involves chemical and physical methods such as direct injection of naked plasmid DNA (particle bombardment), receptor-mediated endocytosis and gene transfer through liposomes, polymers, nano particlesetc.

***Some non viral methods***

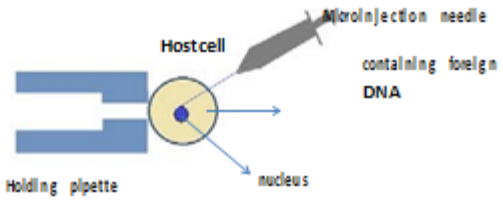
- **Direct injection/particlebombardment:**

DNA can be injected parenterally which can be considered for Duchenne muscular dystrophy (DMD). An alternative approach uses particle bombardment ('gene gun') technique, in which DNA is coated on to metal micro particles and fired from a ballistic gun into cells/tissues. This technique is used to transfer the foreign DNA and its transient expression in mammalian cells *in vitro* and *in vivo* as well. It can cross the physical barriers like skin, muscle layer for which it is used for vaccination. Particle bombardment is used to deliver drugs, fluorescent dyes, antigenic proteins etc.

*Advantage:* Simple and comparatively safe.

*Disadvantage*

- Poor efficiency of gene transfer.
- A low level of stable integration of the injected DNA. Repeated injection may cause damage in the proliferating cells.




MICROINJECTION

- **Microinjection** involves the delivery of foreign DNA, by the help of glass micropipette into a living cell. The cell is held against a solid support or holding pipette and micro neeedle containing the desired DNA is inserted into the cell. The tip of the pipette used is about 0.5 to 5 micro meter diameter which resembles an injection needle. For this, glass micropipette is heated until the glass becomes somewhat liquefied and is quickly stretched to ressemblea injection needle. The delivery of foreign DNA is done under a powerful microscope(micromanupulator).

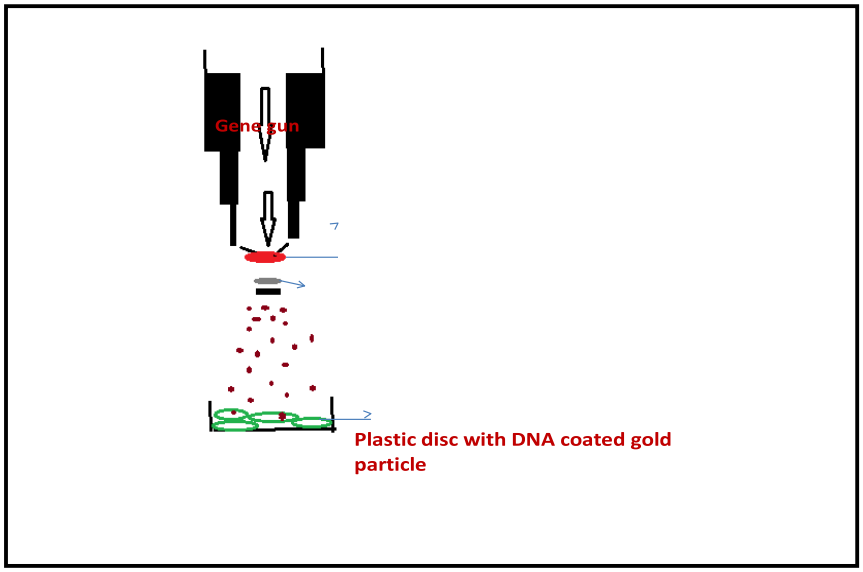



**Figure 8-1.7.2.2 Gene gun mediated gene transfer**

In **particle bombardment** method, the tungsten or gold particles (micro projectiles) are coated with the foreign DNA. Micro-projectile bombardment uses high-velocity metal particles to deliver biologically active DNA into the target cells. The macroprojectile is coated with the coated particles and is accelerated with air pressure and shot into plant the target tissue. A perforated plate is used, which allows the micro-projectiles to pass through to the cells on the other side of the plate and stops the macropojectile. Particle coated with the foreign gene releases the foreign gene when enters into the target cell and integrates into the chromosomal DNA. This technique is also used to transfer genes in mammalian cells. Mammalian cell lines like HEK 293, MCF7 showed gene expression when transfected with luciferase and green fluorescent genes and their gene expression was dependent on helium pressure, size and amount of gold particle and DNA load on each particle. Cell viability depends on heliumpressure.

- ***Liposomes Mediated***

  Liposomes are spherical vesicles which are made up of synthetic lipid bilayers which imitate the structure of biological membranes. DNA to be transferred is packaged into the liposome *in vitro* and transferred to to the targeted tissue. The lipid coating helps the DNAtosurvives*invivo*andentersintothecellbyendocytosis.Cationicliposomes, where the positive charge on liposomes is stabilized by binding of negatively charged DNA, are popular vehicles for gene transfer *in vivo.*

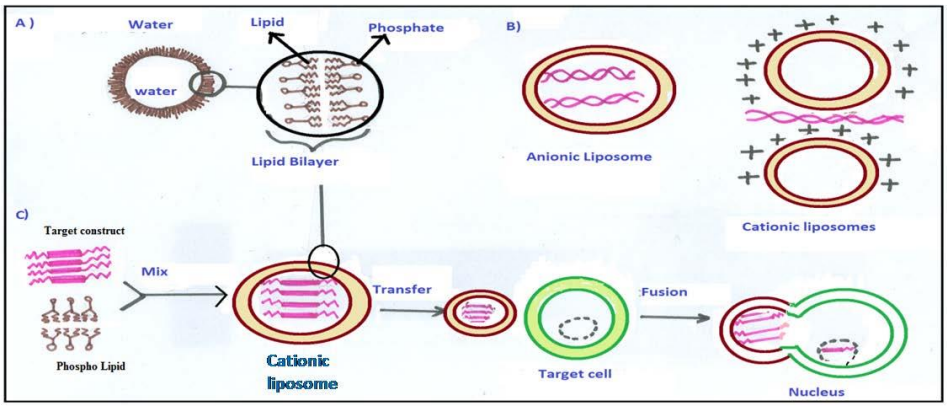



**Figure: In vivo liposome mediated gene transfer- (A) formation of lipid bilayer in water (B) Structure of anionic and cationic liposome (C) Use of liposome to transfer genes into cells.**

Advantage:

- The liposomes with the foreign DNA are easy toprepare.
- There is no restriction in the size of DNA that is to betransferred.

Disadvantage:

- Efficiency of gene transfer is low and transient expression of the foreign gene is obtained as they are not designed to integrate into the chromosomalDNA.

➢ ***Electroporation***

In electroporation, the external electric field is applied to the protoplast, which changes the electrical conductivity and the permeability of cell membrane; and thus the exogenous molecules found in the medium are taken up to either the cytoplasm (transient transfection) or into the nucleus (stable transfection).The efficiency of electroporation canbeincreasedbygivingthecellaheatshock,priortotheapplicationofelectricfield or by using small quantity of PEG while doing electroporation.

*Advantage:*

- By electroporation large numbers of cells can be processed at once, and thus the amount of time spent processing cells can be cutdown.

*Disadvantages:*

- If the voltage applied is not calculated properly, the cells maydamage.
- If electroporation does not occur in controlled environment, the potentially harmful substances can enter the cell or the impurities from solution may enter. This is because there is no way to control what enters the cellmembrane.

***Advantages of Gene Therapy***

- Gene therapy can cure genetic diseases by addition of gene or by removal of gene or by replacing a mutated gene with correctedgene.
- Gene therapy can be used for cancer treatment to kill the cancerouscells.

- Gene expression can becontrolled.

- Therapeutic protein is continuously produced inside the body which also reduces the cost of treatment in longterm.

## NEURODEGENERATIVE DISEASES

Neurodegenerative Diseases are characterized by progressive dysfunction and death of neural cells and tissue, which results in various forms of movement disorders and cognitive decline over time. Such diseases include Parkinson's disease, Alzheimer's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis (Lou Gehrig's disease), each of which is described further below.

Parkinson's Disease is the result of dying dopaminergic neurons in the substantia nigra of the brain, and it is characterized by resting tremor, bradykinesia, and rigidity, and symptoms eventually progress towards dementia. Although most cases of Parkinson's disease have not yet been linked to genetic causes, mutations in genes encoding synuclein and LRRK2 have been shown to cause inherited forms of Parkinson's disease, and synuclein mutations have also been associated with other neurodegenerative diseases such as Lewy body dementia. With the loss of dopaminergic signals, the intricacy and meaning of information encoded in certain neural activity is lost and neural activity becomes more affected by the activity of cholinergic pathways in the basal ganglia. While certain drugs (like dopamine agonists and anticholinergics) and deep brain stimulation can significantly improve some symptoms of the disease, they cannot cure it, and it remains a progressive downhill neurodegenerative disease. Approximately 1 million people in the United States live with the disease, and about 60,000 more cases are diagnosed each year .

Alzheimer's Disease is a neurodegenerative disease involving the frontal lobes of the brain where many cognitive processes occur. Therefore the manifestations of the disease may include a variety of symptoms, including memory loss, impaired judgment, confusion, mood swings, and progressive deterioration in many bodily functions and the ability to care for oneself. Like all neurodegenerative diseases, this is currently a progressive and irreversible affliction. It was previously believed that Alzheimer's disease was caused by amyloid plaque formations in the brain, but some evidence suggests that these plaques may be a downstream effect rather than a cause and may not be directly associated with the cognitive dysfunctions of the disease. Several areas of the brain may be affected in this disease, including frontoparietal cortex, hippocampus, and other nuclei of the brain, and there is generally decreased cholinergic activity in the forebrain. Drugs for Alzheimer's tend to increase cholinergic activity by blocking cholinesterase action, and these drugs may slow the progression of dementia in some patients. Some forms of Alzheimer's disease have been found to be due to inherited mutations in genes like Apo E4, presenilin, amyloid precursor protein, and others. Over 5 million people in the United States have Alzheimer's disease, and there are nearly half a million new cases diagnosed each year.

Amyotrophic Lateral Sclerosis (ALS) is the result of death of neurons involved in movement (motor neurons). The death of lower motor neurons in the spinal cord causes

weakness and atrophy in muscles, while death of upper motor neurons causes spasticity and dyscoordination. This eventually leads to difficulty moving, speaking, swallowing, and breathing, which can often result in skin ulcers and lung infections similar to spinal cord injury patients. Many cases are related to inherited alterations in the C9orf72 gene (alterations of nucleotide expansions with associated cytoplasmic inclusions, which is a disease mechanism similar to Huntington's Disease or many other pathogenic repeat expansion diseases, although in this case the repeat expansion is a non-coding segment, which likely causes flawed transcriptional, translational, or post-translational processes), or to inherited mutations in the superoxide dismutase gene, which normally functions to remove oxygen free radicals from the body, but when mutated results in higher levels of oxidative damage and inflammation. Riluzole is a drug that may suppress excitatory signaling in neurons, and it is the sole drug approved for ALS in the United States. Although the incidence and prevalence of the disease are not known for certain in the United States, it is estimated that at least 4 people per 100,000 have the disease and approximately 2 people per 100,000 are diagnosed with the disease annually, meaning that about 6,000 people in the United States are diagnosed with the disease each year .

Huntington’s Disease is the result of loss of neuronal tissue in the frontal lobe cortex and basal ganglia, particularly the caudate nucleus. The manifestations of the disease include cognitive, behavioral, and movement disturbances. The sudden and dramatic uncontrollable movements of Huntington’s disease are called “chorea,” referring to the “dance-like” movements of swinging arms, and patients may also suffer dystonia, rigidity, memory loss, mood swings, and decline in judgment and cognitive abilities, eventually resulting in dementia. The underlying genetic cause of the disease has been pinpointed as related to the length of CAG repeats (>40 repeats), which make a polyglutamine string on one end of the huntingtin protein, but many details of the pathophysiology of the disease still remain to be understood. Antipsychotic medications are often used to manage the symptoms of the disease, and the prevalence of the disease is estimated to be approximately 5-10 per 100,000 .

Multiple Sclerosis is sometimes characterized as a neurodegenerative disease, but it may be more fitting to classify it as a neuroimmunological disease since the demyelination appears to be mediated by T-cells and an IgG antibody response in the nervous system and effective treatments include various forms of immunomodulatory agents. However, the cause and effect mechanisms of the disease are not yet fully elucidated. The disease is characterized by demyelination (loss of myelin sheaths around nerve fibers) and the formation of demyelinated plaques in the brain or spinal cord as seen on neuroimaging. Symptoms can include weakness, paralysis, spasticity, ophthalmic nerve problems, bowel and bladder incontinence, and visual disturbances. These symptoms may become worse with stressful or warm environments, and they may remit and relapse over time. It is estimated that over 2 million people in the world have multiple sclerosis, with over 300,000 of those living in the United States .

# SPINAL CORD INJURY

**Spinal Cord Injury** is damage to the spinal cord, which is a cylindrical column of central neural tissue that descends from the brain down the spinal canal, from which peripheral nerves arise to deliver signals to every part of the body. Spinal cord injury occurs in about 12,000 people each year in the United States and in over 300,000 people each year in the world, and nearly 300,000 people in the United States are living with a spinal cord injury, along with over 3,000,000 people living with this injury in the world. In addition to increased difficulty with activities of daily living, spinal cord injured patients also generally have medical complications involving chronic pain, bowel and bladder dysfunction, ulcers, and infections like pneumonia, urinary tract infections, and skin ulcer infections.

Injury to the spinal cord may occur from stretching, contusion, laceration, lack of blood flow (as in a stroke), or even from certain genetic or infectious diseases. The injury may be partial (only affecting some neural function below the lesion) or complete (destroying all neural function below the lesion). In traumatic injuries vertebrae are typically fractured or displaced, exerting compressive, tensile, and shear forces on the spinal cord tissue, and vascular injury (including hemorrhage or thrombosis) can further worsen the injury. Symptoms generally include paralysis, weakness, loss of sensation, and disturbances of autonomic function (such as that involving blood pressure, bowel and bladder function, or sexual function). Some temporary spinal incapacitation can result from swelling and inflammation after an acute injury, while additional neural death may also result from excitotoxicity, swelling, inflammation, and secondary messenger cascades after an injury, and therefore time is needed to assess the full extent and outcome of the injury. After time, glial scarring, demyelination, and cyst formation may also occur in the neural tissue. Efforts to find neuroprotective agents that can prevent further cell death and damage after the primary injury have been promising, but as of yet no drugs have been proven clincally effective for this approach in humans. Research is also underway to find innovative and useful therapeutic interventions that will improve or cure spinal cord injury, and, in particular, neural tissue engineering holds much potential to restore function to these patients. This includes unique and innovative efforts to reconstruct spinal cord pathways using 3-dimensional neural tissue with stem cells, biomaterials, and nanotechnology .

## Ethics

1. **Respect for human life**

   Respect for human life requires that we show respect for human embryos. Some people believe that embryonic stem cell research violates this principle, as an embryo is destroyed during the process of stem cell line derivation. Others argue that the potential benefits of stem cell research (e.g. alleviating human suffering) represents a way of showing respect for human life.

Additional concerns rest on the belief that the creation of embryos for research purposes and the derivation of stem cell lines might lead to the de-sensitisation of human life and to potentially uncontrolled commercialisation or instrumentalization of the human body.

One must keep in mind that these issues are directly linked with another key issue: the moral and legal status of the human embryo.

2. **Human dignity**

The concept of human dignity is a difficult one because it is unclear what it means exactly; and this is exacerbated by the fact that it has been employed to justify fundamentally opposing views. Most authors understand the notion of human dignity as our essential humanity, what makes us human. Consequently this concept is closely related to beliefs regarding the moral status of the human embryo.

3. **Status of the human embryo**

This is a delicate question with various answers according to each individual's conception of the embryo. On the one hand, some consider embryos to be cell masses having no more value than any other biological cell or tissue. On the other hand, some confer the human embryo full personhood status; that is, they consider the human embryo to have the same moral status as a human being that has been born.
Finally, many people hold a gradualist view; they consider moral status to be a continuous process; as the embryo proceeds through stages of development, it gradually gains moral value. This position emphasizes the embryo's potential to become a human being and, hence, affords the embryo "special respect".

## Invitro Organogenesis

## Eight tiny organs grown by scientists

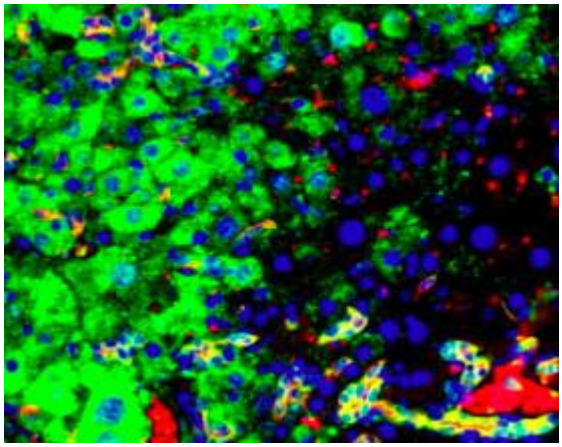
On 20 July researchers at the MRC Centre for Regenerative Medicine announced that they had regrown damaged livers in mice. It's just one example of scientists growing tiny versions of organs in animals and in the lab to study development and disease, and test potential treatments. Many of these organs also represent the first steps towards growing whole organs – or parts of organs – for transplant. MRC Science Writer **Cara Steger** rounds up progress.

Why might you want to grow a tiny organ? Small organs, or parts of them, are useful for studying both development and disease, and for toxicity testing or testing new treatments. In some cases, mini organs will be able to replace research using animals.

But they also offer a tantalising glimpse of a world in which we can grow complex solid organs for transplant. These tiny organs – often more like proto-organs with just some of an organ's functions – are quite literally 'starting small', first seeing if it's even possible.

Here we list eight tiny organs that have been grown so far.

**Little livers**



*Transplanted hepatic progenitor cells can self-renew (yellow) and differentiate into hepatocytes (green) to repair the damaged liver (Image: Wei-Yu Lu, MRC Centre for Regenerative Medicine, The University of Edinburgh')*

The MRC Centre for Regenerative Medicine researchers used liver stem cells, called hepatic progenitor cells, to regrow damaged livers in mice. After extracting the stem cells from healthy adult mice and maturing them in the lab, the researchers transplanted the cells into mice with liver failure.

In three months the cells had grown enough to partly restore the structure and function of the animals' livers, providing hope that this technique could one day replace the need for liver transplants in humans.

**Itty-bitty intestines**

In a study at the Cincinnati Children's Hospital Medical Center, researchers used induced pluripotent stem cells to grow human intestinal tissue in the lab. They then connected the tissue to the kidney of a mouse, providing it with a blood supply to allow it to mature into a piece of human intestine. This technique could provide a useful way of studying and ultimately treating gastrointestinal diseases in the future. Other work involving organoid intestines has been pioneered by EuroStemCell partner Hans Clevers.

**Compact kidneys**

Working lab-grown kidneys have been transplanted into rats by researchers from the Center for Regenerative Medicine in the US. The team stripped down a rat kidney to a scaffold-like structure, before introducing rat kidney and blood vessel cells that grew into a

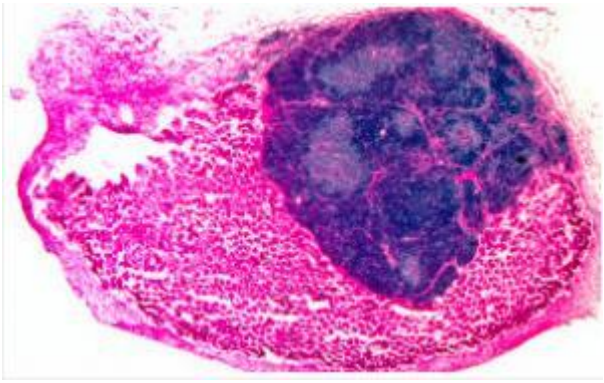
new functioning kidney. They then transplanted the organ into rats where it successfully filtered blood and produced urine.

**Small skin**

An MRC-funded team led by King's College London and the San Francisco Veteran Affairs Medical Center has grown a 3D piece of skin in the lab. Using induced pluripotent stem cells, they produced an unlimited supply of skin cells, some of which were then used to grow a small piece of skin. The lab-grown skin has a working natural barrier that protects it from losing moisture, and prevents it from absorbing chemicals and toxins. This makes it particularly useful for studying a range of skin conditions, and for testing drugs and cosmetics.

**Tiny thymi**

The thymus is an immune system organ which sits just in front the heart. Another group of researchers at the MRC Centre for Regenerative Medicine have reprogrammed mouse cells called fibroblasts, which normally become connective tissue, to instead become thymus cells. When mixed with other thymus cell types and transplanted into mice, the cells grew into a functioning mouse thymus.



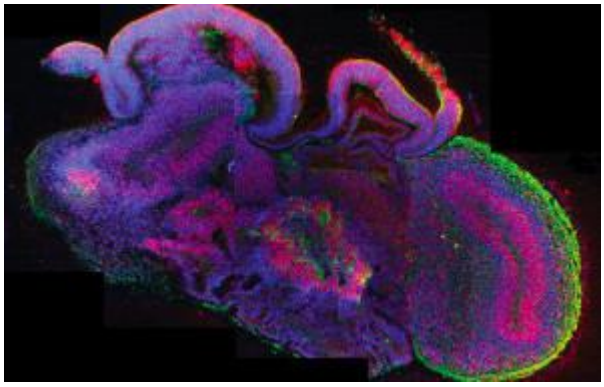
Thymus cells (dark blue) against a background of kidney cells (pink) (Image: MRC Centre for Regenerative Medicine, the University of Edinburgh)

**Teeny tickers**

Miniature human hearts have been grown in the lab using a mouse heart 'scaffold'. Researchers from the University of Pittsburgh removed all the cells from a mouse heart, leaving a skeleton-like structure, before reintroducing immature human heart cells. After just a few weeks the cells developed into beating heart tissue .

**Small-scale stomachs**

Three-dimensional human gastric tissue has been grown by a team at the Cincinnati Children's Hospital Medical Center using human pluripotent stem cells that were coaxed into becoming stomach cells. The structures are only three millimetres in diameter, but could turn out to be useful disease models for understanding how the stomach develops and is affected by different diseases. Plans are already underway to use these tiny organs for studying how the bacterium, *H. pylori*, causes stomach ulcers and gastric disease.



A cross-section of a cerebral organoid (Image copyright: IMBA/ Madeline A. Lancaster)

**Bijou brains**

A team of scientists from the Institute of Molecular Biotechnology in Austria, in collaboration with scientists at the MRC Human Genetics Unit at the University of Edinburgh, has grown miniature brain-like 'organoids' with distinct brain regions, including a cerebral cortex and retina.

The team used human embryonic and human induced pluripotent stem cells that were provided with the oxygen and nutrients needed to mature into brain organoids. No one's going to be growing brains – or even parts of brains for transplant – but the work will help us to understand the brain and any diseases and disorders that affect it: already, the team has grown organoids with a disorder called microcephaly.

**Liver Buds to the Rescue**

Some 16,000 ailing Americans are waiting to receive a liver transplant. But due to a shortage of viable livers, it's likely that fewer than 7,000 transplants will be performed in 2013. In Japan, where the shortage is worse, the number of people in need of new livers is 10 times as great as the number of deceased donors who could provide one.

That gap motivated stem cell biologist Takanori Takebe and his colleagues at the Department of Regenerative Medicine at Yokohama City University in Japan to find an alternate solution. This year they succeeded in generating mini-livers, or liver buds, from stem cells that were taken from human skin and reprogrammed to an embryonic state. (Embryonic stem cells are notable because they can morph into virtually any cell type in the body.)

When mixed with two other types of cells, the fabricated primitive liver cells organized themselves into three-dimensional structures, complete with blood vessels. In effect, Takebe's team re-created the process by which a human embryo begins to form a functioning liver.

Transplanted into a mouse, the human liver buds, about 5 millimeters long, exhibited many functions of the mature organ, such as metabolizing sugars and drugs. When the scientists disabled the mouse's own liver, the human buds kept the animal alive for two months. A person with liver failure would require an infusion of "tens of thousands" of liver buds, Takebe says.

Until the buds can be generated from the skin of each individual patient, recipients will have to rely on immune-suppressing drugs to avoid rejection, just as they would with the transplant of an entire organ. Replacement liver buds might be available to human patients in a decade or less. — Jeff Wheelwright

**Growing Brain Organoids**

Scientists can't yet grow spare parts of the human brain to fix neurological injuries or defects, but they have recently used stem cells to create brain organoids, formations of cells that mimic some of the brain's regions. A team led by neuroscientist Jürgen Knoblich of Austria's Institute of Molecular Biotechnology developed the organoids to help them simulate disease.

Two types of stem cells were used to produce the mini-brains: embryonic cells and adult cells that had been reprogrammed to a starter state. The cells were put into a special culture and then suspended in a gel and stimulated by nutrients, all geared to turn them into neurons like those found in the cortex.

The neurons literally "self-organized," according to Knoblich, and after several weeks formed three-dimensional structures about one-tenth of an inch in diameter.

"If you zoom out and look at the whole, it's not a brain," Knoblich says. "But our cultures contain individual brain regions that have a functional relationship with one another." Besides the dorsal cortex, researchers were able to grow, among other regions, parts of the ventral forebrain, which makes neurons that connect to the cortex, and the choroid plexus, which generates spinal fluid.

In their most impressive experiment, the scientists derived organoids from the skin cells of a person affected by microcephaly. This genetic disorder causes a drastic reduction in brain size and stature. The microcephalicorganoids were smaller than the organoids grown from healthy people, apparently because the patient's stem cells had divided too early and became depleted.

"What our organoids are good for is to model the development of the brain and to study anything that causes a defect in development," Knoblich says. For example, by taking neural stem cells from a patient with schizophrenia, researchers might turn back the clock and track the onset of the condition in an organoid. Knowing how schizophrenia starts might help prevent it. — Jeff Wheelwright

**Human Stem Cells Made From Eggs**

It was 1996 when biologists first fused a mammalian skin cell with an egg cell, cloning Dolly the sheep. That was the start of the race to make a human embryo the same way. The method, called somatic cell nuclear transfer (SCNT), replaces the DNA in an egg cell's nucleus with the genetic material from the nucleus of a skin cell, then tricks the egg cell to start dividing as if it had been fertilized with sperm.

**Liver Buds to the Rescue**

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Until the buds can be generated from the skin of each individual patient, recipients will have to rely on immune-suppressing drugs to avoid rejection, just as they would with the transplant of an entire organ.

## Using Stem Cells for Gene Therapy

There are many reasons why stem cells hold great potential for successful use in gene therapies. Stem cells have the ability to self-renew, which means that the need to provide repeated administrations of gene therapy can be reduced or possibly even eliminated. In particular, hematopoietic stem cells are an ideal choice because they can easily be removed from the blood, bone marrow or umbilical cord. They are not as difficult to identify and isolate as other stem cells and can be more readily coaxed to differentiate in a laboratory setting before injection into the patient.

Other stem cells that suggest promise for gene therapy include myoblasts and neural stem cells. Researchers have found that myoblasts work particularly well for injection into muscle tissue because they readily join with other muscle fibers and therefore integrate well into the network of muscle fibers.

Neural stem cells appear to hold potential for treating gliomas, which are a difficult type of brain tumour to treat. When a patient suffers from a glioma, the tumour cells attack the healthy brain

tissue and travel throughout the brain. In studies thus far, scientists have been able to take neural stem cells and then genetically modify them to create a protein that activates a precursor drug that is not toxic to one that destroys tumour cells. After injecting these genetically modified stem cells into mice who had human-derived gliomas, there was a significant reduction in tumour size within only two weeks.

Research involving embryonic stem cells and gene therapy rather than the previously mentioned adult stem cells is another area that is currently under investigation. Because embryonic stem cells have a greater potential for self renewal as opposed to adult stem cells, repeated administrations of gene therapy are less likely to be necessary. This means that over the long-term, embryonic stem cells could provide better maintenance of therapeutic effects in comparison with adult stem cells.

Overall, there are benefits and challenges to both embryonic and adult stem cell use in gene therapies. Both sources of stem cells, however, hold potential for treating diseases in this manner and the continuation of research will hopefully yield tangible therapies one day soon.

## Tissue Engg for Diabetes

**Islet & Pancreas Transplants**

Transplantation has been an important field of diabetes research for decades. Therapies include whole-pancreas transplants and various ways of transplanting just the insulin-producing islets of Langerhans of the pancreas. For now, transplants are usually recommended for people whose diabetes is not well controlled by external injected insulin or who have low hypoglycemic awareness (the ability to recognize when blood sugar is low).

About 1,500 *pancreas transplants* are performed in the U.S. each year. It's a complex and costly surgical procedure in which a second pancreas is added without removing the first. Because the new pancreas contains fresh islets and beta cells, this can eliminate the need for insulin therapy—and indeed most patients with a successful pancreas transplant are able to be insulin-independent for many years.

A less invasive (but also expensive) method of providing the body with a new supply of beta cells is *islet transplantation*. Typically this has been done by infusing purified human islets through a major vein that feeds into the liver. Once established, the islets begin producing insulin in response to changing blood sugar levels. Thus far the most clinically advanced islet transplantation technique has been the Edmonton Procotol, introduced in 2000 by a team at the University of Alberta, which has allowed patients to stay insulin-independent for a year or more.

**Where Do the Islets Come From?**

All forms of islet or pancreas transplants are currently limited by the availability of human islets. Cadavers are the commonest source. Researchers (in New Zealand especially) are using pig islets, which closely resemble the human kind. Future prospects include creating beta cells from other cells in the patient's own body or—ideally—synthesizing them from stem cells.

**Immune Suppression—The Great Challenge**

The overwhelming obstacle to success in any kind of transplant therapy for diabetes is the body's own immune response—the same basic response that provokes type 1 disease in the first place. Even in perfectly healthy people, the introduction of a "foreign" body or substance prompts our immune system to reject the invader, a problem all transplant surgeries must overcome.

An array of powerful immune-suppressing drugs has been developed to counter this natural reaction of the body. In the case of both pancreas and infused-islet transplants, these drugs do work to a greater or lesser extent, allowing the new islets to go on doing their job of producing insulin. People who received pancreas transplants as long ago as the 1980s still have functioning islets, and the drugs also extend the effective life of infused islets.

But for most type 1 diabetics, and those working on cures, using immune-suppressing drugs is not a satisfactory long-term solution. The drugs are hugely expensive and highly toxic by their very nature, with potentially risky side effects. They must be used rigorously and indefinitely for the life of the transplant. And because they act very broadly, they can sometimes injure the new islets and reduce the benefits of transplantation.

Work on improving immune-suppressing drugs—a major research area in itself—is progressing. But it's not hard to see that a better approach would be to erase the need for them.

"For any type of transplantation procedure, a balance is sought between efficacy and toxicity."

—Jonathan R. T. Lakey and coauthors of the landmark paper on the Edmonton Protocol, published in the *New England Journal of Medicine*, 2000

**Encapsulating Islets**

To protect the islets and beta cells from autoimmune attacks, researchers are exploring several ways to "encapsulate" or wall off islets before they are transplanted. Some kind of semi-permeable barrier surrounds the islets, excluding large molecules (like the immune system's soldiers) yet allowing oxygen and other nutrients to reach the beta cells and insulin to be released.

Islet encapsulation and the bioartificial pancreas are different ways to describe the same line of research. There are two main approaches: macro- and microencapsulation; each has its pros and cons. Within these categories, the protective devices take various forms: coatings, capsules, hollow fibers, and sheets.

**The Bioartificial Pancreas**

A bioartificial pancreas—a device that encapsulates and nurtures islets of Langerhans— replaces the islets and beta cells destroyed by type 1 diabetes. Implanted in the peritoneal cavity or under the skin, it contains about a million islets. It responds to changing blood glucose levels by releasing hormones, chiefly insulin.

Any bioartificial pancreas is fabricated from living and nonliving components. The living component is the islets, which sense glucose levels and secrete insulin according to normal physiology. The nonliving component shields the islets from the diabetic's body and its destructive immune mechanisms, yet permits the islets inside to thrive.

**Several Approaches**

Medical technology firms, working with researchers, have tried various ways to create a bioartificial pancreas that performs as it should, using physical configurations such as coatings, capsules, hollow fibers and sheets. *Microencapsulated*, or "coated," islets are the first-generation bioartificial pancreas. Their advantage is that nutrients can easily move into the islets and insulin can move out. Among the drawbacks is that they are hard to remove because they don't stay in one place.

*Macroencapsulation*, another approach, groups islets cells together in a larger package. Such devices tend to be more stable and easily extracted, but—at least in their earlier capsule form—may restrict the free passage of nutrients and insulin. This "starves" the beta cells and impedes the goal of controlling blood glucose.

**Technical Challenges**

As you can see, the technical requirements for a bioartificial pancreas are exacting, and they have proven very hard to solve. The critical issues are:

- *Avoiding the foreign-body response.* Most often the surface of the bioartificial pancreas provokes a *fibrotic reaction* that walls off the device, so the islets cannot get nutrition and the bioartificial pancreas dies of starvation.

- *Enabling oxygen to penetrate to the core of the device.* The dimensions of most bioartificial pancreases don't allow free passage of vital oxygen.
- *Fabricating the device without damaging the islets.* Sometimes the process destroys too many islets for the bioartificial pancreas to function.
- *Placing the device in close proximity to blood vessels,* which deliver oxygen and nutrients to the islets and carry secreted insulin to the rest of the body.

Various techniques are being explored to extend the life and efficacy of transplanted islets. Some researchers are attempting to build into the device helpful biochemical nutrients that release slowly. Other are focusing on the shape and configuration of the bioartificial pancreas.

**An Islet "Sheet"**

The research project supported by Hanuman has advanced the field with a radically thin device that meets most of the technical challenges.

About the size of a business card, the Islet Sheet is the product of 30 years of experimentation and study by Islet Sheet Medical Company and its research associates. It consists of a layer of human islets "macroencapsulated" within a membrane of ultra-pure alginate (derived from seaweed). The sheet membrane is reinforced with mesh and coated on the surface to prevent contact between the cells inside and the host's immune attackers. Oxygen, glucose, and other nutrients diffuse readily into the sheet, keeping the islets alive; insulin, hormones, and waste products diffuse out. The Islet Sheet may be removed or replaced at any time. And except for a brief period after transplantation, no immune suppression drugs are needed.

# Targeted Molecular Therapy

Targeted molecular therapy is a type of personalized medical therapy designed to treat cancer by interrupting unique molecular abnormalities that drive cancer growth. The drugs used in targeted therapy are designed to interfere with a specific biochemical pathway central to the development, growth, and spread of that particular cancer.

Targeted molecular therapy is personalized to meet each person's individual needs because cancer develops differently in everyone. In some cancers the molecular targets are known but in others the targets are still being identified. In some cases, the same types of cancer have different molecular targets. Identifying the molecular targets in any given patient's cancer requires working closely with pathologists to carefully identify the correct cancer pathology.

Targeted molecular therapy provides medical oncologists a better way to customize cancer treatment. Advantages of molecular targeted therapy include:

- Potentially less harm to normal cells
- Potentially fewer side effects
- Improved effectiveness
- Improved quality of life

Types of Targeted Molecular Therapy

Some types of targeted molecular therapy include:

- Selective BRAF inhibitor vemurafenib for BRAF mutant melanoma targets the protein (BRAF) involved in normal cell signaling. Mutations in the gene for BRAF are found in about half of melanomas as well as some other adult cancers. In melanoma the cancers uses the mutated BRAF protein to grow and spread. By interfering with the functions of the mutated BRAF protein

with drugs such as vemurafenib, the melanoma tumors often stop growing and spreading, and in some cases even shrink.

- Imatinib and nilotinib target a protein (BCR-ABL) that is critical to the growth of chronic myelogenous leukemia cells

- Erlotinib targets a protein called the epidermal growth factor receptor or EGFR. This protein is involved in cell signaling and is mutated in up to 25 percent of lung cancers, especially in cancers developing in never or light smokers.

- Trastuzumab targets a cell signaling protein called HER2 that is overactive in about 25 percent of breast cancers.

- Other examples of targeted therapies include lapatinib for breast cancer; crizotinib for lung cancer; bevacizumab for lung and colon cancer; and sorafenib for liver and kidney cancer.

Diseases Treated with Targeted Molecular Therapy

Targeted molecular therapy is not indicated for all patients and all types of cancer. Currently, targeted molecular therapy is used to treat:

Brain cancer

- Breast cancer
- Gastrointestinal (GI) cancer
- Head and neck cancer
- Kidney cancer
- Leukemia
- Lung cancer
- Melanoma
- Mesothelioma
- Myeloma
- Prostate cancer
- Thyroid cancer

# UNIT – I

## PART – A

**1. Define Bioinformatics.**

Bioinformatics is the utilization of computer networks & databases for data storage & solving problems in molecular biology.

"Bio" means living & 'Informatics' means Information science. It is also called computational molecular Biology.

**2. Abbreviate.**

a) ASN – Abstract syntax Notation

ASN – Abstract syntax Notation
HTTP- Hyper Text Transfer protocol
TCP – Transmission control protocol.
PIR – Protein Information resource.

**3. What is an IP number ladders mentioning its components?**

Each hast on a TCP/IP internet is assigned a unique 32-bit internet address that is used in all communication with that host. In the simplest case, each host attached to an internet is assigned a single 32-bit universal identifier as its internet address. A prefix of an IP address identifies a network. IP addresses in all hosts on a given each address is a pair of netid & n

Netid – identifies a network
Hostid – identifies a host on that network.

**4. Describe the terms Homologous, Analogous, orthologous, paralogous, xenologous.**

Homologous – Characters are similar due to common ancestry
Analogous - Characters are similar due to convergent evolution
Orthologous – Characters are homologous with conserved function.
Paralogous – Characters are homologous with divergent function.
Xeneologous – Sequence identity due to horizontal transfer.

**5. Mention various data mining tools, their description & classification.**

1) ENTEREZ (Enterez sequence retrieval system)
   Search multiple databases like GenBank, swiss protect.

2) BLAST (Basic Local alignment search tool)

It is used to compare a moved sequence with those contained in nucleotide & protein databases by aligning the novel sequence with previously characterized genes.

3) FASTA (Fast A)

Compares a protein sequence to another protein sequence or a protein library or a DNA sequence to another DNA sequence to another DNA sequence or to a DNA sequence library.

**6. Give an account on components of Bio informatics.**

**It comprises 3 components**

**1. Creation of databases**

This involves the organizing, storage & management of biological datasets. The databases are accessible to researches to know the existing information & submit new entries.

Eg: Protein sequence data bank for molecular structure.

Databases will be of no use until analysed.

**2. Development of algorithms & statistics**

this involves the development of tools & resources to determine the relationship among the members of large data sets. Eg: comparison of protein sequence data with the already existing protein sequences.

**3. Analysis of data and interpretation**

The appropriate use of components 1 & 2 (given above) to analyse the data & interpret the results in a biologically meaningful manner. This includes DNA, RNA & protein sequences, protein structure, gene expression.

**7. What is biological Databases?**

The collection of the biological database a computer which can be manipulated to appear in varying arrangements & subsets is regarded as a database. The biological information can be stored in different database. Each database has its own website with unique navigation tools.

Eg: Nucleotide sequence Database
Protein sequence Database etc.

**8. Mention some applications of Bioinformatics.**

- Sequence mapping of biomolecules (DNA, RNA, Proteins).
- Production of functional gene products.
- Molecular modeling of biomolecules.
- Designing of drags for medical treatment.
- Finding sites that can be cut by restriction enzymes.

**9. Define search engine with example.**

A search engine is an interactive tool that helps us to access information available via the world wide web. They are actually databases that contain references from thousands that contain references from thousands of resources. They serve as interaction between users & distant web servers.

Eg: Google, Yahoo, Lycos, Alta vista etc.

**10. Define meta search engines.**

They do not build a database of their own. Instead, they submit the search simultaneously to multiple search engines & then refine & amalgamate the results.

**11. What is TELNET?**

The TCP/IP protocol suite includes a simple textual remote terminal protocol called TELNET that allows a user to log into a computer across an internet.

**12. What is the use of these commands?**

(i) cp
(ii) rm

(iii) mv

(i) cp – Makes copies of your files

Eg: cp file one file two
Result: copies the contents of file one to file named file two.

(ii) rm – Deletes specific files

Eg: rm new file
Result: Deletes the file named "new file".

(iii) mv – changes the identification of one or more files.

Eg: mv old file new file
Result: changes the name of the file "old file" to "New file"

**13. Specify the use of**

(i) more
(ii) cat
(iii) with example

(i) more

Enables examination of a continuous text one screenful at a time on a terminal.

Eg: more new file
Result: Displays the contents of "new file" on screen at a time

(ii) cat – Displays the contents of a file on your terminal

Eg: cat new file
Result: Displays the contents of the file "new file" your terminal

**14. Write short notes on Needleman-wunsch algorithm.**

This algorithm sets up the scoring matrix in which the homologous sequence is added to the to row & the query sequence is added to the left column. Each element in the matrix represents the relationship b/w. two residues a particular position. The first row & column is

filled up with gap penalties of residues in respective sequences. This step is called matrix initialization.

**15. Write short notes on UNIX.**

The UNIX operating system was pioneered by ken Thompson & Dennis Ritchie at laboratories in the late 1960's. One of the primary goals in the design of this operating system was to create an environment that promoted efficient program development. Also important was that the operating system be small & memory efficient & that it be easily to maintain. The UNIX version could be easily transferred to different computer systems.

# PART – B

**1. Explain**

- **What is Bio informatics? & its History**
- **Components of Bioinformatics**
- **Some Database with its salient features**
- **Applications of Bioinformatics**

**Bioinformatics**

- Bioinformatics is the combination of biology and information technology. It is a recently developed science using information to understand biological phenomenon.

- It broadly involves the computational tools and methods used to manage, analyze and manipulate volumes & volumes of biological data.

- Bioinformatics is large by a computer based discipline. Computers are infact very essential to handle large volumes of biological data, their storage & retrieval.

**History of Bioinformatics**

The term bioinformatics was first introduced in 1990's originally, it dealt with the management and analysis of the data pertaining to DNA, RNA and protein sequences. As the biological data is being produced at an unprecedented rate, its management and interpretation invariably requires bioinformatics. Thus, bioinformatics now includes many other types of biological data some of the most important ones are:

- Gene expression profiles
- Protein structure
- Protein interactions
- Micro arrange (DNA chips)
- Functional analysis of bio molecules
- Drug designing

Bio informatics covers many specialized areas of biology.

**1. Functional Genomics**

Identification of genes and their respective functions.

**2. Structural Genomics**

Predictions related to functions of proteins.

**3. Comparative Genomics**

For understanding the genomes of different species of organisms.

**4. DNA micro arrays**

These are designed to measure the levels of gene expression in different tissues, various stages of development and in different diseases.

**5. Medical informatics**

This involves management of biomedical data with special reference to bio molecules in vitro assays and clinical trials.

**Components of Bioinformatics**

It comprises 3 components.

**1. Creation of databases**

This involves the organizing, storage and management of biological data sets. The databases are accessible to researchers to know the existing information and submit new entries.

Eg: Protein sequence data bank for molecular structure

Data base will be o no use until analyzed.

**2. Development of algorithms and statistics.**

This involves the development of tools and resources to determine the relationship among the members of large data sets Eg: comparison of protein sequence data with the already existing protein sequences.

**3. Analysis of data and interpretation:**

The appropriate use of components 1 and 2 (given above) to analyse the data and interpret the results in a biologically meaningful manner. This includes DNA, RNA and protein sequences, protein structure, gene expression profiles and biochemical pathways.

**Biological Databases**

The collection of the biological data on a computer which can be manipulated to appear in varying arrangements and subsets in regarded as a database. The biological information can be stored in different databases. Each database has its own website with unique navigation tools.

**Nucleotide sequence Databases**

The nucleotide sequence data submitted by the scientists and genome sequencing groups is at the database namely GenBank, EMBL (European Molecular Biology Laboratory) and DDBJ (DNA Data Bank of Japan).

Primary nucleotide Database Gen Bank EMBL DDBJ

**Protein Sequence databases**

Protein sequence data bases are usually prepared from the existing literature & in con salutation with the experts. These database represent the translated DNA databases.

**Molecular structure of databases**

3D structures of macro molecules are determined by X-ray crystallography and nuclear magnetic resonance (NMR). PDB and SCOP are primary databases of 3-D structures of biological molecules.

**Other databases**

KEGG data base is an important one that provides information on the current knowledge of molecular biology and cell biology with special reference to information on metabolic pathways, interacting molecules and genes.

| Database | Salient Features |
|---|---|
| Primary nucleonic sequence databases<br>Gen Bank<br>(www.ncbi.nih.gov/Genebank)<br>EMBL<br>(www.ebi.ac.uk/emb1/) | Provides nucleotide sequence databases maintained by NCBI (National centre for Biotechnology Information), USA<br><br>European Molecular Biology laboratory (EMBL) maintains nucleotide sequence databases under the aegis of European Bioinformatics Institute (EBI), OK. |

| | |
|---|---|
| Other nucleotide sequence Data bases<br><br>UniGene<br>www.nebi.nih.gov/uniGenel<br><br>Genome Biology<br>www.ncbi.nih.gov/Genomel<br><br>EBI Genomes<br>www.ebi.uk./geneome | The nucleotide sequences of Gen Bank in the form of clusters, representing genes are available.<br><br>The information about the completed genomes is available.<br><br>Provides data and statistics for the completed genomes, besides the information on the ongoing projects. |
| Protein Sequence data base<br><br>SWISS – PROT<br>www.expasy.ch/sport<br><br>PIR<br><br>(pir.george/own.edu) | Provides the description of the structure of a protein, its domains structure, post translational modifications, variants etc.<br><br>Protein Information resource (PIR) is a database provided by the NBRF (National Biomedical Research Foundation in USA. |
| Protein sequence motif databases<br><br>PROSTIE<br>www.expasy.ch/prosite/)<br><br>Pfam<br>www.cgr.ki.se/pfam/)<br><br>Ma cromolecular Database<br>PDB<br>www.rcsb.org/pdb)<br><br>SCOP<br>www.mrc./mb.cam.ac.uk/scop/)<br><br>Other databases<br><br>KEGG<br><br>www.genome.ad.jp/kegg/) | Provides information on protein families and domains.<br><br>A database of protein families defined in the form of domains.<br><br>This is the primary database for 3D structures of biological macro molecules (determined by x-ray and NMR studies)<br><br>Provides information on structural classification of proteins (SCOP).<br><br>Proteins are classified on the basis of 3D structures.<br><br>The kyto Encyclopedia of Genes and Genomus (KEGG) is a database with latest computerized information on biomolecules and cell biology. KEGG provides details on information pathways, interacting molecules and the connecting links with genes. |

**Applications of Bioinformatics**

- Sequences mapping of biomolecules (DNA, RNA, Proteins)
- Identification of nucleotide sequences of functional genes.
- Finding of sites that can be cut by restriction enzymes.
- Designing of primer sequence for polymerase chain reaction.
- Prediction of functional gene products.
- To trace evolution any trees of genes.
- For prediction of BD structure of proteins
- Molecular modeling of biomolecules.
- Designing of drugs for medical treatment.
- To handle vast biological data.
- Development of models for the functioning of various cells, tissues & organs.

**2. Write the basic properties of the Unix Operating systems such as unix shell, file system. Full & relative path names & given few frequently used unix commands.**

**UNIX**

The UNIX operating system was pioneered by ken Thompson & Dennis Ritchie at Bell laboratories in the late 1960's. One of the primary goals in the design of this operating system was to create an environment that promoted efficient program development. Also important was that the operating system be small memory efficient & that it be easily to maintain.

The UNIX version would be easily transferred to different computer systems.

**UNIX FILE SYSTEM**

UNIX implements a hierarchical file system. In this scheme a directory can have a no. of files & subdirect ories underneath it. A disk can be divided in to multiple partitions. Each of the partitions heists own file system. A file system starts with a root director at the top of the inverted tree. The root directory contains a number of directories, which in turn contain a number of files / subdirectories & soon.

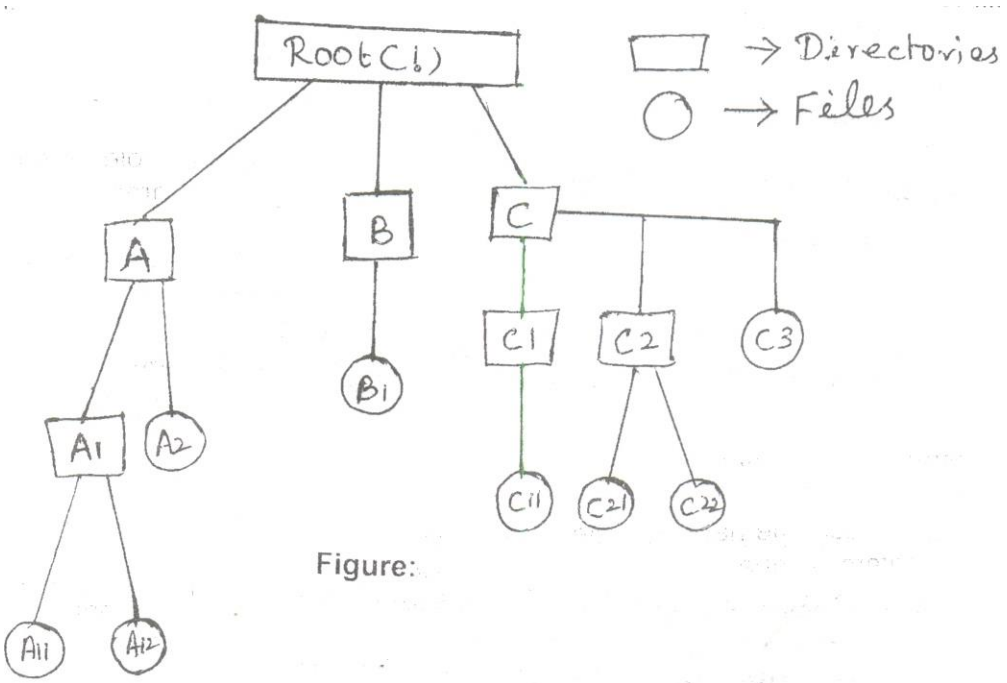



**Figure.**

**Components of UNIX**

Kernel
Shell
File system
Commands

**Structure of UNIX system**

**User**


**Figure:**

**File System**

The basic unit used to organize information in the UNIX system is called a file. The UNIX file system provides a logical method for organizing, storing, retrieving, manipulating & managing information. Files are organized in to a hierarchical file system, with files grouped together into directories. An important simplifying feature of the UNIX system is the general way it treats files. For Eg: physical devices are created as files; this permits the same commands to work for ordinary files & for physical devices i.e., printing a file con a printer) is treated similarly to displaying it on the terminal screen.

**Shell**

Shell reads your commands & interprets them as requests to execute a program which it then arranges to have carried out. Because shell plays this role, it is called common interpreter. Besides being a common interpreter, the shell is also a programming language which permits to control & carry out the commends.

**Kernel:**

It is the part of OS that interacts directly with the hardware of a computer through device drivers that are built in kernel.

**Displaying a Directory**

Is-Lists the names of files in a particular UNIX directory. If you type the is command with no parameters or qualifiers, the command lisp lays the file listed in your current working directory. When you give the 1s command, you can add one or more modifiers to get additional information.

**Example: 1s**

Result: Lists the names of files in your default directory, in alphabetical order.

**Example: Is-a**

Result: Causes all your files to be listed, including those files that begin with a period (i.e., hidden files).

**Displaying and concatenating (Combining) Files**

More – Enables examination of a continuous text one screenful at a time on a terminal. It normally pauses after each screenful, printing – more – at the bottom of the screen. Press <RETURN> to display one more line. Press the <SPACE BAR> to display another screenful. Press the letter Q to stop displaying the file.

Example: more newfile

Result: Displays the contents of "newfile" one screen ("page") at a time.

Cat – Displays the contents of a file on your terminal.

Example: cat newfile

Result: Displays the contents of two files – "newfil" and "oldfile" – on your terminal as one continuous display.

While a file is being displayed, you can interrupt the output by pressing <CTRL/C> and return to the UNIX system prompt. CTRL/S> suspends the terminal display of the file and the processing of the command. To resume display, press <CTRL/Q>. The interrupted command displayed lines beginning at the point at which processing was interrupted.

The cat command is also used to concatenate (Combine) files and put them into another files. If you concatenate files to another one the at already exists, the existing contents are permanently lost.

Example: cat fileone filtwo filethree > newfile
Result: Links together three files – fileone, filetwo and filethree – into a new file called "newfile". The original files remain intact.

**Copy Files**

Cp—Makes copies of your files. You can use it to make copies of files in your default directory, to copy files from one directory to another directory, or to copy files from other devices.

Example: cp fileone filetwo

Result: Copies the contents of fileone to a file named filetwo. Two separate files now exist.

Example: cp/usr/neighbor/testfile.
Result:Copies the file testifile from the directory/user/neighbor to your UNIX account. The period ( . ) at the end of the command line indicates that the file is to be copied to your current working directory and the name will remain the same.

To copy a file from another user's directory on UNIX, you must know the person's username.

Example: cp ~ username/file1 yourfile
Result: Copies the file "filel" from user to your UNIX account. The name of the file in your directory becomes yourfile. (Protections must be set for file to be readable by you in user abcst's directory in order to be able to copy the file).

**Deleting files**

rm – ces. Deletes files. You can enter more than one file specification on a command line by separating the file specifications with

Example: rm newfile
R1lt: Deletes the file named "newfile".
Example: rm newfile oldfile
Rult: Deletes two file – "newfile" and "oldfile".
Result
Example: rm new*
Rlt: Deletes all files that begin with the prefix new.

**Renaming Files**

This command changes the identification (name) of one or more files.

Eg: mv oldfile bin/newfile

Changes the name of the file "oldfile" to "newfile" and places it in the directory/bin. Only one file will exist.

**Printing from UNIX**

The Ipr command prints files on UNIX. Use the-Pqueuename option to select a printer, use the-D option below to get PostScript

**Ipr-PDLH sample.file**

is is the default output. Single-sided output, two pages' worth of text per side, landscape format (132 characters per line by 66 lines per page). Output is queued to printer in (David) Lawrence Hall (DLH) computing lab.

**3. (i) What is the scope of Bioinformatics? Why is it a multidisciplinary field?**

**Applications of Bioinformatics**

Bioinformatics is being used in the following fields:

- Molecular medicine
- Personalized medicine
- Preventative medicine
- Gene therapy
- Drug development
- Microbial genome applications
- Waste cleanup
- Climate change studies
- Alternative energy sources
- Biotechnology
- Antibiotic resistance
- Bioweapon creation
- Evolutionary studies
- Crop improvement for

    - Insect resistance
    - Improved nutritional quality
    - Drought resistant varieties
    - Veterinary Science

**Molecular Medicine**

The human genome has several applications in the fields of biomedical research and clinical medicine. Every disease has a genetic component. This may be inherited or by a result of the body's response to an environmental stress which causes alterations in the expression of certain genes in the genome.

With the completion of the human genome project now we can search for the genes that are directly associated with different diseases and understand the molecular basis of these diseases more clearly. This new knowledge of the molecular mechanisms of disease will be a must for better treatments, cures and development of efficient diagnostic tests.

**Personalized Medicine**

Clinical medicine will become more personalized with the development of the field of pharmacogenomics. Pharmacogenomics is the study of how an individual's genetic inheritance affects the body's response to drugs.

At present, some drugs fail to make them to the market because a small percentage of the clinical patient population show adverse effects to a drug due to sequence variants in their DNA. As a result, potentially life saving drugs never make them to the market place.

**Gene Therapy**

**Gene therapy** is the treatment of genetic diseases by changing the expression of a person's gene.

In the near future, the potential for using genes themselves to treat disease may become a reality. Currently, this field is in its infantile stage with clinical trials for different types of cancer and many other diseases. Without the knowledge of bioinformatics, it is not at all possible to design a gene drug to treat any dreadful disease in man.

**Drug development**

At present all drugs on the market target only about 500 proteins. More knowledge of proteomics and disease mechanisms enable us to identify and validate new drug targets, on which the drugs act selectively. These highly specific drugs promise to have fewer side effects than many of today's medicines.

**Microbial Genome Applications**

In fact, all activities and properties of microbes are determined by their genomes. Therefore, microbial genomics offers a greater insight into the microbial world.

Its capacities have broad and far reaching implications on environment, health, energy and industrial applications.

By studying he genetic material of these organisms, scientists begin to understand these microbes at a very fundamental level. They isolate the genes that give them their unique abilities to survive under extreme conditions to have a thorough knowledge.

**Waste Cleanup**

Deinococcus radiodurans is the world's toughest bacterium and it is the most radiation resistant organism known. Scientists are interested in this organism because of its potential usefulness in cleaning up waste sites that contain radiation and toxic chemicals. Database of microbial diversity is useful for screening such useful microbes.

**Climate change studies**

Extensive use of fossil fuels as energy source is the major cause for carbon dioxide pollution in the atmosphere. Increasing $CO_2$ level has much adverse effects in the global climate. Recently, the DOE (Department of Energy, USA) has launched a program to decrease atmospheric carbon dioxide levels. In this first attempt, the genomes of microbes that use carbon dioxide as their sole carbon source were screened to use them as remedial agents to reduce $CO_2$ level in the atmosphere.

**Alternative Energy Sources**

Scientists are studying the genome of the microbe chlorobium tepidum which has an unusual capacity for generating energy from light, as an alternative energy source.

**Biotechnology**

Biotechnology database offers much more information about microbial biodiversity and usefulness of different microbes that can be used in biotechnology industry. Understanding the physiology and genetic make-up of the organisms will help food manufacturers as well as the pharmaceutical industry. For example,

The archaeobacterium Archaeoglobus fulgidus and true bacterium Thermotoga maritime have much potential for practical applications in industries and government funded environmental remediation.

**Antibiotic Resistance**

Genomics databases provide fine details about the genetic control of antibiotics resistance and mode of its transmission. Scientists have been examining the genome of Enterococcus

faecalis-a leading cause of bacterial infection among hospital patients. They have discovered a virulence region made up of a number of antibiotic-resistant genes taking part in the transformation of harmless gut bacteria in to pathogenic invaders. This region is referred as a pathogen city island. It provides useful markers for detecting pathogenic strains and helps to establish methods to prevent the spreading of infection in wards.

**Bioweapon Creation**

Genomics database provide complete information for artificial synthesis of viruses. Scientists have recently built the Poliomyelitis virus by artificial methods. They did this using genomic data available on the Internet and materials from a mail-order chemical supply. The research was financed by the US Department of Defense as part of a biowarfare response program to the world the reality of bioweapons.

**Evolutionary Studies**

The sequencing of genomes from all three domains of life, eukaryota, bacteria and archaea, means that evolutionary studies can be performed. These studies are performed in order to determine the evolutionary tree of life and the last universal common ancestor.

**Crop improvement**

Information about plant genomes is essential for planning better breeding programs, leading to crop improvement. Comparative Genetics of the plant genomes has shown that the organization of their genes has remained more conserved over evolutionary time than was previously believed. These findings suggest that information obtained from the model crop systems can be used to suggest improvements to other food crops. At present the complete genomes of Arabidopsis thaliana (water cress) and Oryza sativa (rice) are available.

**Veterinary Science**

Sequencing project in farm animals including cows, pigs and sheep will provide a better understanding of the biology of these animals. These projects will have huge impacts on the production and heath of livestock's, which ultimately have benefits for human nutrition.

**(ii) Explain different methods of connecting to Internet & give 5 important domain names.**

If we want to connect our computer to the Internet, we can do so only through an Internet server. There are Companies which have Internet servers and provide Internet connectivity called Internet Service Providers (ISP). They have satellite connections with the Internet. We must apply

to an ISP and ask for an Internet connection. They give a web page, an e-mail box and other facilities. We must have a telephone connection to communicate with the ISP's office, and a device called Modem. Our computer gets connected to the Internet. We connect our computer to the modem, and the modem is connected to the telephone line. This is illustrated in Fig.

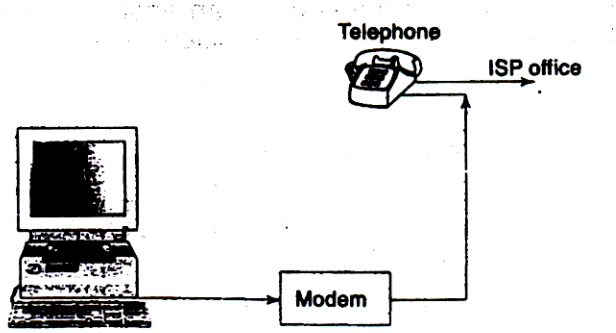



**Figure: Internet Connectivity**

There are various types of Internet connections. The following are some of them.

- Shell Account (text only).
- TCP/IP Account text, graphics, sound and animation).

Shell accounts enable us to browse through text items only; the graphics and multimedia elements cannot be viewed. So the shell account does not permit full use of Internet capability. Using a shell account, we can make use of the e-mail facility. (From June 1999 shell accounts too have multimedia).

Transfer control protocol/Internet Protocol (TCP/IP) is very powerful and most popular. With a TCP/IP connection, we can enjoy Internet fully.

**MODEM**

Conversion of digital signals into signals transmittable through a telephone is called modulation. Converting telephone signals into digital signals is called demodulation. A modem is a device used for modulation and demodulation (Modulator-Demodulator). Modems are classified according to their rate of transfer of data, measured in terms of bytes per second (BPS). If the data transfer capacity of a modem is 14.4 kilobytes, it means that the rate of transfer of data through the modem is 14.4 kilobytes per second. Baud is a synonym BPS, named after J.M.Baudot, the inventor of the telegraph code. So the data transfer rate of a 14.4 K modem means that its data transfer rate is 14.4 kilobytes. Common modems are of 14,400 BPS; there are also 28 800 BPS modems are called V.34. An international forum, the CCITT (Committee for Consultation on Interantional Teleraph and Telephone), has defined a series of standards for modems. V.32, V32 bis are some examples of CCITT standards.

**INTERNET ADDRESSING**

Millions of computers have been connected to the Internet. When we want to communicate with a computer through the Internet, we need to specify the address of that computer, every computer connected to the Internet is given an address, called the IP Address [Internet Protocol Address]. The IP address of a computer consists of four groups of number separated by periods or dots (.). For example, 18.181.0.24 is an IP address.

All users have text-based addresses. Which, an Internet service called DNS (Domain Name System) converts into the actual numerical address. Now let us look further into the text-based Internet address. This address is also called the Uniform Resource Locator (URL). The Internet address has two parts. They are the:

- Name of the user (user ID)
- Name of the server (server ID)

The @ symbol is given in between the above two names. For example, our address is stxavier@md2.vsnl.net.in

All addresses on the Internet are classified into several top-level domains. The classification may either be geographical or organizational. Table shows some examples of codes used for geographical classification.

**Table**

**Geographical domains**

| Domain | Country |
|---|---|
| At | Austria |
| Au | Australia |
| Ca | Canada |
| Ch | (Confederatio Helvetical) |
| De | Germany, (Deutschland) |
| Dk | Denmark |
| As | Spain (Espana) |
| Fr | France |
| Gr | Greece |
| Ie | Republic of Ireland |
| In | India |
| Jp | Japan |
| Uk | English (United Kingdom) |
| Us | USA |

Another top-level domain classification is based for the nature of the organization. For example, all universities and educational institutions are given "edu" as the top-level domain name. other top-level domain names for organizations are given in Table.

**Organization-based domains**

| Domain | Organization |
|---|---|
| Com | Commercial organization |
| Edu | Educational institution |
| Gov | Government |
| Int | International organization |
| Mil | Military |
| Net | Networking organization |
| Org | No-profit organization |

The first part of the URL specifies the protocol used. Table shows some URLs.

**Physical Connections**

As we have already seen, Internet connectivity is done through a modern and a telephone line as shown in Fig. The path of the data is as follows.

1. The user's node sends the digital data.
2. The modem of the user coverts the digital data into analog signals.
3. The telephone line transmits the data to the modem of the ISP.
4. The modem of the ISP converts the analog signals received into digital data.
5. The ISP's node receives the message in digital form.

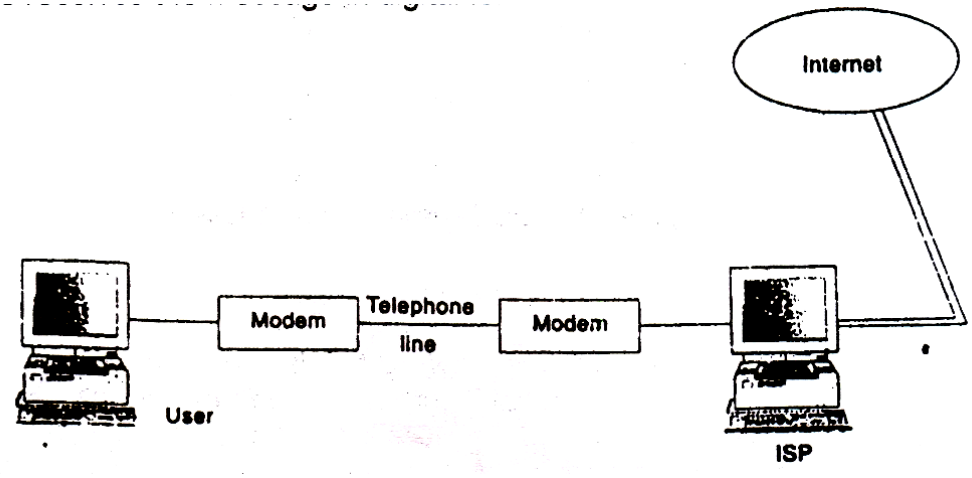



**Figure: Internet Connectivity**

In this communication process, two important devices work together.

- Modem
- Telephone line

The quantity of data transferred by a device is called its throughput or data transfer rate. The throughput is measured in terms of KBPS (KiloBytes Per Second). For example, the speed of two popular types of modems are shown in Table.

**Two modems**

| Modem | Speed |
|---|---|
| V.32 bits modem | 14.4 KBPS |
| V.34 bits modem | 28.8 KBPS |

Most of the V.34 modems can compress the data in ratio of 4:1, they can achieve a throughput of 28.8 x 4 – 115.2 KBPS in an ideal situation. However, practically they can achieve a speed of at least 56 KBPS.

**Telephone Lines**

Usually, an ordinary Internet account holders gets only a dial-up connection. This means that several users share one port of the ISP Server and at one time, only one user can work. The port is connected to a particular telephone number. When one user dials the telephone number get physical connection to the server, if the port is already in use by another user, an engaged tone will be heard. When the port is free, the user gets connected to the ISP and can work on the Internet. Figure shows the dial-up connection of users.

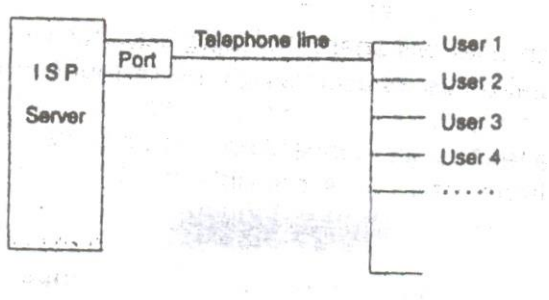



**Figure: Dial-up Connections of Users**

**Leased Lines**

Instead of a dial-up connection, one can get a leased line connection, which is very expensive. In this case, the user is given full freedom on a port of the ISP server. One port is for

his exclusive use. So he can use it around the clock without any interruption. Whenever the user dials, he gets a connection and can work on the Internet. Figure shows the leased line of a user.

**Speed of Telephone Lines**

One can achieve a speed of 56 KBPS only in the case of a leased line. In the case of dial-up connection, the telephone connection is so designed that three or four users can simultaneously use the same port. This is possible by getting four connections on the same number.

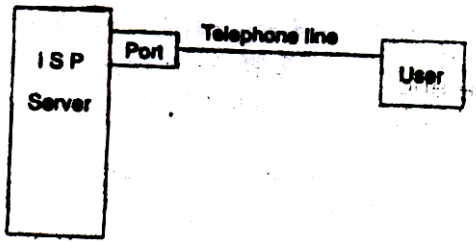



Figure: Leased Line of a User

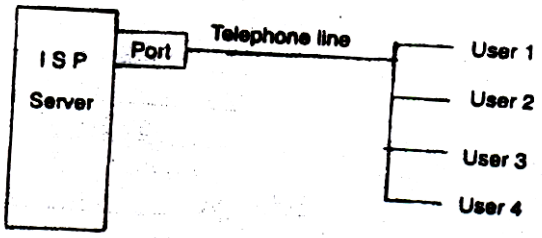



**Figure: Dial-up Accessing a Port Simultaneously**

In such a case, each user will share the line. So, any user can get a speed of only 56/4 – 14 KBPS. Figure shows dial-up users accessing a port simultaneously.

It a company gets a leased line connection, it is possible for several units of the company to access the Internet simultaneously. It is possible to have several types of media in telephone connections, as shown in Table.

**4. Define search engines & explain it in detail with examples.**

**Searching Engines**

A search engine is an interactive tool that help access information available via the World wide Search engines are actually databases that contain referrer from thousands of resources. They serve as interfaces between users and distance web servers.

Users can submit a query that ask the database it if certain resources that matches the query.

Thus search engines give a list of topics like New, B ness, Education, Entertainment, Arts and Sports and Popular search engines are Google, Yahoo, Lycos, Alta etc.

A word, phrase, title, date or some other criteria submitted as a query to the search engines. The search engine run the search string against the database and return list of resources that match with the criterion to the use. They present the list in the form of hyperlinks. The users link it to get enough information.

To start a search engine, the URL of the search engine typed in the address box. For example, to use the Yahoo se engine, its URL http://www.yahoo.com is typed in the address box and 'GO' button or Enter key is pressed. The main page Yahoo is displayed in the screen.

**Table: Some popular search engines and their URLs.**

| Search Engines | URLs |
|---|---|
| Google | www.google.com |
| Yahoo | www.yahoo.com |
| Excite | www.excite.com |
| Lycos | www.lycos.com |
| HotBot | www.hotbot.com |
| WebCrawler | www.webcrawler.com |
| infoSeek | www.infoseek.com |
| Alta Vista | www.altavista.com |
| Ask Jeeves | www.askjeeves.com |
| LookSmart | www.looksmar.com |

**Google (http://www.google.com/)**

**Google** is one of the most popular search engines to interact with World wide Web. It was selected as the most Outstanding Search Engine four times by Search Engine Watch Readers.

Google was originally a Stanford University project Back Rub by students Larry Page and Sergey Brin. By 1998, the name had been changed to Google and the project jumped off campus and became the private company google. It remains privately held today.

Google has a well-deserved reputation as the top choice for searching in web. The crawler-based service provides both comprehensive coverage of the web along with great relevancy.

Google has facilities to help the users to search sites from local country as well as world resources. If local information is required, local country is selected before proceeding the search. Otherwise, search engines look for world resources in web.

Using on the top of the search box on the Google home page, one can easily seek out images from across the web:

- Discussions that are taking place on Usenet newsgroups,
- Locating new information
- Perform product searching.

Google is also offers a wide range of features, such as

a. excellent spell checking,
b. easy access to dictionary definitions,
c. integration of stock quotes,
d. street maps, telephone number and more

The Google Tool bar has also won a popular following for the easy access from the Internet Explorer browser.

**Yahoo (http://www.yahoo.com/)**

Yahoo is the web's oldest "directory," launched in 1994. However, in October 2002, Yahoo made a giant shift to crawler based listings for its main results. These came from Google until February 2004. Now, Yahoo uses its own search technology.

In addition to excellent search results, one can use tabs above the search box on the Yahoo home page to seek images. Yellow Page listings or use Yahoo's excellent shopping search engine.

Like Googel, Yahoo sells paid placement advertising links that appear on its own site which are distributed to others. These are sold through Overture. Yahoo purchased Overture in a company in October 2003.

**AltaVista (http://www.altavista.com/)**

AltaVista was a premier search engine opened in December 1995 and was originally owned by Digital. It was taker over by Compaq, when that company purchased Digital in 1998. Alta Vista was later spun off into a private company, controlled by CMGI. It shows the following features:

- It includes a vast amount of inclusive indices.
- It helps to search web as well as usenet new news groups.
- It searches words, phrases, Boolean operators, URLS page titles and related links.
- It returns the results very quickly.
- The search results are standard, compact and in details format.
- It has facilities to give simple and advanced researches.
- Whole word searching is very precise and sensitive.
- It is most likely for searching scientific words.

**Lycos (http://www.lycos.com/)**

Lycos is one o the oldest search engines on the web, launched in 1994. it ceased crawling the web for its own listings in April 1999. it provides access to human-powered results from LookSmart for popular queries and crawler-based results from Yahoo for others.

Lycos is owned by Terra Lycos, a company formed with Lycos and Terra Networks merged in October 2000. Terra Lycos also owns the HotBot search engines.

**HotBot (http://www.hotbot.com/)**

HotBot provides easy access to the web's three major crawler-based search engines: Yahoo, Google and Teoma. Unlike meta search engine, it cannot blend the results from all of these crawlers together. Nevertheless, it's a fast, easy way to get different web search "opinions" in one place.

HotBot's "choose a search engine" interface was introduced in December 2002. however, HotBot has a long his as a search brand before this data.

HotBot, debuted in May 1996, gained a strong following among serious searchers for the quality and comprehensives of its crawler-based results. It also caught the attention of experienced web users and techies, especially for the annual colors and interface it continues to sport today.

**5. (i) What is the central Dogma of molecular Biology? How can Molecular biology be considered as an information science.**

**Central dogma of molecular biology**

The expression of biomolecules is governed by the central dogma of molecular biology. Central dogma can be stated as "DNA makes RNA makes Protein". The processes related to

central dogma are replication, transcription and translation. The creation of multiple copies of DNA itself is called replication. The schematic of the central dogma is given in Figure:

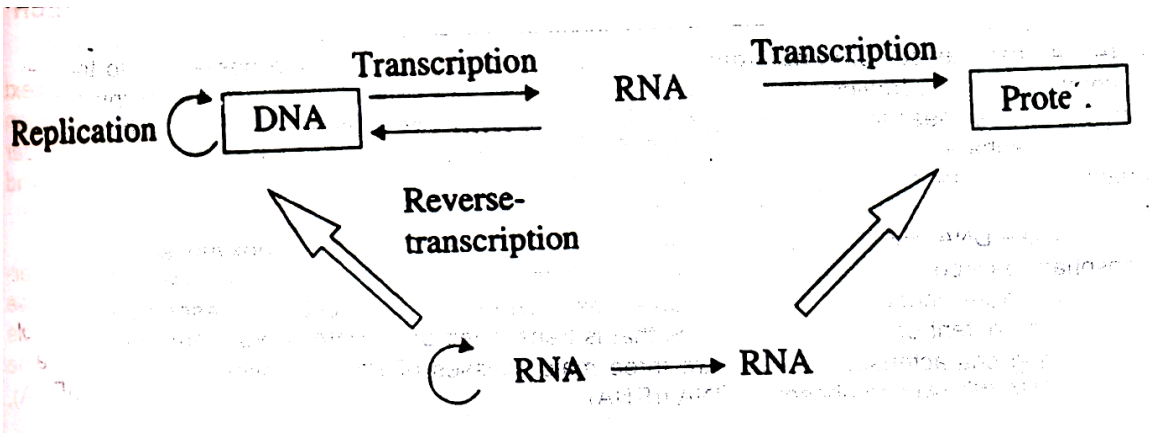



**Figure: the central dogma is about the flow of biological information and explains the expression of the biological information into function units.**

As mentioned above, the biomolecules playing the critical role in the central dogma are the nucleic acids and proteins. Nucleic acids play various roles and they have various functional sites that enable them to play these roles. There re other molecules that interact with this information flows system. The molecules, their roles and the summary of the process are summarized in Table.

**Table: Molecules Participating in Information flow and the Functional Sites**

| Molecule | Role | Functional Sites | Interacting Molecules |
|---|---|---|---|
| DNA | Replication<br>Transcription | Replication origin Promotor Enhancer Operator and other prokaryotic Regulators | Original recognition complex RNA polymerase Transcription factor Repressor, etc |
| RNA | Post-transcriptional processing<br>Translation | Splice site<br><br>Translation initiation site | Spliceosome<br><br><br>Ribosome |
| Protein | Post-translational processing | Cleavage site Phosphory lation and other modification sites ATP binding sites | Protease<br>Protein kinase, etc. |
| | Protein sorting | Signal sequence, localization signals | Signal recognition particle DNA |
| | Protein function | DNA binding sites<br>Ligand binding sites<br>Catalytic sites | Ligands<br>Various other molecules |

Various terms that are commonly used in relation to the central dogma, are briefly explained below in the following sections.

**Nucleic acids: DNA and RNA**

All plants, animals, bacteria and some viruses contain genetic information in the form of DNA. A characteristic of living organisms is that DNA is reproduced and passed on to the next generation. DNA contains the instructions for making proteins. It is a blueprint of information composed of a linear array of nucleotides, each of which a has a base plus a deoxyribose sugar and a phosphate. There are four types of bases: cytosine (C), thymine (T), adenine (A) and guanine (G). DNA is in the form of a double helix.

Unlike DNA, RNA is single-stranded. It contains ribose instead of deoxyribose in its sugar-phosphate backbone, and uracil (U) instead of thymine (T) in its pyramidine bases. It can be assembled from ribonucleotides using DNAS as a template. Transcription preserves the whole information content of the DNA sequence that is transcribed on to RNA, since RNA has the same base-pairing characteristics. There are three major classes of RNA: messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

**DNA replication**

DNA replication or DNA copying mechanism is a semi-conservative process. It involves unwinding of the two strands of a parental DNA duple, with each strand serving as the template for the synthesis of a new strand, complementary to and wound about the parental strand. At each base of the new strand, the complementary base to the parental strand is present and is held in position during polymerization by base pairing.

**Genes**

A gene is a segment of DNA representing nucleotides required for the production of a functional protein or a functional RNA molecule. Genes range in size from small (1.5 kb for glob in gene) to large (approximately 2000 kb for Duchenne muscular dystrophy gene). A gene includes not only the actual coding sequences but also adjacent nucleotide sequences required for the proper expression of genes. The structure and function of genes is discussed in a later chapter.

**Genome**

Genome constitutes the total genetic material of an organism. The human genome is highly complex and contains about three billion nucleotides. The human genome has been sequenced recently; there are several other organisms that have been sequenced already.

**Chromatin**

The eukaryotic chromosome is composed of DNA and proteins, together called chromatin. The proteins include positively charged basic proteins called his tones as well as non-his tonic proteins. The basic structural unit of chromatin is a nucleosome, which is a complex o DNA with a core of his tones.

Nucleosomes are further compacted to form solenoids, which are packed into loops, and each of these contains about 100,000 base pairs of DNA. The loops are the fundamental units of DNA replication and/or gene transcription. The protein machine that copies the chromosomes also plays a direct role in preserving the developmental state of cells. Chromatin is also reproduced for each gene to remain active or inactive as it was in the mother cell. The same complex of proteins that copies the DNA also transfers at least some of the special marks to the new chromosome as well.

**(ii) Explain the following briefly**

**(i) Sequence submission methods**

1. Sequin. A data submission tool hat includes ORF Finder, and alignment viewer/editior, and a link to PowerBLAST.
2. Banklt. It is a www submission tool for one or simple sequence submissions.

SRS as discussed in the earlier chapter is a system that allows you to browse the contacts of database through a web interface, exploring links to other databases and lunching other programs on the retrieved database records. It accesses the following databases:

**(a)** **EMBL Nucleotide Database.** Europe's primary collection of nucleotide sequences is maintained din collaboration with Genbank (USA) and DDBJ (Japan).
**(b)** **Swiss-**Prot. A complete annotate protein sequence database.
**(c)** **Macromolecular Structure Database.** European Project for the management and distribution of data on macromolecular structures.
**(d)** **Array Express.** For gene expression data.
**(e)** **ENSEMBL.** Providing up to date completed met azoic genomes and the best possible automatic annotation.

DBGEET (http://www.geneome.ad.jp/dbget/) is an integrated database retrieved system. DBGET has three basic commands:

1. bget, which performs the retrieval of database entries specified by the combination of dbname: identifier.
2. bfind, which is used for searching entries by keywords.
3. blink, which is the LinkDB search used to retrieve related entries in a given database or all databases in GenomeNet.

DBGET operates differently from Entrees and SRS in the manner that no keyword indexing is performed when a database is installed or updated. Instead, selected fields are extracted and stored in separate files for find searches. This results in rapid database updates. Sequence similarity searches by BLAST and FASTA, sequence motif searches by MOTIF, and biological searches in KEGG are all linked to the DBGET system.

Biology Workbench (http://workbench.sdsc.edu/) is an integrated tool for performing searches on protein and nucleotide databases. The home page is shown in Figure.

BioRs (http://mips.gsf.de/projects/biors) is a retrieval system for biological data developed by Biomax Informatics AG to perform various retrieval tasks. The system retrieves specific biological data from different data sources and allows easy integration of heterogeneous public proprietary database.

**(ii) Genbank & FASTA formats.**

**GenBank**

GenBank (Genetic Sequence Databank) is one of the lowest growing repositories of known genetic sequences. It flat structure that is an ASCII text file, readable by humans and computers. In addition to sequence data, Gen files contain information like accession numbers, gene phylogenetic classification and references to public literature. There are approximately 28, 507, 990, 166 bases in 22, 318, 883 sequence records as of January 2003.

URL: http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html

**GenBank format**

A sequence file in GenBank format can contain several sequences. One sequence in GenBank format starts with a line containing the world LOCUS and a number of annotation lines. The starts of the sequence is marked by a line containing "ORI-GIN" and the end of the sequence is marked by two slashes ("//").

**An example sequence in GenBank forat is:**

LOCUS AB000263 368 mRNA linear PRI 05-FEB-1999
DEFINITION Home sapiens mRNA for prepro cortication like peptide, complete cads
ACCESSION AB000263.

**(iii) PHYLIP multiple alignment format**

**PHYLIP**

PHYLIP is Phylogenetic Inference Package. It can infer phylogenies by parsimony, compatibility, distance matrix methods and likelihood. It can also,

i. compute consensus tree,
ii. compute distance between trees,
iii. draw trees, etc.

Consensus trees are a convenient way to summarize the agreement between two or more trees.

It can handle data of nucleotide sequences, protein sequences, restriction sites or restriction fragments.

**6. Define Hardware & explain its components in detail.**

Hardware is a general term that refers to the physical artifacts of technology. It may also mean the physical components of a computer system, in the form of computer hardware.

What is Hardware?

Your PC (Personal Computer) is a system, consisting of many components. Some of those components, like Windows XP, and all your other programs, are software. The stuff you can actually see and touch, and would likely break if you threw it out a fifth-story window, is hardware.

Note everybody has exactly the same hardware. But those of you who have a desktop system, like the example show in figure 1, probably have most of the components shown in that same figure. Those of you with notebook computers probably have most of the same components. Only in your case the components are all integrated into a single book-sized portable unit.

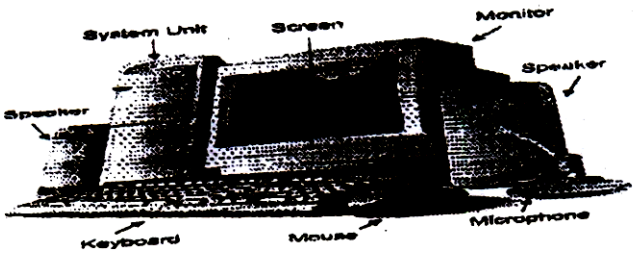



**Figure:**

The system unit is the actual computer, everything else is called a peripheral device. Your computer's system unit probably at least one floppy disk drive, and one CD one CD or DVD drive, into which you can insert floppy disks and CDs. There's another disk drive, called the hard disk inside the system unit, as shown in Figure 2. You can't remove that disk, or even see it. But it's there. And everything that's currently "in your computer" is actually stored on that hard disk. (We know this because there is no place else inside the computer where you can store information!)

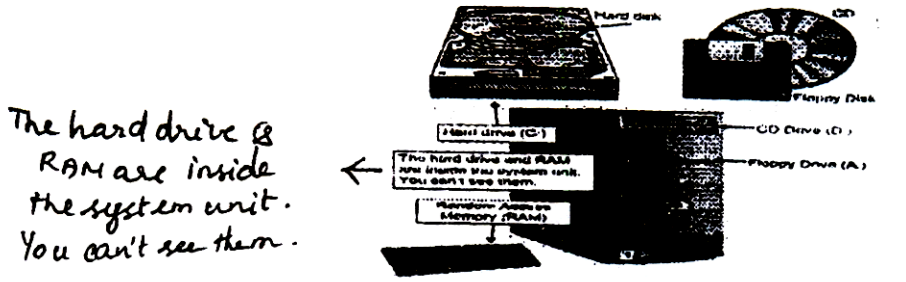



**Figure:**

They floppy drive and CD drive are often referred to as drive with removable media or removable drives for short, because you can remove whatever disk is currently in the drive, and replace it with another. Your computer's hard disk can store as much information as tens of thousands of floppy disks, so don't worry about running out of space on your hard disk any time soon. As a rule, you want to store everything you create or download on your hard disk. Use the floppy disks and CDs to send copies of flies through the mail, or to make backup copies of important items.

**Random Access Memory (RAM)**

There's too much "stuff" on your computer's hard disk to use it all at the same time. During the average session sitting at the computer, you'll probably use only a small amount of all that's available. The stuff you're working with at any given moment is stored in random access

memory (often abbreviated RAM, and often called simply "memory"). The advantage using RAM to store whatever you're working on at the moment is that RAM is very fast. Much faster than any disk. For you, "fat" translates to less time waiting and more time being productive.

So if RAM is so fast, why not put everything in it? Why have a hard disk at all? The answer to that lies in the fact that RAM is volatile. As soon as the computer is shut off, whether intentionally or by an accidental power outage, every thing in RAM disappears, just as quickly as a light bulb goes out when the plug is pulled. So you don't want to rely on RAM to hold everything. A disk, on the other hand, holds its information whether the power is on or off.

**The Hard Disk**

All of the information that's "in your computer", so to speak , is stored on your computer's hard disk. You never see that actual hard because it's sealed inside a special housing and needs to stay that way. Unlike RAM, which is volatile, the hard disk can hold information forever – with or without electricity. Most modern hard disks have tens of billions of bytes of storage space on them. Which, in English, means that you can create, save, and download files for months or years without using up all the storage space it provides.

In the unlikely event that you do manage to fill up your hard disk, windows will start showing a little message on the screen that reads "You are running low on disk space" well in advance of any problems. In fact, if that message appears, it won't until you're down to about 800 MB of free space. And 800 MB of empty space is equal to about 600 blank floppy disks. That's still plenty of room!

**The Mouse**

Obviously you know how to use your mouse, since you must have used it to get here. But let's take a look at the facts and buzzwords anyway. Your mouse probably has at least two buttons on it. The button on the left is called the primary mouse button, the button on the right is called the secondary mouse button or just the right mouse button, I'll jus refer to them as the left and right mouse buttons. Many mice have a small wheel between the two mouse buttons, as illustrated in Figure.

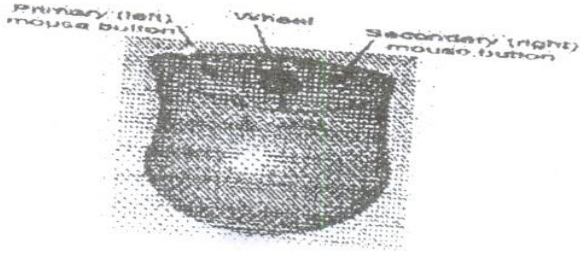



**Figure:**

The idea is to rest your hand comfortably on the mouse, with your index finger touching (but not pressing on) the left mouse button. Then, as you move the mouse, the mouse pointer (the little arrow on the screen) moves in the same direction. When moving the mouse, try to keep the buttons aimed toward the monitor don't "twist" the mouse as that just makes it all the harder to control the position of the mouse pointer.

If you find yourself reaching too far to get the mouse pointer where you want it to be on the screen, just pick up the mouse, move it to where it's comfortable to hold it, and place it back down on the mouse pad or desk. The buzzwords that describe how you use the mouse are as follows:

- Point: To point to an item means to move the mouse pointer so that it's touching the item.
- Click: Point to the item, then tap (press and release) the left mouse button.
- Double-click: Point to the item, and tap the left mouse button twice in rapid succession-click-click as fast as you can.
- Right-click: Point to the item, then tap the mouse button on the right.
- Drag: Point to an item, then hold down the left mouse button as you move the mouse. To drop the item, release the left mouse button.
- Right-drag: Point to and item, then hold down the right mouse button as you move the mouse. To drop the item, release the right mouse button.

**The Keyboard**

Like the mouse, the keyboard is a means of interacting with your computer. You really only need to use the keyboard when you're typing text. Most of the keys on the keyboard are laid out like the keys on a typewriter. But there are some special keys like Esc (Escape), Ctrl (Control), and Alt (Alternate). There are also some keys across the top of keyboard labeled F1, F2, F3, and so forth. Those are called the function keys, and the exact role they play depends on which program you happen to be using at the moment.

Most keyboards also have a numeric keypad with the keys laid out like the keys on a typical adding machine. If you're accustomed to using an adding machine, you might want to use numeric keypad, rather than the numbers across the top of the keyboard, to type numbers. It doesn't really matter which keys you use. The numeric keypad is just there as a convenience to people who are accustomed to adding machines.


**Figure:**

Most keyboards also contain a set of navigation keys. You can use the navigation keys to move around through text on the screen. The navigation keys won't move the mouse pointer. Only the mouse moves the mouse pointer.

On smaller keyboards where space is limited, such as on a notebook computer, the navigation keys and numeric keypad might be one in the same. There will be a Num Lock key on the keypad. When the Num Lock key is "on", the numeric keypad keys type numbers. When the Num Lock keys is "of", the navigation keys come into play. The Num Lock key acts as a toggle. Which is to say, when you tap it, it switches to the opposite state. For example, if Num Lock is on, tapping that key turns off. If Num Lock is off, tapping that key turns Num Lock on.

**7. Define topology & explain its types in detail.**

**Topology**

Network topology is the study of the arrangement or mapping of the elements (links, nodes, etc.) of a network, especially the physical (real) and logical (virtual) interconnections between nodes. [1][2]+ A local area network (LAN) is one example of a network that exhibits both a physical topology and a logical topology. Any given node in the LAN will have one or more links to one or more other nodes in the network and the mapping of these links and nodes onto a graph results in a geometrical shape that determines the physical topology of the network. Likewise, the mapping of the flow of data between the nodes in the network determines the logical topology of the network. It is important to note that the physical and logical topologies might be identical in any particular network but they also may be different. Any particular network topology is determined only by the graphical mapping of the configuration of physical and/or logical connections between nodes. LAN Network Topology is, therefore, technically a part of graph theory. Distance between nodes, physical interconnections, transmission rates, and/or signal types may differ in two networks and yet their topologies may be identical.

**Basic types of topologies:**

The arrangement or mapping of the elements of a network gives rise to certain basic topologies which may then be combined to form more complex topologies (hybrid topologies). The most common of these top right of this basic types of topologies are (refer to the illustration at the page).

- Bus (Linear, Linear Bus)
- Star
- Ring
- Mesh

- Partially connected mesh (or simply 'mesh')
- Fully connected mesh

- Tree
- Hybrid
- Point to Point

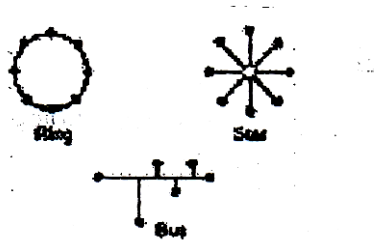


**Figure:**

**Topology**

The physical layout of a network, referred to as its topology, is a function of the practical constraints imposed by the environment, the protocols that must be supported, and the cost of installation. The most common protocols used with LANs, Ethernet and token ring, assume a bus and ring topology, respectively. The star topology is often used as a hub to connect several networks and in wireless networks, where multiple devices connect via radio frequency, it links to a central wireless access point or wireless hub.

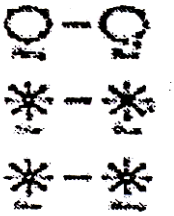
The three pure topologies-ring, bus, and star-illustrated in Figure, rarely exist alone. More likely, they are part of a hybrid network such as small workgroup connected by bus to a star network in another workgroup, perhaps supporting computers running under a different operating system as well.

The practical implication of network topologies are material and labor costs associated with running cable and purchasing and installing the new network electronics. For example, in establishing a laboratory with a new network, running cables from one workstation position to the next to support a bus topology is usually cheaper and les labor-intensive than running cables from each workstation and device position to a central closet to support a star topology. However, although wiring a laboratory to support a star topology is much more expensive in terms of cable required and the labor involved in pulling all cables to a central closet, the cables pulled for a star topology can be easily reconfigured in a bus to support Ethernet or token ring protocols.

Similarly, a network wired in a ring topology can easily be converted to support an Ethernet bus by breaking the ring and installing the appropriate electronics. These modifications are illustrate in. This conversion of topologies is most difficult going from ring or bus topology to

a star topology, because the electronics in the hub or center of the star typically controls each spoke of the hub individually, normally requiring a separate cable from the hub to each device.

**Figure Network Topology Conversion. Network topologies initially configured to support one protocol can be modified to support others. For example, a star topology can be converted to a bus or ring topology, and a ring can be converted to a bus topology.**



**Classification of network topologies**

There are also three basic categories of network topologies:

- physical topologies
- signal topologies
- logical topologies

The terms signal topology and logical topology are often used interchangeably even though there is a subtle difference between the two and the distinction is not often made between the two.

**Physical topologies**

The mapping of the nodes of a network and the physical connections between them – i.e., the layout of wiring, cables, the locations of nodes, and the interconnections between the nodes and the cabling or wiring system

**Signal topology**

The mapping of the actual connections between the nodes of a network, as evidenced by the path the signals take when propagating between the nodes.

Note: The term 'signal topology' is often used synonymously with the term 'logical topology' however, some confusion may result from this practice in certain situations since, by definition, the term 'logical topology' refers to the apparent path that the data takes between nodes in a network while the term 'signal topology' generally refers to the actual path that the signals (e.g., optical, electrical, electromagnetic, etc.) take when propagating between nodes.

Example

In an 802.4 Token Bus network, the physical topology may be a physical bus, a physical star, or a hybrid physical topology, while the signal topology is a bus (i.e., the electrical signal propagates to all nodes simultaneously [ignoring propagation delays and network latency]), and the logical topology is a ring (i.e., the data flows from one node to the next in a circular manner according to the protocol).

**Logical topology**

The mapping of the apparent connections between the nodes of a network, as evidenced by the path that data appears to take when traveling between the nodes.

**8. Explain TELNET in detail.**

**Telnet Protocol**

The TCP/IP protocol suite includes a simple textual remote terminal protocol called TELNET that allows a user to log into a computer across an internet. TELNET establishes a TCP connection, and then passes keystrokes from the user's keyboard directly to the remote computer as if they had been typed on a keyboard attached to the remote machine. TELNET also carries textual output from the remote machine back to the user's screen. The service is called transparent because it gives the appearance that the user's keyboard and display attaché directly to the remote machine.

TELNET offers three basic services. First, it defines a network virtual terminal that provides a standard interface to remote systems. Client programs do not have to understand the details of all possible remote systems; they are built to use the standard interface. second, TELNET includes a mechanism that allows the client and server to negotiate options, and it provides a set of standard options (e.g., one of the options controls whether data passed across the connection uses the standard 7-bit ASCII character set or an 8-bit character set). Finally, TELNET tears both ends of the connection symmetrically. In particular, TELNET does not force client input to come from a keyboard, nor does it force the client to display output on a screen. Thus, TELNET allows an arbitrary program to become a client. Furthermore, either end can negotiate options.

Figure. illustrates how application programs are used to implement a TELNET client and a TELNET server.

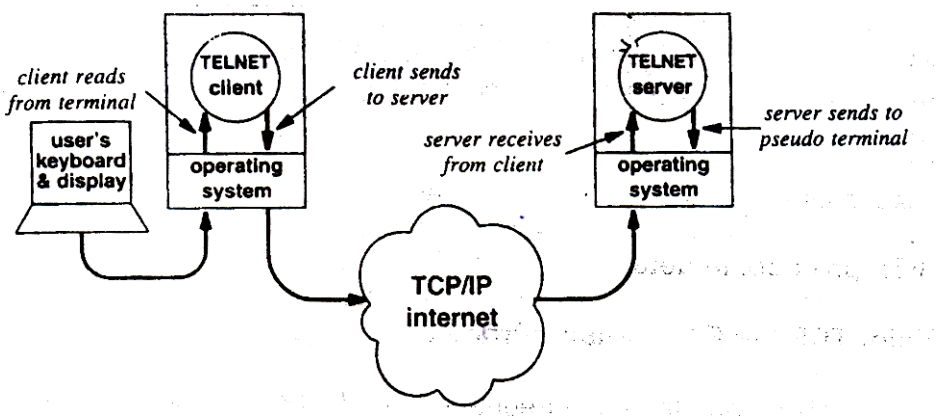



**Figure: The path of data in a TELNET remote terminal session as it travels from the user's keyboard to the remote operating system.**

As the figure shows, when a user invokes TELNET, an application program on the user's machine becomes the client. The client establishes a TCP connection to the server over which they will communicate. Once the connection has been established, the client accepts keystrokes from the user's keyboard and sends them to the server, while it concurrently accepts characters that the serer sends back and displays them on the user's screen. The server accepts a TCP connection from the client, and then relays data between the TCP connection and the local operating system.

In practice, the server is more complex than the figure shows because it must handle multiple, concurrent connections. Usually, a master server process waits for new connections and creates a new slave copy to handle each connection. Thus, the 'TELNET server', show in Figure, represents the slave that handles one particular connection. The figure does not show the master server than listens for new requests, nor does it show the slaves handling other connections.

We use the term pseudo terminal * to describe the operating system entry point that allows a running program like the TELNET server to transfer characters to the operating system as if they came from a keyboard. It is impossible to build a TELNET server unless the operating system supplies such a facility. If the system supports a pseudo terminal abstraction, the TELNET server can be implemented with application programs. Each slave server connects a TCP stream from one client to a particular pseudo terminal.

Arranging for the TELNET server to be an application-level program has advantages and disadvantages. The most obvious advantage is that is makes modification and control of the server easier than if the code were embedded in the operating system. The obvious disadvantage is inefficiency. Each keystroke travels from user's keyboard through the operating system to the client program, from the client program back through the operating system and across the underlying internet to the server machine. After reaching the destination machine, the data must travel up through the server's operating system to the server application program and from the

terminal entry point. Finally, the remote operating system delivers the character to the application program the user is running. Meanwhile, output (including remote character echo if that option has been selected) travels back from the server to the client over the same path.

Readers who understand operating systems will appreciate that for the implementation shown in figure, every keystroke requires computers to switch process context several times. In most systems, an additional context switch is required because the operating system on the server's machine must pass characters from the pseudo terminal back to another application program (e.g., a command interpreter). Although context switching is expensive, the scheme is practical because users do not type at high speed.

**9. Explain FTP protocol in detail.**

**FTP: The Major TCP / IP File Transfer Protocol:**

File transfer is among the most frequently used TCP/IP applications, and still accounts for a nontrivial amount of Internet traffic. Standard file transfer protocols existed for the ARPENT before TCP/IP become operational. These early versions of file transfer software evolved into a current standard known as the File Transfer Protocol (FTP).

**FTP Features:**

Given a reliable end – to – end transport protocol like TCP, file transfer might seem trivial. However, as the previous sections pointed out, the details of authorization, naming, and representation among heterogeneous machines make the protocol complex. In addition, FTP offers many facilities beyond the transfer function itself.

- Interactive Access. Although FTP is designed to be used by programs, most implementations also provide an interactive interface that allows humans to interact with remote servers.
- Format Specification. FTP allows the client to specify the type and representation of stored data. For example, the user can specify whether a file contains text or binary data and whether text files use the ASCII or EBCDIC character sets.
- Authentication control. FTP requires clients to authorize themselves by sending a login name and password to the server before requesting file transfers. The server refuses access to clients that cannot supply a valid login and password.

**FTP Process Model**

Like other servers, most FTP server implementations allow concurrent access by multiple clients. Clients use TCP to connect to a server. As described in chapter, a single master server

process awaits connections and creates a slave process to handle each connection. Unlike most servers, however, FTP slave process does not perform all necessary computation. Instead, the salve accepts and handles a control connection from the client, but uses an additional process and an additional TCP connection to handle each data transfer operation. The control connection carriers commands that tell the server while file to transfer. A new TCP connection and a new process on both the client and server side is created for each data transfer operation (i.e. each file transfer). While the exact details of the process architecture depend on the operating systems used, Figure illustrates the concept.

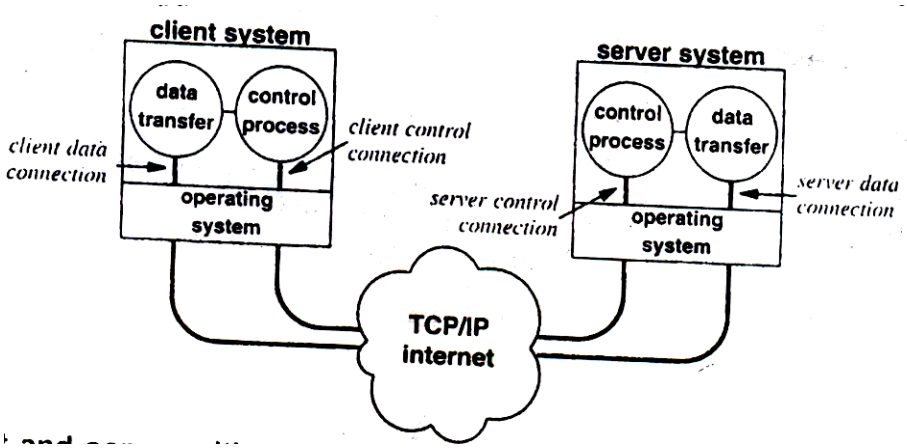



**Figure: An FTP client and server with a TCP control connection between them a separate TCP connection between their associated data transfer processes.**

As the figure shows, the client control process connects to the server control process using one TCP connection, while the associated data transfer process use their own TCP connection. In general , the control processes and the control connection remain alive as along as the user keeps the FTP session active, and a data transfer connection persists for one file transfer. The idea can be summarized.

Data transfer connections and the data transfer processes that use them are created dynamically when needed, but the control connection persists throughout a session. Once the control connection disappears, the session is terminated and the software at both ends terminates all data transfer processes.

**10. Explain HTTP in detail.**

**Hypertext Transfer Protocol**

The protocol used for communication between a browser and a web server or between intermediate machines and web servers is known as the HyperText Transfer Protocol (HTTP) . HTTP has the following set of characteristics:

*Application Level.* HTTP operates at the application level. It assumes a reliable, connection-oriented transport protocol such as TCP, but does not provide reliability or retransmission itself.

*Request/Response.* Once a transport session has been established, one side (usually a browser)must send an HTTP request to which the other side responds.

*Stateless.* Each HTTP request is self-contained ; the server does not keep a history of previous requests or previous sessions.

*Bi-Directional Transfer.* In most cases, a browser requests a web page, and the server transfers a copy to the browser. HTTP also allows transfer from a browser to a server (e.g. when a user submits a so-called "form").

*Capability Negotiation.* HTTP allows browsers and servers to negotiate details such as the character set to be used during transfers. A sender can specify the capabilities it offers, and a receiver can specify the capabilities it accepts.

*Support For Caching.* To improve response time, a browser caches a copy of each web page it retrieves. If a user requests a page again, HTTP allows the browser to interrogate the server to determine whether the contents of the page has changed since the copy was cached.

*Support For Intermediaries*. HTP allows a machine along the path between a browser and a server to act as a proxy server that caches web pages and answers a browser's request from its cache.

**HTTP GET Request**

In the simplest case, a browser contacts a web server directly to obtain a page. The browser begins with a URL, extracts the hostname section, uses DNS to map the name into an equivalent IP address, and uses the IP address to form a TCP connection to the server. Once the TCP connection is in place, the browser and web server use HHHTTP to communicate; the browser sends a request to retrieve a specific page, and the server responds by sending a copy of the page.

A browser sends an HTTP GET command to request a web page from a server+. The request consists of a single line of text that begins with the keyword GET and is followed by a URL and an HTTP version number. For example, to retrieve the web page in the example above from server www. Cs. Purdue.edu, a browser can send the following request.

GET http://www. Cs.purdue.edu/people/comer/ HTTP/1.1

Once a TCP connection is in place, there is no need to send an absolute URL- the following relative URL will retrieve the same page:

GET /people/comer/HTTP/1.1

To summarize

The Hypertext Transfer Protocol (HITP) is used between a browser and a web server. The browser sends a GET request to which a server responds by sending the requested item.

## UNIT – II

## PART – A

**1. Define Database with example.**

A collection of data arranged in a way suitable for locating, adding, removing & changing the data is called database.

Eg. PIR database is a collection of data about protein sequences & structures.

**2. Define Biological Database with its types.**

Biological Database are the collection of data & information about different biological aspects. Information in these databases can be searched, compared, returned & analyzed.

Types:

1) Generalized Databases
2) Specialized Databases.

**3. What is active data?**

Active data are referenced on a regular basis during day-to-day business operations. Overtimes, this data loses its importance & is accessed less often, gradually losing its business value and ending with its archival or disposal.

**4. What is inactive data?**

Inactive data are put out to pasture once they are not longer active i.e., there are no longer needed for critical business tasks or analysis.

Data life cycle management (DLM) is a policy based approach to manage the flow of an information system's data throughout its life cycle: from creation & initial storage to the time when it becomes absolute & is deleted. DLM products automate the process involved, typically organizing data in to separate tiers according to specified policies & automating data migration from one tier to another.

Eg: Hierarchical storage management (HSM) is one type of DLM product.

**5. Distinguish between DLM & ILM**

| DLM | ILM |
|---|---|
| Data life cycle management | Information life cycle management |
| DLM products deals with general attributes of files such as their Lype, size sage. | ILM products have more complex capabilities |
| DLM product allow you to search stored data for a certain file type of a certain age. | ILM product would let you search various type of stored files for instances of a specific piece of data such as a customer number. |

**6. What is Database Technology?**

It provides an Internal Representation (model) of the External world of interest. Examples are the representation of particular data/time/flight /aircraft in airline reservation or item code/item description/quantity on hand /re-order level/reorder quantity in a stock control system.

**7. Give the objectives of Biological Databases.**

(1) To make all relevant data available at one place.
(2) To store all relevant information easily
(3) To make biological data available to scientists.
(4) To update the existing information easily

**8. Define Generalized Database.**

The database that has a wide range of related information (data) is called generalized database. Databases of DNA , proteins , mRNAs, structural databases are generalized databases. They include sequence database & structure databases.

**9. Define specialized Database.**

Databases created to meet some special needs are called specialized databases. They include databases of the genomes of various organisms, its biochemical pathways, diseases of human beings etc.

## PART – B

**1. What is (i) SRS (ii) RDBMS. Explain their role in Data retrieval & Data management?**

**Sequence Retrieval System (SRS)**

The SRS is a software tool for data integration, analysis and display for bioinformatics, genomic and related data. It was created by the Swiss Institute of Bioinformatics and European Bioinformatics Institute (EBI). SRS is a comprehensive data warehouse with powerful querying and linking capacity. It is linked with about 200 biological databases including nucleotide databases, protein sequence databases, secondary and tertiary structure databases, metabolic pathway databases, ontologies, literatures, patient abstracts, etc. Therefore, data can be retrieved from any of these databases. The link option allows us to get all entries in one databases which are linked to entries of other databases. Hyperlinks help to access other database via a database in use. The URL of SRS in http: //srs.ebi.ac.uk/srsbin/cgi.bin/

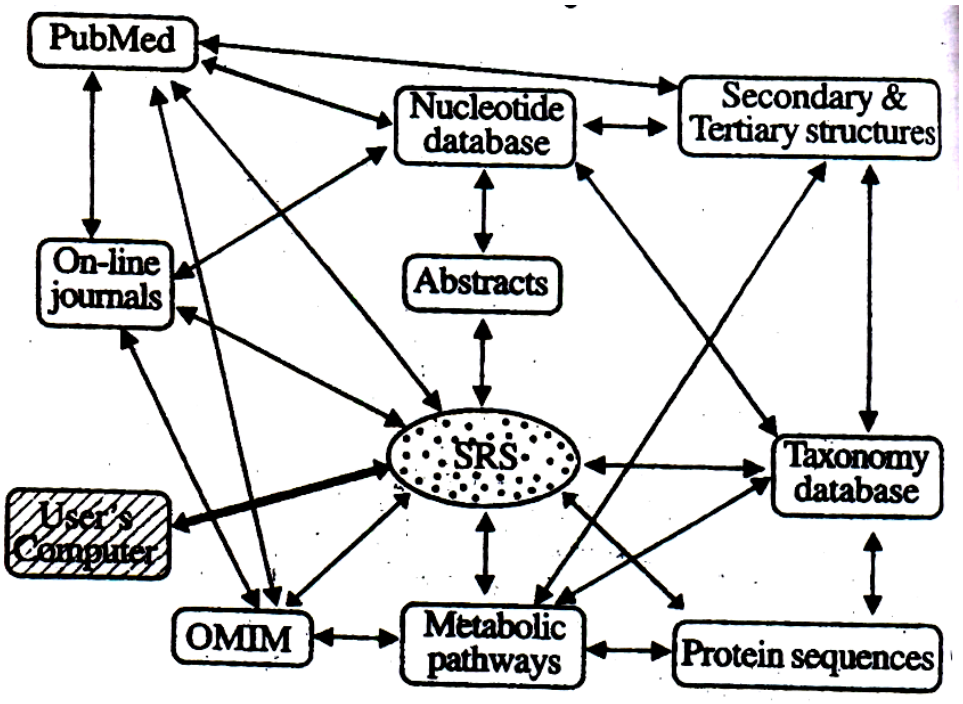



**Fig: SRS map showing hard disk connection with other databases.**

The SRS web interface is very useful for-

- ★ Retrieving entries of DNA , RNA and protein sequences
- ★ Performing a query
- ★ Linking our query result to other databases
- ★ Displaying our query result

- ★ Analysis of query results
- ★ Querying databases using command line SRS such as getz.

SRS helps to change the display option so that a viewer an look at in a different aspect.

After creating a new view, the SRS can be used to analyzed the results by using bioinformatics analysis tools. It gives more information relevant to the initial query. BLAST and FAST can be used within SRS for sequence analysis and comparison.

SRS command line interface is called getz. It is available through UNIX window. One can query a databank from the command line.

The best and nowadays the most commonly used database model is the relational model (figure). The data are all placed in several tables, each one constructed specifically according to the relationships between the data. It therefore becomes equally easy to retrieve and arrange the data according to any relationship. If a table for a particular relationship does not already exist, a new table is easily constructed. Such a relational database management system (or RDBMS) is constructed according to a set of twelve rules first stated by E.F. Codd. As the name suggest, the pride of place in relational database is given to the relations between the data. The words relation, here, is used as mathematical concept, which in ordinary language is a table of data. Thus a relation may consist of two or more columns. Each column has column name. The column-names are called the attributes of the table. Each row of the table is called a tuple and specifies the connection between a set of data. A more detailed explanation of the concepts underlying an RDBMS will not be attempt here. However, even this elementary description will make clear the enormous utility of the relational method of arranging the data. Apart from concepts such as minimizing redundancy, maximizing data integrity and so on, it is evident that almost any question regarding the data may be answered with equal ease. In more formal words, querying such a database is easy. Among the several commercial relational database packages are Oracle, Sybase , etc. These package come with not only the set of rules on how to arrange the data, but also software that will allow the user to rearrange any data in the relational format. Also included in the package is software to create reports, software to manage the data, i.e., top update, insert to delete data as required, and software to create user interface that make it easy to use a particular implementation of the database. An important part of such any such RDBMS is the query language, or the software that allows all the questions to the framed. Many of the biological databases are implemented as relational models, though this may not always be apparent, since the query language acts as an interface to retrieve the required data in the required format.

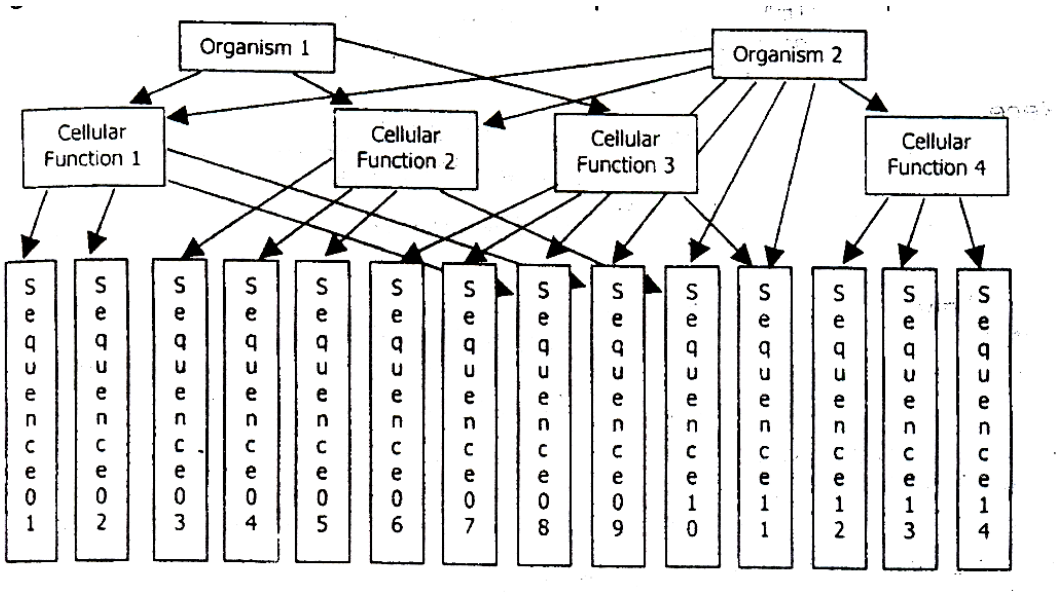



Figure A sequence database arranged on the network model. As in figure the boxes indicate data or categories of data, and the arrows indicate their relationships. Note that now there is only one non-redundant set of data corresponding to 'cellular function', with relationships to both organisms.

**2. What are the various types of protein databases? Which are the most important examples these types? Explain in detail.**

**Protein Sequence Databases**

The database in which amino acid sequence of proteins are stored in known as Protein sequence database. There are many different protein databases to assist the researchers in getting amino acid sequence of different proteins. EXProt, NCBI Protein Database, PIR , Swiss-Prot, TrEMBL and UnitProt are some important protein sequence databases. The names, main features and URL of these data bases are given in the table.

**Protein sequence databases**

| Database name | Full name and/ or description | URL |
|---|---|---|
| EXprot | Sequences of proteins with experimentally verified function | http://ww.cmbi **kun.nl /EXP** |
| NCBI Protein database | All protein sequences: translated from GenBank and imported from other protein databases | http://wwwni ml m.nih. gov/entr.z query,fcgi? Db=Protein |
| Swiss-Prot | Now UniProt/Swiss-Prot: expertly acurated protein sequence database, section of the UnProt knowledgebase | http://www **org/sprot** |

| | | |
|---|---|---|
| TrEMBL | Now UniProt/TrEMBL: Computer-annotated translations of EMBL nucleotide sequence entries: section of the UniProt knowledge base | http://www expary org/sprot |
| UniProt | Universal protein knowledge base: merged data from Swiss –Prot, TrEMBL and PIR protein sequence databases. | http://www. Uniprot org/ |

Recently, Swiss –Prot, TrEMBL and PIR have been co-existing as Protein databases in UniProt consortium. The main goal of UniProt knowledge base is to support biological research by maintaining a high quality database with extensive cross references and querying interfaces freely accessible to the scientific community.

Given below are the salient features of protein sequence data in databases:

- ★ Sequences are present in a form as they are in biological state in vivo.
- ★ The sequence data may be originally submitted to the database or abstracted from one or more papers appeared in journals.
- ★ Sequence data corresponds to wild type individuals.
- ★ Amino acids in the sequence are numbered starting from 1 at the N-terminal end to COO-terminal end.
- ★ Sequence data represent unmodified amino acids.
- ★ Each modification of amino acids in a sequence is given in the feature table.
- ★ Single-letter code is used to denote amino acids.
- ★ Two-letter codes are used for modified amino acids in the sequence.
- ★ Regions of α-helix, β-sheet, turns , hairpins are given separately in relation to residue's number participating in them.
- ★ Regions of hydrogen bonding, S-S bonds and other are given separately.
- ★ Domain structure and catalytic sites, if any are noted in relation to the corresponding amino acids.
- ★ Function of the sequence (Protein) is given in separate line.

**Examples of Protein Database**

**SWISS –PROT**

SWISS-PROT is an annotated protein sequence database. It was established by Amos Bairoch at the Department of Medical Biochemistry at the University of Geneva in 1986. It has been maintained collaboratively by Swiss Institute of Bioinformatics (SIB) and European Bioinformatics Institute (EBI) since 1987. It is available at the website ww.expasy.ch/sprot/

This database is generally considered one of the best protein sequence databases in terms of the quality of the annotation.

It consists of a wide array of sequence entries. Each entry is composed of many lines as has been given in the structure of protein sequence database. The general structure of SWISS-Prot and EMBL protein databases are more or les the same. The salient features of protein sequence entry in Swiss-Prot protein database are:

- ★ Complete amino acid sequence of stored protein are included
- ★ Taxonomic data of source organisms are given
- ★ Post-transcriptional modifications with carbohydrates, phosphate groups, acetyl group, metals, GPI anchor, etc are given
- ★ Domains and sites are given in proper lines. They include-

    - Calcium binding region
    - ATP –binding region
    - Zinc fingers
    - Homeoboxes

- ★ Secondary structures along the protein sequence is given
    - α-helices
    - β-sheet
    - Hairpins

- ★ Quaternary structure is explained well
- ★ Similarities of the entry with other sequences are given in a separate line.
- ★ Diseases associated with the protein are mentioned properly
- ★ Sequence conflicts and variation between individuals are listed in the entry.
- ★ Details of references, bibliographic references, cross-references to other databases are also given.
- ★ All information is well documented.

The SWISS-PROT database has some legal restrictions, the entries themselves are copyrighted, but freely accessible and usable by academic researchers. Commercial companies must buy a license fee from SIB.

**TrEMBL**

TrEMBL is a computer-annotated supplement of SWISS-PROT. It contains all the translation of EMBL nucleotide sequence entries not yet integrated with SWISS-PROT. It was

developed by Rolf Apweiler. The annotation of an entry in TrEMBL has not reached the standards required for its inclusion into SWISS-PROT.

The information for TrEMBL is derived from DDJB. EMBL and Gen Bank nucleotide entries or directly from submitter's data. The information is then upgraded by automatic annotation with computers. Sequences of the same organism, which have 100% identify are merged together into a single entry. It helps to reduce redundancy.

The databases can be accessed and searched through the SRS system at ExPASY or one can download the entire database as one single flat file.

**PIR**

The PIR (Protein Information Resources, http://(pir.georgetwon.edu) database is an outgrowth of the protein Sequence Database, originally created by Margaret Dayhoff. It is currently maintained at the Georgetown University in collaboration with Munich Information Center for protein Sequences (MIPS) Germany and the Japanese International Protein Information Database.

The advantage of PIR is in its hierarchical organization. The June 2002 release of PIR contained 293.236 entries that were classified into ~ 100,000 protein families and ~ 30,000 super families. Recently, PIR has intensified its protein classification efforts with the creation of IProClass.(http://pir .georgetown.edu/iproclass), a protein classification database.

**UniProt**

Uniprot is a knowledgebase formed by UniProt consortium set up by Swiss-Prot, TrEMBL and PIR protein sequence database. All these three databases differing in protein sequence coverage and annotation are co-existing in Uni-Prot knowledgebase. The main goal of UniProt is to support biological research by maintaining a high quality database. It assists the researchers in getting fully annotated protein sequences, extensive cross references and querying interfaces at free of cost. The entries are accurate, consistent and rich in sequence data.

Uniprot has three major sections: One is Swiss-Prot that gives manually annotated records about sequences; these second one is TrEMBL that provides annotated records assembled with computers from literatures and other databases; and the third one is PIR that acts as a data repository. All these three databases can easily be accessed via UniProt.

**3. Explain Biological Databases in detail.**

A collection of data arranged in a way suitable for locating, adding, removing and changing the data, is called database. The database in which biological data is stored, is called a biological database. For instance, PIR Database is a collection of data about protein sequence and structures.

Biological databases are the collection of data and information about different biological aspects. Information in these databases can be searched, compared, retrieved and analyzed.

Biological databases have becomes important tools in assisting scientists to understand several biological phenomena:

- The structure of biomolecules and their interaction.
- The whole metabolisms of organisms
- The evolution of species.
- Fight against diseases.
- Development of medications.
- Discovering basic relationships amongst species in history of life.

**Objectives of Biological Databases**

There are four main objectives of biological database.

- **To make all relevant data available at one places**

It is difficult to collect all relevant information at journal or book or article. Biological databases II in all relevant information available at one place. So it is easy to browse all related information at one site.

- **To store all relevant information easily**

It is not possible to preserve all information by printing books. Storage and management of database are in jobs.

- **To make biological data available to scientists**

To process the experimental data, it is necessary analyze it with the light of previous data. Database make the previous data available to the researcher help to interpret the new data.

- **To update the existing information easily**

The knowledge on biological sciences is expanding day-by day. It is difficult to add new information to already printed books. In databases, the new information be added at any time without much risks. It is useful for easy updating.

**Properties of Databases**

The various properties of the database are listed below:

- A database stores data of relevant information. The data may be-
    - Nucleotide sequences
    - Protein sequences
    - Protein sequence patterns or motifs
    - Macromolecular 3D structure
    - Gene expression data
    - Metobolic pathways.

- **It has facilitates for Data entry and quality control:**

- Scientists (teams )deposit data directly
- Appointed curators add and update data

- **It may be formed of primary data or secondary data or other databases:**

- Primary databases: experimental results directly into database
- Secondary databases: results of analysis of primary databases
- Aggregate of many databases

- **Date Files are stored in specific form.** They may be-

- Flat-files
- Relational database (SQL)
- Object-oriented database(Eg. CORBA, XML

- **It may be maintained by Government or Institute**
- **Databases are available to public with or with restriction**:

**Classification of Biological Databases**

Biological Databases can be broadly classified into types:

a. Generalized databases and
b. Specialized databases.

**Generalized Databases**

The database that has a wide range of related information (data) is called Generalized Database. Database DNA, proteins, mRNAs, structural databases are general databases. They include sequence databases and structural databases.

Sequence databases are those that have the record either nucleotide sequence or amino acids sequence. Structure databases are the ones that contain the records of solved structures of macromolecules.

Generalized Databases are of the following types:

- DNA Molecular databases
- RNA Sequence databases
- Protein Sequence databases
- Proteomics databases
- Structural databases

**Specialized Databases**

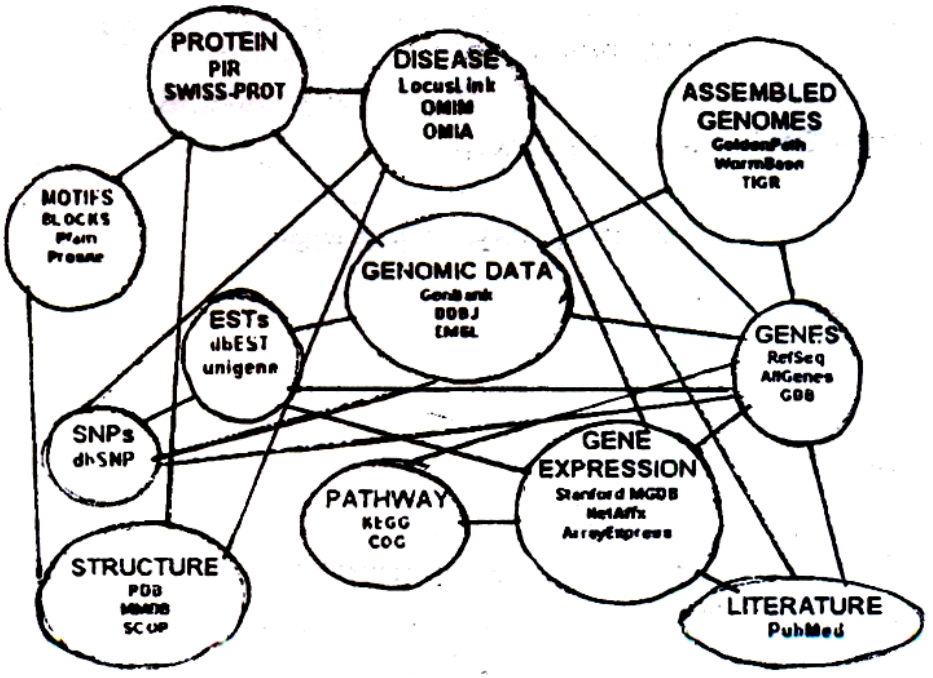
Databases created to meet some special needs are called specialized databases. They include databases of the genomes of various organisms, its biochemical pathways, diseases of human beings, etc.

Following categories of databases are grouped under Generalized Databases:

- Genomics Databases (non-vertebrate)
- Metabolomic and Signaling Pathways
- Human and other Vertebrate Genomes
- Human Genes and Diseases
- Microarray Data and other Expression

**Hard-link Relationships Between Databases**

No one database exists as in individual system. Because or rapid development in the field of information technology, it is now possible to interact with all the databases. All these databases are linked with each other for their efficient use. This is called hard-link connection.




**Map showing hard-link relationships between Databases.**

**DNA Molecular Databases**

DNA sequence database were first assembled at Los Alamos National Laboratory (LANL), New Mexico, by Walter Goad and colleagues in the Gen Bank database and at the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany.

The nucleic acid databases may be divided into primary databases and secondary databases or value added databases. The Primary databases contain the data of original sequences taken from the sources. The resource may be Gen Bank (NCBI) DNA, PIR/NBRF Protein, SWISS-PROT Protein or PDB 3D structure.

The secondary databases or value added databases contain annotated data and information. Eg. OMIM- Online men-delian Inheritance in man – gene and clinical data. GDB – Genome Data Base-human, SNPs –Single Nucleotide Polymorphisms.

**Nucleotide Sequence Databases**

The database in which sequence data of DNA, cDNA, mRNA and tRNA are stored is said to be nucleotide sequence database. Nucleotide sequence data are given in the database as they were originally submitted to the database or appeared in the paper. The base sequence is always listed from 5′ to 3′ direction and numbered sequentially starting with 1.

Salient features of sequence data stored in nucleotide sequence databases are given below:

- ★ Sequences are present in a form as they are in the bio-logical state in vivo
- ★ cDNA sequences are considered as RNA sequences
- ★ For genomic DNA, only coding strand is stored.
- ★ The sequence data generally correspond to wild type individuals.

**Protein Sequence Databases**

The database in which amino acid sequence of proteins are stored in known as Protein sequence database. There are many different protein databases to assist the researchers in getting amino acid sequence of different proteins. EXProt, NCBI Protein Database, PIR , Swiss-Prot, TrEMBL and UnitProt are some important protein sequence databases. The names, main features and URL of these data bases are given in the table.

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**4. Explain Data Life Cycle in detail.**

**Data Life cycle:**

Data becomes active as soon as it is of interest to an organization. Data life cycle begins with a business need for acquiring data. Active data are referenced on regular basis during day-to-day business operations. Over time, this data loses its important and is accessed less often, gradually losing its business value, and ending with its archival or disposal.

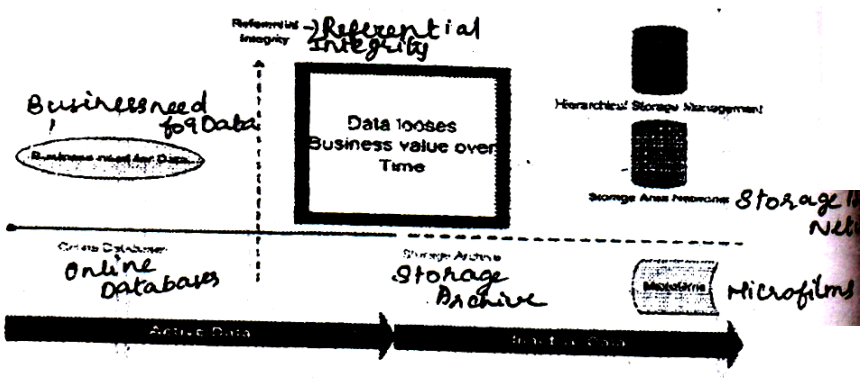



**Fig: Data Life Cycle in Enterprises**

**Active Data**

Active Data is business use to an organization. The ease of access for business users to active data is an absolute necessity in order to run an efficient business.

The simple, but critical principle, that all data moves through life-cycle stages is key to improving data management. By understanding how data is used and how long it must be retained, companies can develop a strategy to map usage patterns to the optimal storage media, thereby minimizing the total cost of storing data over its life cycle. The same principles apply when data is stored in a relational database, although the challenge of managing and storing relational data is compounded by complexities inherent in data relationships. Relational databases are a major consumer of storage and are also among the most difficult to manage because they are accessed on a regular basis. Without the ability to manage relational data effectively, relative to its use and storage requirements, runaway database growth will result in increased operational costs, poor performance , and limited availability for the applications that rely on these databases. The ideal solution is to manage data stored in relational databases as part of an overall enterprise data management solution.

**Inactive Data**

Data are put out to pasture once they are no longer active. i.e. there are no longer needed for critical business tasks or analysis.

Prior to the mid-neneties, most enterprises achieved data in Microfilms and tape back-ups.

There are now technologies of data archival such as storage Area Networks (SAN), Network attached Storage (NAS) and Hierarchical Storage Management. These storage systems can maintain referential integrity and business context.

Data life cycle management (DLM) is a policy-based approach to managing the flow of an information system's data throughout its life cycle:from creation and initial storage to the time when it becomes obsolete and is deleted. DLM products automate the processes involved, typically organizing data into separate tiers according to specified policies, and automating data migration from tier to another based on those criteria. As a rule, newer data, and data that must be accessed more frequently, is stored on faster, but more expensive storage media, while less critical data is stored on cheaper, but slower media.

Hierarchical storage management (HSM) is one type of DLM product. The hierarchy represents different types of storage media, such as RAID (redundant array of independent disks) systems, optical storage, or tape, each type representing a different level of cost and speed of

retrieval when access is needed. Using an HSM product, an administrator can establish and state guidelines for how often different kinds of files are to be copied to a backup storage device. Once the guideline has been set up, the HSM software manages everything automatically. Typically, HSM applications migrate data based on the length of time elapsed since it was last accessed, while DLM applications enable policies based on more complex criteria.

The terms data life cycle management and information life cycle management (ILM) are sometimes used interchangeably. However, a distinction can be made between the two. According to Karen Dutch, vice president of product management at Fujitsu Softek, DLM products deal with general attributes of files, such as their type, size and age; ILM products have more complex capabilities. For example, a DLM product would allow you to search stored data for a certain file type of a certain age, for example, while an ILM product would let you search various types of stored files for instances of a specific piece of data, such as a customer number.

Data management has become increasingly important as business face compliance issue in the wake of legislation, such as HIPAA and the Sarbanes-Oxley Act, that regulates how organizations must deal with particular types of data. Data management experts stress that data life cycle management is not a product, but a comprehensive approach to managing an organization's data, involving procedures and practices as well as applications.

**5. Explain Database technology in detail.**

**Database technology**

**1. What is Database Technology?**

The essential feature of database technology is that it provides an INTERNAL Representation (model) of the EXTERNAL world of interest. Examples are the representation of a particular date/time/flight/aircraft in airline reservation or of item code/item description/quantity on hand/reorder quantity in the stock control system.

The TECHNOLOGY involved is concerned primarily with maintaining the internal representation consistent with external reality; this involves the results of extensive R & D over the past 30 years in areas such as user requirements analysis, data modeling, process modeling data integrity, concurrency, transactions, file organization, indexing, rollback and recovery, persistent programming, object orientation, logic programming, deductive database system, active database system…..and in all these (and other) areas there remains much to be done.

**2. Why is it Important?**

Business in much of world depends on database technology. For example:

**Finance:** The UK clearing banks have calculated that if their database systems were removed it would take every person in UK working 24 hours per day 7 day per week to process all the financial transactions manually. The London stock exchange relies on compute systems for recording buying and selling of stock which happens very quickly and in large quantities. The amount of money involved in these transactions is enormous.

**Transport:** The airlines all use online seat reservation system and have systems for scheduling aircraft for building and maintaining timetables, for handling the in-fight catering and for mechanical servicing of the planes. Similar systems exist for rail, sea and road transport. They all use database technology extensively.

**Utilities:** the major utilities (water, electricity, gas) all have generation /distribution system based on database technology.

**Resources:** The mineral exploration /extraction companies, and governments who regulate them (especially for oil exploration/extraction) have extensive databases which have complex data structures (usually including GIS (geographical information system) components.

**Production engineering:** from scheduling workflow through the production lines of machines to stock control and order processing, database technology underpins all activity in this area.

**Environment:** Protection and control of the environment by government agencies depends heavily on database systems with GIS facilities, together with databases of toxic substances and clean-up recommendations.

**Tourism:** Hotel systems and local tourist attractions information and booking facilities rely on database system, and the major package tour operators have extensive databases for holiday planning and booking, together with financial system for payment and invoicing.

**Leisure:** the entertainment industry uses database system extensively for theatre, concert and cinema ticket bookings.

**Culture:** Museums, art galleries, history exhibitions-all utilize database technology (and especially multimedia database technology) for cataloguing their collections and recording access to them.

**Education:** Courses, materials and assessment all rely heavily on database technology in all sectors of education. Increasingly the linking of database technology with hypermedia delivery systems allow courseware to be maintained up-to-data and delivered to the consumer.

**Healthcare:** primary healthcare has long relied on database technology to schedule hospital beds or appointments at clinics. The patient health record has been the subject of intensive study (and R& D resources) over many years because of its complexity of structure , content and media and also because of the security and privacy issues. Epidemiology utilizes database technology to hold and organize key information from many patients in order to allow statistical processing to detect trends and to alert medical practitioners to possible epidemics. More recently, data mining techniques have been applied to this area –relying again on database technology.

Government administration would be paralysed without database technology, the collection of taxes and the payment of social security benefits depends totally on database technology.

**Retail:** the major retail stores utilize database technology in stock control and PoS (Point of Sale) systems. Modern retailers use advanced data mining techniques to determine trends in sales and consumers preference to optimize stock control, retail performance, customer convenience and profit.

The essential point is that database technology is a CORE TECHNOLOGY with links tio:
information management/processing
data analysis/statistics
data visualization/presentation
multimedia and hypermedia
office and document systems
business process, workflow ,CSCW (Computer-supported cooperative work)
But modern DB systems depend on an infrastructure of:
Networks both LAN (local area network) and WAN (wide area network)
Client –server computing architecture
Skilled data analysis and DB design
Skilled system development methods(s)
For them to be effective and therefore used in any sector of activity.

**3. Which Applications Areas?**

From the programme of the workshop there is clearly interest in:
Culture & scientific information
Tourism
Telemedicine

Natural resources management
Production engineering
all of which have been mentioned above as typical DB application areas. There is not enough time in the presentation to describe in any detail how DB technology is used in these application areas.

**4. What Relevant Technologies?**

Relational DBMS is the modern base technology for many business applications. It offers flexibility and easy-to-use tools at the expense of ultimate performance. More recently relational systems have started to extend their facilities in the directions of information retrieval, object-orientation and deductive /active systems leading to the so-called 'Extended Relational System'.

Information Retrieval Systems started with handling library catalogues and extended to full free-text utilizing inverted index technology with a lexicon or thesaurus. Modern systems utilize some KBS (knowledge –based systems) techniques to improve retrieval.

Object-Oriented DBMS started for engineering applications where objects are complex, have versions and need to be treated as a complete entity. OODBMSs share many of the OOPL features such as identity, inheritance, late binding, overloading and overriding. OODBMSs have found favour in engineering and office systems but have not yet been successful in traditional application areas.

Deductive/Active DBMS have emerged over the last 20 years and combine logic programming technology with database technology. This allows the database itself to react to external events an to maintain dynamically its integrity with respect to the real world.

## UNIT – III

## PART - A

**1. Define sequence alignment.**

Arranging the query sequence with its homologous sequence from a sequence database to assign residue –residue correspondences between them is called sequence alignment. It is the basic tool of bioinformatics.

**2. What are the criteria for sequence alignment?**

- Uniformative alignment
- Alignment without gaps
- Alignment with gaps on both sequences
- Alignment with gaps on query sequence.

**3. Define optimal alignment.**

Optimal alignment is the best alignment according to a defined set of rules & parameter values. It is ideal for comparing different sequences.

**4. Define Global alignment with example.**

An alignment that matches the residues of a relatively short sequence with residues over the entire length of other sequence is called global alignment.

Eg: V P K T A L I L L A V E L (Sequence 1)
V - - T A L – L L A – E L (Sequence 2)

Here, the short sequence is stretched up to the ends of the long sequence by introducing gaps. Therefore there is no overhang in the long sequence.

**5. Define Local alignment with example.**

An alignment that matches the segment of the two sequences that match well us called local alignment.

Eg: F T F T A L I L L – A V A V
- - F T A L – L L A V - -

Here, gaps are introduced at the ends of short sequence and within the sequence. The overhangs are prominent in the long sequence. Only conserved regions are matched well.

**6. Define pairwise sequence alignment.**

Arranging the query sequence with only one homologous sequence from a data base at a time is called pairwise sequence alignment.

**7. What are the 3 methods by which on can align a pair of sequences?**

1. Dot Matrix method
2. Dynamic programming
3. Word method (or) K-tuple method.

**8. Define multiple sequence alignment.**

The alignment of the query sequence with more than two homologous sequence with more than two homologous sequences form a sequence data is called multiple sequence alignment.

**9. Define dynamic programming.**

The process of solving problems where one needs to find the best decision one after another is called dynamic programming.

**10. What are the methods by which multiple sequence can be performed?**

1. Dynamic programming
2. Progressive method
3. Iterative method

**11. Define BLAST.**

BLAST is Basic Local Alignment search Tool. It is a homology & similarity search tool it is a set of search programs designed for the windows platform and is used to perform fast similarity searches regardless of whether the query is for protein or DNA. It is provided by NCBI.

**12. Define FASTA.**

FASTA is FAST homology search all sequences. This tool is very much similar to the BLAST, but this tool will speed up sequence comparison when compared with BLAST FASTA is a

DNA & protein sequence alignment software package first described (as FASTP) by David J. Lipmar, and William R. pearson in 1985.

**13. Define Hidden markor models (HMMs).**

This is computational method to describe the suttle patterns in families of hemologues the query sequence. HHMS perform fold recognition as done by CASP programs and predicting protein folding patterns. The Hidden markov models were discovered by A. A. Markow

**14. Define Neural net.**

An interconnected assembly of simple processing elements that together determine interactions between molecules.

**15. Define substitution matrices.**

A set of numbers or symbols arranged in a grid and used as a single quantity in calculations is called matrix. The matrix of numbers dealing with probable substitution of different residues for every residues is called substitution matrix.

**16. Write short notes on Dot Matrix Method.**

The matrix formed of dots numbers is called dotmatrix. The dots are uniform throughout the matrix. When values are added to the matrix according to respective position of residue, the dots kitted by these values give a line graph called dot plot.

# PART - B

**1. (I) Write about the BLAST programs listed in NCBI with their query & database used & explain how will you find the compositional complexity of a sequence.**

**a. BLAST**

BLAST is Basic Local Alignment Search Tool. It is a homology and similarity search tool it is a set of search programs designed for the Windows platform and is used to perform fast similarly searches regardless of whether the query is for protein or DNA. It is provided by NCBI.

It is used to compare a novel sequence with those contained in nucleotide and protein databases by aligning the novel sequence with previously characterized genes. The emphasis of this tool is to find regions of sequence similarity. This will yield functional clues about the structure and function of this novel sequence.

The following are the salient features of BLAST:

- BLAST finds out patches of sequence similarity rather than best alignments.
- BLAST produces ungapped alignment between the query and database.
- BLAST reports multiple local alignments between the query and database.
- BLAST is based on an explicit statistical theory.
- BLAST is rapid.
- Program can be run locally or by sending queries to NCBI using the Email ID. **blast @ ncbi. nlm. nih. gov.**
- BLAST is not guaranteed to detect best alignment between the query and database.
- Reports from BLAST can be analyzed further with tools such as XNU, SEG and X BLAST.
- A substitution matrix is used in all phases of protein searches.

**BLAST Services (From NCBI)**

There are a number of services available from NCBI related to BLAST (**http**://www.**ncbi.nlm.nih.** gov/BLAST/) Some of these are discussed here.

1. **Nucleotide BLAST.** Nucleotide BLAST searches allow one to input nucleotide sequences and compare these against other nucleotides.
2. **Standard nucleotide-nucleotide BLAST.** It takes nucleotides sequence in FASTA format, GenBank Accession numbers or GI numbers and compares them against the NCBI nucleotide databases.

3. **MEGABLAST:** This program uses a "greedy algorithm" for nucleotide sequence alignment searches and concatenates many queries to save time spent scanning the database. It is optimized for aligning sequences that differ slightly and is up to 10 times faster than more common sequence similarity programs. It can be used to compare two large sets of sequences against each other and gives the results very quickly.
4. **Protein BLAST.** Protein BLAST allows one to input protein sequences and compare these against other protein sequences.
5. **Standard protein – protein BLAST.** This takes protein sequences in FASTA format, GenBank Accession numbers or GI numbers and compares them against the NCBI protein data bases.
6. **Pattern hit initiated BLAST (PHI-BLAST).** PHI- BLAST combines matching of regular expression pattern with a Position Specific iterative protein search. **PHI-BLAST** can locate other protein sequences that both contain the regular expression pattern and are homologous to a query protein sequence.

The schematic diagram of PHI- BLAST is given in figure.

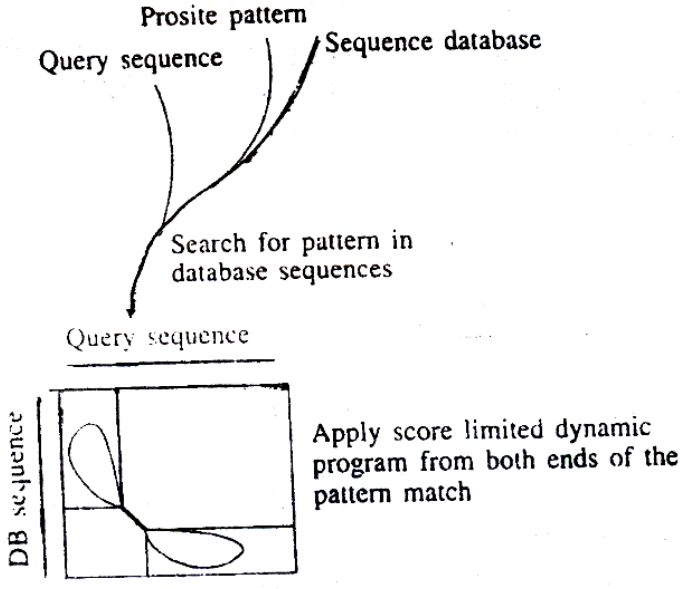



**Fig. Schematic of PHI- BLAST.**

**Search for short, nearly exact sequences**

This search is similar to the standard protein- protein BLAST with the parameters set automatically to optimize for searching with short sequences. A short query is more likely to occur by chance in the database. Therefore increasing the expected value threshold, and also lowering the word size is often necessary before results can be returned. "Low Complexity" filtering has also been removed since this filters out larger percentage of a short sequence, resulting in little or

no query sequence remaining. Also for short protein sequence searches the matrix is changed to PAM-30 that is better suited to finding short regions of high similarity.

**Translating BLAST.** Translating BLAST searches translate either query sequences or database from nucleotides to proteins so that protein – nucleotide sequences can be performed.

**Translated query – Protein db [blastx].** Converts a nucleotide query sequence into protein sequences in all 6 reading frames. The translated protein products are then compared against the NCBI protein databases.

**Protein query – Translated db [tblastx].** Takes a protein query sequence and compares it against an NCBI database that has been translated in all six reading frames.

**Translated query – Translated db [tblastx].** Converts a nucleotide query sequence into protein sequences in all six reading frames and then compares this to an NCBI nucleotide data base which ahs been translated in all six reading frames.

**Pair – wise BLAST.** Pair – wise BLAST performs a comparison between two sequences using the BLAST algorithm. Note that the program considers a "Sequence 1" to be the Query sequence and "Sequence 2" to be the Subject sequence. The program options available are given in Table.

| Program | Query Sequence | Database | Type of Alignment |
|---|---|---|---|
| Blastp | Protein | Protein | Gapped |
| Blastn | Nucleic acid | Nucleic acid | Gapped |
| Blastx | Translate nucleic acid | Protein | Each frame gapped |
| Tblastn | Protein | Translated nucleic acid | Each frame gapped |
| Tblastx | Translated nucleic acid 2 | Translated nucleic acid1 | Ungapped |

**(ii) Explain all the steps followed by BLAST Algorithm to find a matching sequence.**

**BLAST Algorithm**

BLAST, like FASTA, is a word – based method. However, one major difference is that BLAST requires a pre- formatted search database.

BLAST goes through the three steps that are summarized here:

1. Find the list of high scoring words (w). BLAST takes each word form the query sequence (typically w is 3 for amino acids and 11 for nucleotides), and locates all similar words in the current test sequence (fig.)

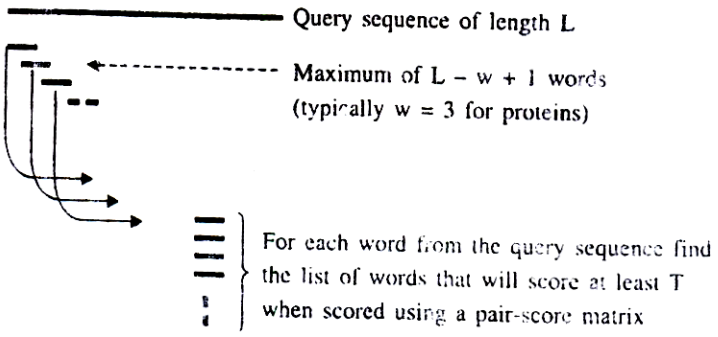



**Fig. Find the list of high scoring words.**

2. Compare the word list to the database and identify the exact matches. It similar words are found. BLAST tries to expand the alignment to the adjacent words, without allowing for gaps (fig.)

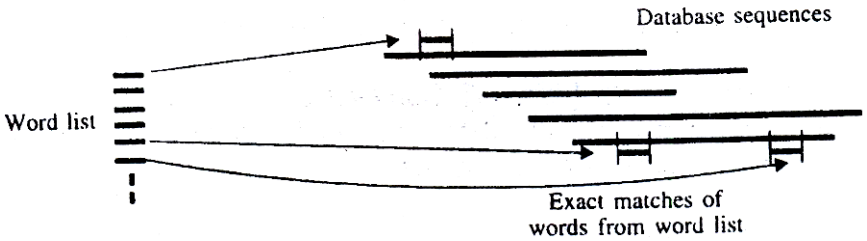



**Fig. Identify the exact matches after comparison.**

3. After all words are tested, a set of Maximal Segment Pairs (MSPs) is chosen for that database sequence. Several short, non – overlapping MSPs may be combined in a statistical test to create a larger, more significant match (fig.)

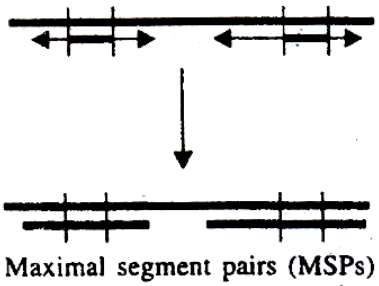



**Fig. Choose the MSPs.**

**BLAST Implementations**

The major Blast implementations available on the WWW are given in Table

**BLAST Implementation Summary**

| Name of the Program | Address (URL) |
|---|---|
| BLAST Network Service on ExPASy | **http://us.expasy.org/tools/blast/** |
| BLAST at EMB net- CH/SIB (Switzerland) | **http://www.ch.embnet.org/software/Bottom BLAST.html?** |
| BLAST at NCBI | **http://www.ncbi.nlm.nih.gov/BLAST/** |
| WU- BLAST at the EBI | **http://www.ebi.ac.uk/blast2/** |
| BLAST at PBIL (Lyon) | **http://npsa-pbil.ibcp.fr/egi-bin/npsa_automat.pl?page=npsa_blast.html** |

**BLAST Output**

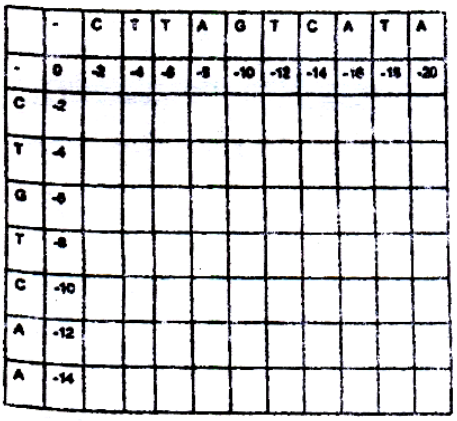
You can use the same protein sequence used for FASTA example above for illustration. BLAST output can be enormous.

First there is a short description of the program and a list of the database and program options chosen. Then there is a list of all of the database sequences that matched your query sequence. Several numbers are assigned to each of these sequence that represent the quality of the match. The list is presented in descending order, so that the best matches are at the top of the list. However, the most biologically significant matches are not always the ones ranked highest in the list. The output for the BLAST query for the sequence is given in

| Sequences producing significant alignments: | Score (bits) | E value |
|---|---|---|
| gi\|6016094\|sp\|043603\|GALS HUMAN Galanin receptor type 2(GA… | 143 | 5e-34 |
| gi\|3023820\|sp\|008726\|GALS RAT Galanin receptor type 2(GAL2… | 133 | 5e-31 |

**2. (i) Needle man wunseh algorithm.**

**(a) Needleman-Wunsch Algorithm:** This algorithm sets up the scoring matrix in which the homologous sequence is added to the top row and the query sequence is added to the left column. Each element in the matrix represents the relationship between two residues at a particular position. The first row and column is filled up with gap penalties of residues in respective sequences. This step is called **matrix initialization.**



| | - | C | T | T | A | G | T | C | A | T | A |
|---|---|---|---|---|---|---|---|---|---|---|---|
| - | 0 | -2 | -4 | -6 | -8 | -10 | -12 | -14 | -16 | -18 | -20 |
| C | -2 | | | | | | | | | | |
| T | -4 | | | | | | | | | | |
| G | -6 | | | | | | | | | | |
| T | -8 | | | | | | | | | | |
| C | -10 | | | | | | | | | | |
| A | -12 | | | | | | | | | | |
| A | -14 | | | | | | | | | | |

**Fig. Initialization of matrix**

From the gap penalty given below the first residue in the column and row, the score of the first right top diagonal is calculated. In this way, all diagonals in the matrix are filled up. It is called **matrix fill.** The matrix is filled up using a recursive scheme. Scoring matrix is calculated by using the equation.

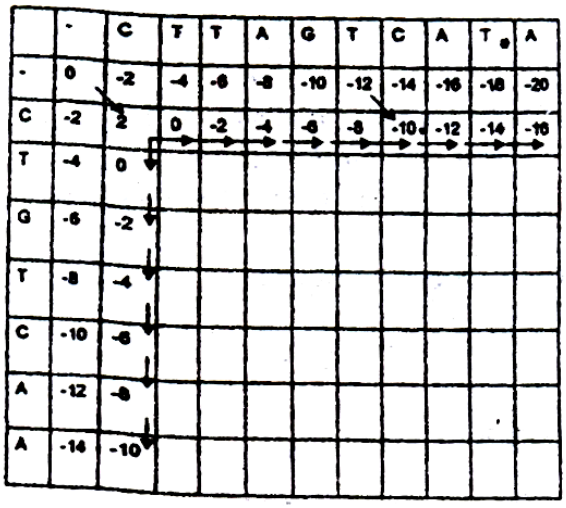
Sij=max Si-1,J-G

Or

Sij=max Si, j-1-G

Where,

Sij is the score of residue at the position I,j G is the gap – score.



| | - | C | T | T | A | G | T | C | A | T | A |
|---|---|---|---|---|---|---|---|---|---|---|---|
| - | 0 | -2 | -4 | -6 | -8 | -10 | -12 | -14 | -16 | -18 | -20 |
| C | -2 | 2 | 0 | -2 | -4 | -6 | -8 | -10 | -12 | -14 | -16 |
| T | -4 | 0 | | | | | | | | | |
| G | -6 | -2 | | | | | | | | | |
| T | -8 | -4 | | | | | | | | | |
| C | -10 | -6 | | | | | | | | | |
| A | -12 | -8 | | | | | | | | | |
| A | -14 | -10 | | | | | | | | | |

**Fig. Matrix filling using the calculation.**

The next step in Needleman-Wunsch algorithm is the **tracing of best alignment** from the scoring matrix. It follows some basic rules:

- ★ Alignment is traced from left top to right bottom or from right bottom to left top.
- ★ If gaps are absent, the matrix shows a straight diagonal line from left top to right bottom.
- ★ Distance occupied by residues is equal to the gap penalty.
- ★ Residues of homologous sequence is written below the matrix.
- ★ While moving across the matrix, gap penalty decreases or increases step-by-step at a constant rate of gap penalty. If gap penalty is -2, it proceeds in -10,-8,-6, -4, -2,0,2,4,6,8,10,12,14, etc.
- ★ If score of a residue falls as intermediate between any of the two adjacent valves of roots of 2, there would be a gap at the position.

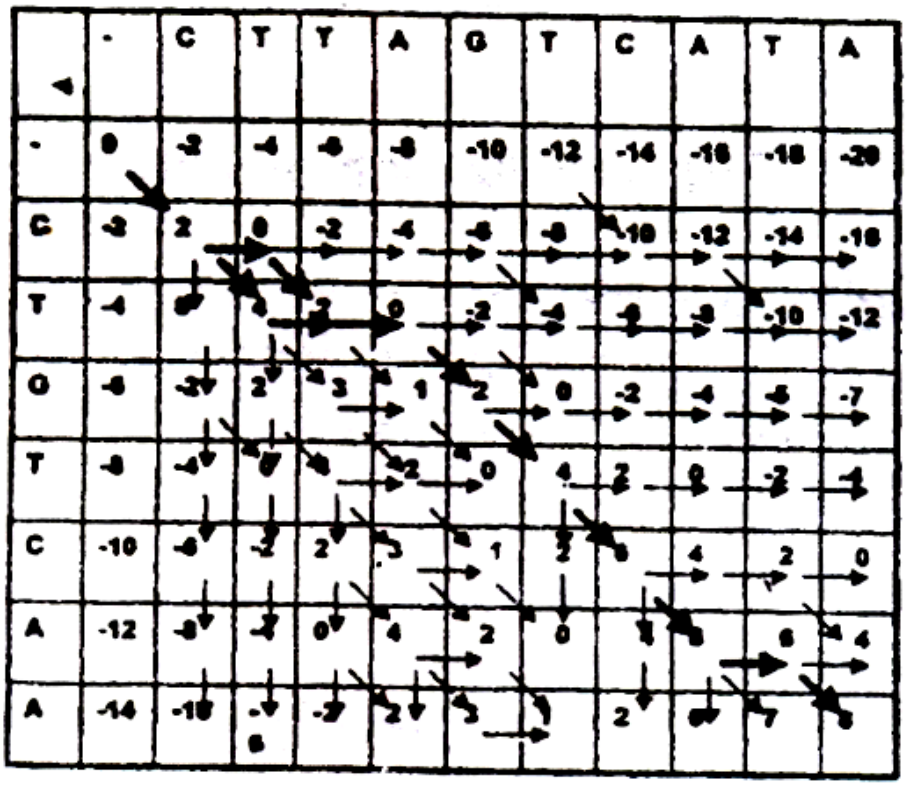



**Fig. Tracing the position of residues.**

**(ii) Smith-waterman algorithm.**

**Smith-Waterman algorithm:** This is the dynamic programming algorithm for local pair wise sequence alignment. It determines similarity regions between two nucleotide or amino acids sequences. It performs optimal local alignment of two sequences based on **substitution matrices** and **gap scoring schemes.** Here, the scores of residues are always **positive** and all negative scores in the matrix cells are set to **zero.** The following equation used to fill the matrix:

$$S = \max \begin{cases} Si-1, j-1^{-S(a,i,bj)} \\ Si-1, j-G \\ Si, j-1-G \\ 0 \end{cases}$$

Here, S is a negative expectation score. It is the average score that the scoring system would yield for random sequence.

Here is a set of rules for local alignment:

- ★ Backtracking should be started at the highest scoring matrix cell and proceeded toward the lowest scoring matrix cell.
- ★ Unrelated sequences tend to form extreme value distribution.
- ★ Low expectation values indicate homologues sequences that have a common ancestry.
- ★ High expectation values shows unrelated sequences
- ★ Too much of mutations in the past had produced variability in the sequence.

**Limitations**

1. Smith-Waterman algorithm is demanding much time and memory.
2. Not suited for aligning lengthy sequences.
3. It needs much space to work
4. Reliable statistical models are necessary for better local alignment.

Because of these limitations, Smith – Waterman algorithm has been put aside and replaced with some modern programmes:

- ★ BLAST algorithm is somewhat more efficient in local sequence alignments than Smith-Waterman algorithm.
- ★ A modified version of smith – Waterman algorithm is used in-
    - SSEARCH programme of FASTA sequence analysis package
    - Altivec programme of Power PC
    - SSE2 algorithm of Intel / AMD processor of Intel Core Micro architecture
    - Biofacet software (1997)
    - FPGA chips
    - Prgeniq
    - GenCore 6 package

**3. Explain in detail about multiple sequence alignment.**

**Multiple Sequence Alignment**

The alignment of the query sequence with more than two homologous sequences from a sequence data is called **multiple sequence alignment.** A database is scanned for the homologues sequences and then the query sequence is aligned with them to identify residue –residue correspondences between them. This is an extension of pairwise sequence alignment. It aligns all the sequences into a query set.

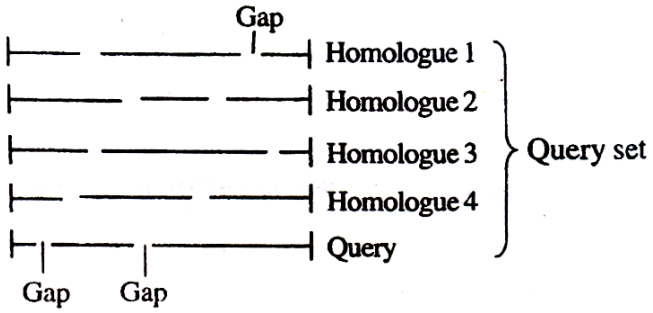



**Fig. Multiple sequence alignment (diagrammatic).**

- Multiple sequence alignment provides basic information for identification of conserved sequence regions.
- This is very useful in designing experiments to test and modify the function of specific proteins.
- It is used in predicting the function and structure of proteins in related organisms.
- New members of protein families are identified with multiple sequence alignment.
- Conserved sequence motifs observed with multiple sequence alignment can be used in conjunction with structural and mechanistic information to locate the catalytic active sites of enzymes.
- Structure prediction tools work mainly based on multiple sequence alignment.
- Multiple sequence alignment helps to build interfaces between closely related sequences.
- Multiple sequence alignments are also used to aid in establishing evolutionary relationships by constructing **phylogenetic trees.**

Strictly speaking, individual amino acids are reluctant to give information abut the sequences.

Multiple sequence can be performed with the following methods:

1. Dynamic programming
2. Progressive method
3. Iterative method

**1. Dynamic programming**

In fact, dynamic programming is applicable to align any number of sequences.

However, because it is time consuming and needs more memory, it is rarely used to align more than three or four sequences. This method requires constructing the n-dimensional equivalent of sequence matrix from two sequences. Here, n is the number of sequences in the query.

Standard dynamic programming is first used on all pain of query sequences and then the "alignment space" is filled in by considering possible matches or gaps at intermediate positions. The paired alignments are once again paired one by one to get the multiple sequence alignment.

Dynamic programming is guaranteed for the global optimum solution for sequences alignment. One method of dynamic programming, relies on the "sum of pairs (objective function) and it has been implemented in the MSA software packages.

**2. Progressive method**

In this method, pairwise global alignment is performed for all the possible pairs and these pairs are aligned together on the basis of their similarity. Thus a **guide tree** is generated. The most similar sequences are aligned together and the less related sequences are added to it progressively one-by one until a complete multiple query set is obtained. This method is also called **hierarchical method** or **tree method.**

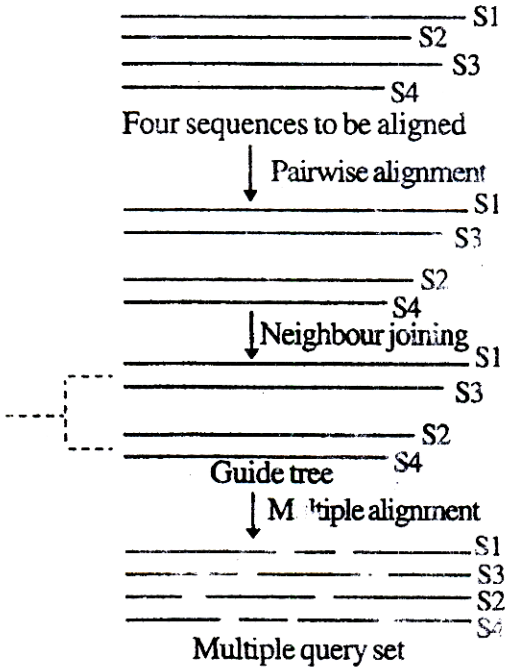



**Fig. Steps involving in progressive method of multiple sequence alignment.**

The results of progressive alignment are depending on the choice of "most related" sequences and accuracies in the initial pairwise alignments. Most powerful MSA methods weight also the sequences in the query set according to their related ness, which reduces the likehood of making a poor choice of initial sequences. Thus it improves alignment accuracy.

Many variants of **Clustal Programmes** are used for multiple sequence alignment, phylogenetic tree construction and **protein structure prediction.** A slower but more accurate variant of this method is known as **T-coffee**. This implementation is also found in **Clustal W** and **T-Coffee**.

**3. Iterative method**

A method of performing a series of steps to produce successively better approximation to align many sequences step y-step is called **iterative method.** Here the pairwise sequence alignment is totally avoided. This method works on the principle of objective function of alignment scoring. Using an appropriate scoring. Using an appropriate scoring method, one sequence is aligned with an other sequence and then the third sequence is aligned with the previous set. This is repeated for many times until a complete query set with enough number of sequences is formed. Here, the sequences are aligned one by one.

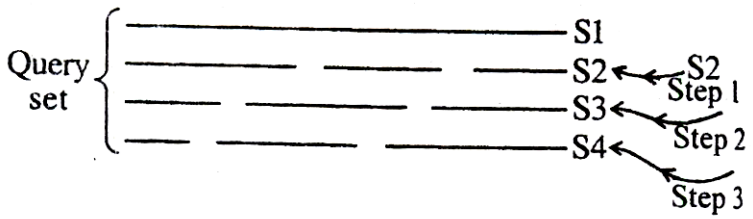



**Fig. Iterative method of multiple sequence alignment.**

Iterative methods attempt to improve on the weak point of the progressive methods, the heavy dependence on the accuracy of the initial pariwise alignments.

**Applications of multiple Sequence Alignment**

One of the major aims of bioinformatics is searching in databases for homologous sequence of a protein. Our goals in using multiple sequence alignment technique are-

i. Selecting true homologues by avoiding sequences that mimic as true homologues
ii. Tracing the distant relationship effectively
iii. Building up of effective procedures that make use of MSA for database searching.
iv. Identification of subtle patterns characteristic to families of proteins.

**4. Explain in detail about substitution matrices.**

A set of numbers or symbols arranged in a grid and used as a single quantity in calculations is called **matrix.** The matrix of numbers dealing with probable substitution of different residues for every residue is called **substitution matrix.** It explains the rate at which each possible residue in a sequence changes to each other residue over time. Substitution matrices are used in amino acids or DNA sequence alignment, where the similarity between sequences depends on the mutation rates as represented in the matrix. They are found to be superior to identity. Genetic code and physical property matrices.

The substitution matrix describes the likehood that two residue types would mutate to each other in evolutionary time. Is used to estimate how well two residues of given types would match, if they were aligned with each other. Eg. PAM and BLOSUM matrices.

The choice of matrix to be used in sequence alignment is determined by nature of sequences that need to be aligned.

i . PAM 250 is used for comparing a variety of distantly related proteins.
ii . PAM 120 is used for comparing sequences that are not known to be related.
iii. BLOSUM 62 matrix is used for ungapped local alignments.

It is generally accepted that no single matrix is the complete answer for all types of sequence alignment and comparison. For best result, it is advised to compliment BLOSUM 62 matrix with comparisons using PET91 PAM 250 matrix and other structurally derived matrices.

**PAM**

One of the first amino acid **substitution matrices,** the PA (Point Accepted mutation) matrix was developed by **Margan Dayhoff** in 1970s. This matrix is calculated by observing differences in amino acids in closely related proteins.

A collection of 1300 sequences in 72 families manual aligned to estimate frequency of substitution of differ amino acids in known structures when 1% mutation occurs is calculated that is takes $10^8$ years to have 1% mutation. If based on the like hood of a particular mutation by comparison similar sequences.

The PAM1 matrix estimates what rate of substitution would be expected, if 1% of the amino acids have change. The PAM1 matrix is used as the basis for calculating other matrices by assuming that repeated mutations would follow the same pattern as those in the PAM1 matrix and multiplying substitutions can occur at the same site. Using this logic Day off derived matrices as such as PAM30,60,90,120,250 and 300.

**Disadvantages of PAM:**

1. Only a few sequences are there in the original sequence alignment by **Dayhoff** matrix.
2. Not suitable to compare too many sequences.
3. Errors are exploded from PAM 1 to PAM 250.

**BLOSUM**

The matrix derived from the probability of substitution of one amino acid for another in different blocks of known proteins is called **BLOck Substitution Matrix** (BLOSUM). It rectifies the drawbacks of PAM matrix in sequence alignment. **S. Henikoff** and **J.G. Henikoff** (1992) constructed these matrices using multiple alignments of evolutionarily divergent proteins.

Dayhoff's methodology is not well suited for aligning evolutionarily divergent sequences. Sequence changes over long evolutionary time scales are not well approximated by computing small changes that occur over short time scales. The **BLOSUM** (BLOck Substitution Matrix) series of matrices rectifies this problem.

**Differences between PAM and BLOSUM**

The following are the differences between PAM and BLOSUM.

1. PAM matrices are based on an explicit evolutionary model whereas the BLOSUM matrices are based on an implicit model of evolution.
2. The PAM matrices are based on mutations observed throughout a global alignment. But the BLOSUM matrices are based only on highly conserved regions in the series of alignments forbidden to contain gaps.
3. Unlike the PAM matrix, the BLOSUM procedure uses groups of sequences with in which not all mutations are counted the same.
4. Higher numbers in the PAM matrix naming scheme denote larger evolutionary distance, while larger numbers in the BLOSUM matrix naming scheme denote higher sequence similarity and smaller evolutionary distance. Example: PAM 150 is used for more distance sequences than PAM 100; BLOSUM 62 is used for closer sequences than Blosum 50.

**5. Explain in detail about FASTA algorithm.**

FASTA uses the Pearson and Lipman algorithm to search for similarities between one sequence (the query) and any group of sequences of the same type (nucleic acid or protein) as the query sequence. FASTA in generally best to make protein – protein comparisons, but it can also compare DNA sequence to DNA databanks. The related program TFASTA allows a protein query sequence to be compared to DNA databanks.

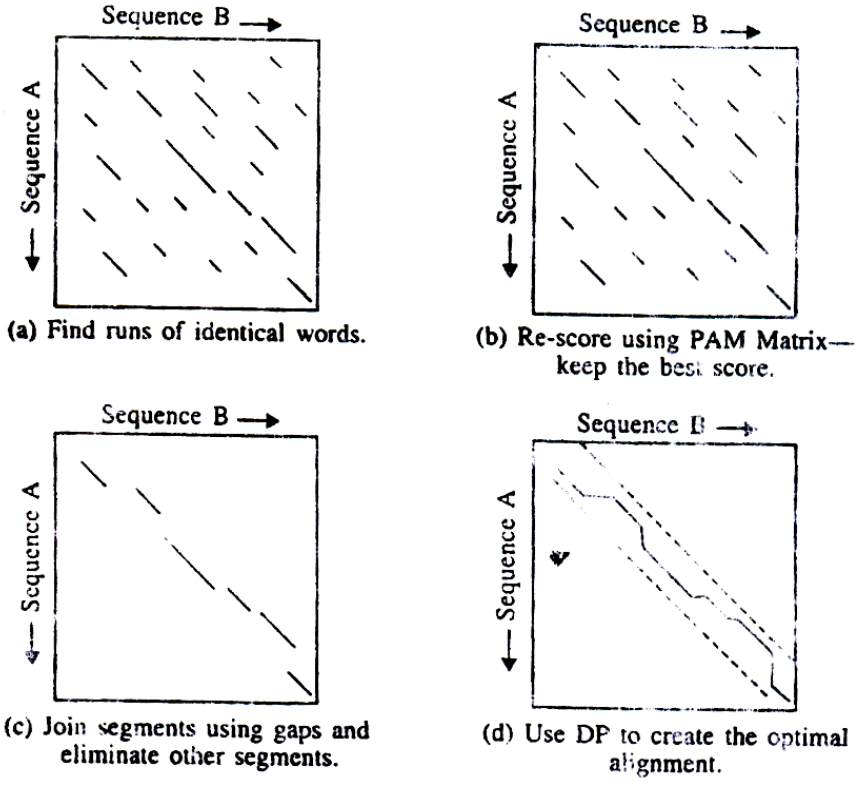
**FASTA Algorithm**

FASTA starts by making a generalization from the concept of dot plots. In a dot plot, regions of similarity between two sequences show up as a diagonals. FASTA goes a step forward and calculates the sum of the dots along each diagonal.

FASTA is a "word" based method. It looks for matching "word" or the sequence patterns called k-tuples. It then builds a local alignment based upon these word matches. FASTA makes a list of all words (sequence patterns of 1 or 2 amino acids, or 5 or 6 nucleotides) in each sequence. It matches identical words from each list, and then crates diagonals by joining adjacent matches.

FASTA then re-scores the highest scoring region using a replacement matrix (e.g. scoring matrices like PAM and Blosum). The best of these scores is called init1. FASTA joins together the high scoring diagonals, allowing for gaps. The best score from that is called initn. FASTA finally uses Smith – Waterman algorithm to identify an optimal local alignment around the regions it has discovered. This last alignment step is a local alignment step and is only applied to a small number of sequences that has high initn values after the database search.

The FASTA algorithm is given in figure. The four steps in FASTA used to calculate similarity scores between a pair of sequences are as follows:




**Figure**

1. **Find runs of identical words.** Identify regions shared by the two sequences that have the highest density of single residue identities (ktup-1) or two – consecutive identities (ktup=2).
2. **Re-score using PAM matrix.** keep the best score. Re- scan the best regions identified in step 1 using the PAM- Matrix. The single best score is stored as init 1 for reporting later.
3. **Join segments using gaps and eliminate other segments.** Determine if gaps can be used to join the regions identified in step 2. If so, determine a similarity score for the gapped alignment, which is reported as initin.
4. **Use DP to create the optimal alignment.** Construct an optimal alignment of the query sequence and the library sequence (Smith-Waterman algorithm). The score is reported as the optimized score. FASTA uses hash – coding method in the initial search for regions of similarity. In hash coding, a lookup table showing the positions of each sequence word of length k (a k-tuple). Is constructed for each sequence. The relative position of each word in the two sequences is then calculated for each sequence. the relative position of each word in the two sequences is then calculated by subtracting the position in the first sequence from that in the second. Words that have the same offset position reveal a region of alignment between the two sequence. The number of comparisons increases linearly in proportion to average sequence length.

The K-tuple length is user – defined and is usually 1 or 2 for protein sequence i.e. either the positions of each of the individual 20 amino acids or the positions of each of the 400 possible dipeptides are located. For nucleic acid sequences, the k-tuple is in the range of 5 to 20. It is much more than the proteins case because short k-tuples are much more common due to the 4 letter alphabet of nucleic acids. The larger the k-tuple chose, the more rapid but less thorough, is the database search.

**FASTA Implementation**

FASTA 3 (http:/www.ebi.ac.uk/fasta33/) at the EBI is one of the most popular FASTA implementations.

Proteomes and genomes FASTA (http:/www.ebi.ac.uk/fasta33/genomes.html) provides sequence similarity and homology searching against complete proteome or genome database using the FASTA programs.

SNP (Single Nucleotide Polymorphisms) sequences can be searched with the FASTA server at the EBI (http://www.ebi.ac.uk/snpfasta3/index.html).

**Recommended Steps for a FASTA Search**

The following strategy is recommended for searches with Fasta for finding the most homologous sequences in a database search while avoiding false negative matches:

1. Comparison should be done using protein sequences, by even translating DNA sequences, if possible.
2. Search non-redundant protein databases like PIR or Swiss-prot with ktup=2.
3. Look for agreement between the real and theoretical distribution of scores.
4. For a match to be significant. E( ) should be < 0.05. If the search has correctly identified homologous sequences, then the corresponding E( ) values should be much less than 0.02, while scores between unrelated sequence should be much greater than this value. If there are no E( ) less than 0.1 then the search has not found any sequences with significant similarity to the probe sequence.
5. If there are no matches with E( ) less than 0.1 then repeat the search with FASTA with ktup=1, or else use the Search. If FASTA now finds matches with E( ) less than 0.02, then the sequences may be homologous, if there is not a low complexity region in the probe sequence. Sequence with score of 0.2 to 10 may also be homologous but have marginal sequence similarity. For further study of this possibility select some of these marginal sequences and use them as probe sequences for additional database searches with FASTA. Additional family members with significant similarity may then be found.
6. Confirm homology of marginal matches by using database sequence many times to calculate the significance of the real alignment. The program Prss (http://fasta.bioch.virginia.edu/fasta/prss.htm) performs the task of sequence shuffling.
7. Protein sequence alignments with 50% identify in a short 20-40 amino acid region are common in unrelated proteins. To be truly significant, the alignment should extend over a longer region.

**6. Explain the following terms:**

**(i) Affine gap penalty:**

Affine gap penalty is a type of gap penalty that penalizes insertions and deletions using a linear function in which one term is length independent, and the other is length dependent. A regular gap extension method would assign a fixed cost per gap. An affine gap penalty encourages the extension of gaps rather than the introduction of new gaps.

Genematcher2 (http://www.paracel.com/products/gm2.html) is a supercomputer for sequence analysis. Its implementation of Smith – Waterman provides three different kinds of gap opening and extension penalties: linear, affine and double affine. In the double –affine model. Two sets of open and delete scores are used for gaps. This allows the user to reduce the penalty of a gap extension, as a gap grows longer. This algorithm is particularly advantageous when comparing chromosomal DNA to ESTs. As introns are not over – penalized when trying to find a match.

**(ii) Log odd score**

- We wish to know it the alignment score is above or below the score of random alignment.
- For gapless alignments we used the log – odds ratio:
  S(a,b)=log (pab/qaqb). Log (pab/qaqb)>0 if the probability that a and b are related by our model is larger than the probability that they are picked at random.
- To adapt this for the HMM model, we need to model random sequence by HMM, with end state. This model assigns probability to each pair of sequences x and y of arbitrary lengths m and n.

**(iii) Baysian rule**

A clinical trail is an experiment carried out to gain knowledge about relative benefits of two or more treatments. Typically, this is part of a gradual accrual of knowledge: a trial to confirm benefits in a large population may follow much careful work on smaller scale studies, or a study may be asking essentially the same question as several other studies. Conventionally, clinical trials are analyzed formally as an individual trial, and their contribution to accruing knowledge then assessed informally, however, increasingly the technique of **meta-analysis** is used to combine the information from similar trails into a formal summary.

More generally, researchers may wish to frame the following questions: " What do we think about the relative benefits of the treatments before knowing the results from this trail? " What information can be gained form the results of this trail? "Considering the results of this trail in the light of previous understanding what do we now think about the relative benefits of the treatments?

If this seems too subjective, an alternative way to casting this frame work is to ask: "What is the previous evidence on the relative benefits of treatment?

"What is the current evidence from this trail? "What is the updated evidence, once we combine the previous with the new evidence?"

The concept of updating to beliefs or evidence is the essence of Bayestan statistics. This article explains the essential concepts through a simple example, and then discusses some of the issues raised, namely the legimate sources of previous beliefs or evidence, including the question of subjectivity, and implications for the design of trials and Bayesian reporting of clinical trials. A particular area of application is data monitoring (see Data and Safety Monitoring).

Most of the discussion is in the context of two group parallel trials, parity for simplicity of exposition, but mainly because of he pre – eminence of this design in practice. The framework is, however, completely general, and applies to more complex designs.

For the combination of results of several trials, possibly with other evidence, Bayesian mere – analysis is outlined. Clinical trials are often used as part of wider decision – making processes. Bayesian statistics is sometimes set in the context of decision – making, and the implications of this are discussed. Finally, there is a note computational issues.

**An example**

Consider the following example: after a heart attack. thrombolviss is often indicated. There is a tension between whether this is done at home once the ambulance arrives which confers the advantage of speed, or in hospital, which is a more optimal environment, but necessitates a delay in treatment. The GREAT trial was run to compare these two strategies[8].

When the trial reported, there were 13 deaths out of 163 patients in the home group, and 23 out of 148 in the hospital group. The authors estimated a reduction in morality of 49%. Some commentators were skeptical that a having of mortality was really possible. Pocock & Sregelhaiter [14] carried out a Bayesian analysis. They judged, ignoring the trial results, that home treatment probably conferred some benefit, say a 15% -20% reduction, but let alone a halving of mortality, was fairly unlikely. These beliefs are termed the prior distribution. ‘

The evidence from the trial is described through the likehood junction. Combining these two gives the posterior distribution of beliefs. This gives an estimate of the reduction mortality of about 25%, but still says that the extremes of no effect or of a halving of mortality are unlikely.

Differences from classical analyses include the incorporation of prior beliefs, the absence of P values, and the absence of any idea of hypothetical repetitions of such studies. The posterior estimate of effect and the absence of any idea of hypothetical repetitions of such studies. The posterior estimate of effect and its surrounding uncertainty via a credibility interval is analogous to a classical point estimate and its associated confidence interval, but has a direct interpretation in terms of belief. As many people interpret a confidence interval as the region in which the effect probably lies, they are essentially acting as Bayesians.

**Bayesian methods** are valuable **inter** alia, whenever there is a need to extract **in**formation from data that are uncerta**in** or subject to any k**in**d of error or noise (**in**clud**in**g measurement error and experimental error, as well as noise or random variation **in**tr**in**sic to the process of **in**terest).**Bayesian methods** offer a number of advantages over more conventional statistical techniques that make them particularly appropriate for complex data. It is therefore no surprise that **Bayesian methods** are becom**in**g more widely used **in** the fields of genetics, genomics, **bioinformatics** and computational systems biology, where mak**in**g sense of complex noisy data is the norm. this review provides an introduction to the grow**in**g literature **in** this area, with particular emphasis on recent developments **in Bayesian bioinformatics** relevant to computational system biology.

**(iv) Smith waterman algorithm**

**Smith-Waterman Algorithm:** This is the dynamic programming algorithm for local pairwise sequence alignment. It determines similarity regions between two nucleotide or amino acid sequences based on **substitution matrices** and gap **scoring schemes.** Here, the scores of residues are always **positive** and all negative scores in the matrix cells are set to **zero.** The following equation is used to fill the matrix:

$$S = \max \begin{cases} Si-1, j-1^{-S(a,i,bj)} \\ Si-1, j-G \\ Si, j-1-G \\ 0 \end{cases}$$

Here, S is a negative expectation score. It is the average score that the scoring system would yield for random sequence.

Here is a set of rules for local alignment:

- ★ Backtracking should be started at the highest scoring matrix cell and proceeded toward the lowest scoring matrix cell.
- ★ Unrelated sequences tend to form extreme value distribution.
- ★ Low expectation values indicate homologues sequences that have a common ancestry.
- ★ High expectation values shows unrelated sequences
- ★ Too much of mutations in the past had produced variability in the sequence.

**Limitations**

1. Smith-Waterman algorithm is demanding much time and memory.
2. not suited for aligning lengthy sequences.
3. It needs much space to work
4. Reliable statistical models are necessary for better local alignment.

Because of these limitations, Smith – Waterman algorithm has been put aside and replaced with some modern programmes:

- ★ BLAST algorithm is somewhat more efficient in local sequence alignments than Smith-Waterman algorithm.
- ★ A modified version of smith – Waterman algorithm is used in-

- SSEARCH programme of FASTA sequence analysis package
- Altivec programme of Power PC
- SSE2 algorithm of Intel / AMD processor of Intel Core Microarchitecture
- Biofacet software (1997)
- FPGA chips
- Prgeniq
- GenCore 6 package

**7. Explain pairwise sequence alignment along with its methods.**

**Pairwise Sequence Alignment**

Arranging the query sequence with only one homologous sequence from a database at a time is called **pairwise sequence alignment.** Region of similarity are identified between the two sequences and then they are aligned so as the similarity regions come in the same column. Thus a pair of sequence is formed in each alignment.

The first alignment is then aligned with yet other homologous sequence. As a result, a tree of sequences are formed.

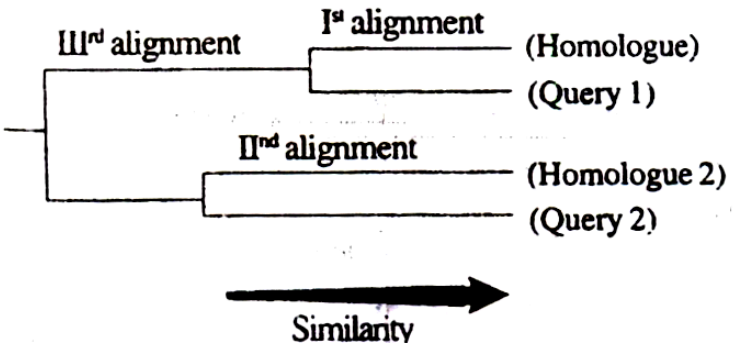



**Fig. Pairwise sequence alignment leading to a tree of sequences.**

Pairwise sequence alignment methods are used to find the best – matching piecewise (local) or global alignments of two sequences. They can only be used between two sequences at a time, but they are efficient to calculate residue – residue correspondences.

The three primary methods of producing pairwise alignments are,

i. Dot Matrix method
ii. Dynamic programming
iii. Word method or k-tuple method

**Dot Matrix method**

The matrix formed of dots instead of numbers is called **dot matrix.** The dots are uniform throughout the matrix. When value are added to the matrix according to respective position of residue, the dots kitted by these values give a line graph called **dot plot.**

The dot matrix approach produces a family of alignments for individual sequence regions. It is qualitative and simple, though time – consuming t analyze on a large scale. From a dot – matrix plot, it sis very easy to visually identify certain sequence features such as

i. **insertions,**
ii. **deletions,**
iii. **repeat or inverted repeats**

To construct a dot matrix plot, the two sequences are written along the top row and leftmost column of a two – dimensional matrix and dot is placed at a point where the characters in the appropriate columns match. In this way, all values are plotted in the matrix as distinct dots. The line appearing in the matrix is called **recurrence plot.**

Some implementations vary the size or intensity of the dot depending on the degree of similarity of the two characters, to accommodate conservative substitutions. The dot plots of very closely related sequences will appear as a **single line along** the matrix's main diagonal.

When homologous sequences from two closely related species are aligned with dot matrix, the similarity regions show a straight line and the inverted sequences show lines perpendicular to the straight line Eg. Genome comparison of Salmonella typhimurium with Salmonella enterica shows straight similarity regions and short perpendicular reverse sequences.

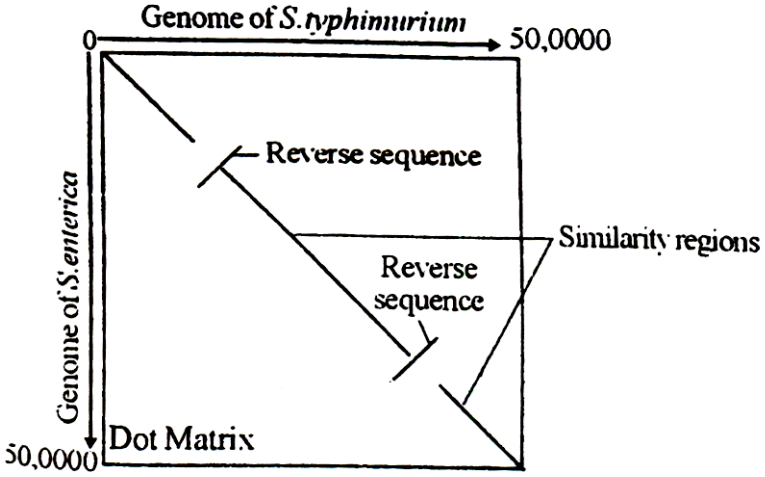



**Fig. Dot plot showing similarity regions and reverse sequence in relation to each other.**

Dynamic programming is a three step process that involves-

i. Breaking of the problem into small subproblems
ii. Solving the subproblems using recursive methods
iii. Construction of optimal solutions for original problem using the optimal solutions.

**Creating Subproblems:** The lengthy sequences to be aligned are split into small sequences to score the individual residues easily. It is done for both nucleotide sequence of DNA and amino acid sequence of protein. Each short sequence is considered to be a **subproblem.**

**Solving the Subproblem:** The problem solving here means aligning the short sequences to detect residue – residue correspondences between the segment of query sequence and of homologous sequence. Scoring matrices for each and every residue in the sequences are calculated from **gap penalty** and **gap score** based on certain algorithms the scores of the residues are so populated as to give optimal alignments; otherwise it is difficult to get precise alignments. Here the residues are matched one after the other step – by – step to get best alignment.

Dynamic programming can produce both **global alignment** and **local alignment.** Global alignment is achieved via **Needleman – Wunsch algorithm** whereas local alignment is obtained via **Smith – Waerman algorithm.** In general, **substitution matrices** are employed to assign the scores of residues for matches and mismatches and **gap penalty** is used to assign gap in one sequence in relation to other.

## UNIT – IV

## PART – A

**1. Define phylogeny.**

Phylogeny refers to ancestral relationship in a set of species or tax a. It illustrates the possible origin and evolution of various species or taxa which took their origin from a common ancestor. The basic principles of phylogeny are that similarity comes from ancestry & divergence leads evolution.

**2. Define Phylogenetic Analysis.**

The study to work out the ancestral relationship among various species or taxa is called phylogenetic analysis or phylogenetics. It demonstrates to which extent various species or taxa are related to each other and whether one of these species or taxa could be the possible ancestor for rest of the species.

**3. Define Phylogenetic tree.**

A phylogenetic tree is a two-dimensional graphic diagram usually shown as a phenogram. It consist of a single root, many nodes and edges. Phylogenetic reactions among various species of a known set is described in the form of a tree called phylogenetic tree.

**4. What are the limitations of phylogenetic trees?**

- ★ The data is noisy
- ★ Data is based on horizontal gene transfer
- ★ Hybridization may have altered the original structure.
- ★ Convergent evolution could change the actual structure.
- ★ Conserved sequence in the data.

**5. Define Node.**

A point of phylogenetic tree at which divergence of new species occurs. Nodes are abstract points of divergence. Each of these nodes represents the most recent ancestor for the descendents. All nodes, except the terminal nodes are called internal nodes.

**6. Define Phenogram.**

Phenogram is a branching diagram that links entities by estimating overall similarity. Here, entities are parallel to each other.

**7. Write short notes on UPGMA.**

**UPGMA –**Unweighted pair group method with arithmetic mean.

It is the simplest method to construct phylogenetic trees. It uses clustering approach and uncorrected data to build a tree. This method is suitable for large datasets consisting lineages with relatively constant rates of evolution.

**8. Define distance matrix.**

The distances between sequences under study are calculated and populated into a matrix called distance matrix.

**9. Write some advantages of UPGMA method.**

- ★ It is useful to construct phylogenic tree of taxa with the relatively constant rate of evolution
- ★ It is a simple & fast method
- ★ It is very common in use

**10. Write short notes on cladistic methods.**

The cladistic methods are character based methods. In these methods, all possible topologies are evaluated and one that optimizes the evolution is chosen as the correct tree.

**11. What is Cladogram?**

Cladogram is a branching, tree-like diagram in which the endopoints of the branches represent specific of organisms. Here, the branches are not parallel to each other.

**12. What are the 2 cladistic methods to construct phylogenetic trees?**

1. Maximum Parsimony method
2. Maximum likelihood method

**13. Distinguish between Maximum parsimony and Maximum likelihood**

| MAXIMUM PARSIMONY | MAXIMUM LIKELIHOOD |
|---|---|
| ★ Shared derived characters | ★ All data is important |
| ★ Slow | ★ Very slow |
| ★ Total distance is minimized | ★ Maximizes free likelihood given in specific parameter values |
| ★ Assumption fail when evolution is rapid | ★ Highly dependent on assumed evolution model |
| ★ Best option for character change in long sequences | ★ Good for very small data sets & for testing trees. |

**14. Write some advantages and disadvantages of Maximum parsimony method.**

**Advantages**

1. It reflects the ancestral relationship
2. It uses all known evolutionary information
3. It is faster than maximum likelihood.

**Disadvantages**

1. It yields little information about branch length
2. It require a long computation time
3. It often yields biased tree under certain conditions.

**15. Write some advantages and disadvantages of Maximum likelihood method.**

**Advantages**

1. It is more accurate than other methods
2. All sequence information is used
3. It evaluates all possible trees

**Disadvantages**

1. Extremely slow
2. Impractical for analyzing large data set.

# PART – B

**1. Explain UPGMA method of phylogenetic tree construction.**

**Unweighted pair group method with arithmetic mean (UPGMA)**

UPGMA is the simplest method to construct phylogenetic trees. It uses clustering approach and uncorrected data to build a tree. This method is suitable for large datasets consisting lineages with relatively constant rates of evolution.

**Steps for building a tree**

The distance between the taxonomic units (species) is calculated on the basis of pairwise differences between them. A distance is the difference in one residue between the order of residues in two sequences. The unit of distance is equal to:

1. The distances between sequences under study are calculated and populated into a matrix called distance matrix.

Let us explain how to calculate the distance, how to make a distance matrix and how to make a distance matrix and how to make use of that matrix to construct a phylogenetic tree with a simple example. Suppose that we want to construct a phylogenetic tree for five species (I,II,III,IV and V) and that a homologous DNA of 10 base-size is taken from each species under study. Imagine the following are the DNA sequences:

| | |
|---|---|
| ATGGCTATCG | Sequence I |
| ATGGCTATGG | Sequence II |
| ATGGCTCAGC | Sequence III |
| ATGGTCAGTC | Sequence IV |
| ATCCTAAGCTA | Sequence V |

From these sequence see, the distances are calculated in the following way:

- ★ There is no difference in residue, if resides of I is matched with those of yet other I. So the difference between sequence I and I is zero. Thus differences between I and I, II and II, III and III, IV and IV , V and V are zero.
- ★ Then the difference between sequence I and II is calculated. The two sequences are written one below other and matched base to base. If the two residues are identical at a corresponding position of these two sequences, they may be darkened with a pencil or sticked off. Then the non-identical residues are counted and given as the mean distance.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| I | A | T | G | G | C | T | A | T | C | G |
| II | A | T | G | G | C | T | A | T | G | G |

Here , there is only one non-identical pair so that difference between I and II is 1.

★ In this way difference is calculated for –

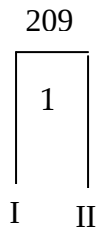
I and I
I and II II and II
I and III II and III III and III
I and IV II and IV III and IV IV and IV
I and V II and V III and V Iv and V V and V.

This calculated distance values are filled up in the matrix called distance matrix.

| 0 | I | II | III | IV | V |
|---|---|---|---|---|---|
| I | 0 | 1 | 4 | 5 | 8 |
| II | | 0 | 3 | 5 | 8 |
| III | | | 0 | 7 | 8 |
| IV | | | | 0 | 6 |
| V | | | | | 0 |

**Distance matrix**

The smallest distance is 1, as shown by the distance matrix, and it is between sequence I and II. Therefore the first graph (or cluster) is drawn for I and II.




Having drawn the first graph(cluster). The matrix is reduced suitably to have a reduced distance matrix. In the reduced matrix, I and II are considered together as a unit (I & II). The distance between (I & II) and III is calculated as-

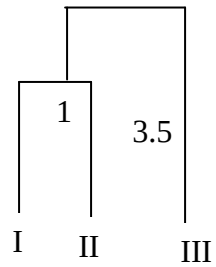
$$\frac{\text{distance betweeen I and III + distance between II and III}}{2}$$

@ $\frac{4+3}{2} = \frac{7}{2} = 3.5$

In this way, all distances between the sequences are calculated and populated in the reduced distance matrix as given below:

| | I & II | III | IV | V |
|---|---|---|---|---|
| (I & II) | 0 | 3.5 | 5 | 8 |
| III | | 0 | 7 | 8 |
| IV | | | 0 | 6 |
| V | | | | 0 |

According to the reduced distance matrix, the smallest distance is between (I & II) and III and the distance is 3.5. So, connected graph is draw between (I and II) and III. The compound graph is denoted as [(I & II) III)]. The path is 3.1-1=2. So, evolutionary distance between (I and II) and III is 2.




In the next step, the first reduced distance matrix is once again reduced by treating [(I & II) III] as a unit. The distance between [(I & II) III)] and IV is calculated as follows:

$$\frac{\text{Distance of } (\text{I and II}) + (\text{II and IV}) + (\text{III and IV})}{3}$$

$$= \frac{5+5+7}{3} = \frac{17}{3} = 5.7$$

The calculated distances are populated into yet other reduced distance matrix as given below

| | (I,II & III) | IV | V |
|---|---|---|---|
| (I,II & II) | 0 | 5.7 | 8 |
| IV | | 0 | 6 |
| V | | | 0 |

According to this matrix, the smallest distance is between (I,II & III) and IV. Hence, a graph for IV is added to the previous compound graph. Then, the V$^{th}$ one is added to its based on the distance 6.

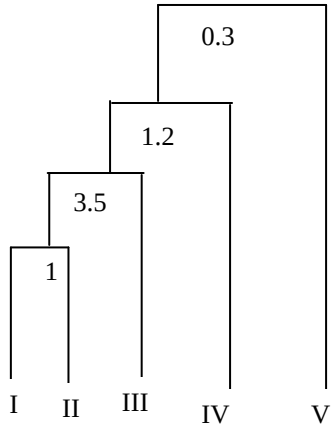



**Fig: A phylogenetic tree**

The percentage of non-identical residues is calculated for each two relatives and given near the edge. It represents the rate of evolution of the species.

$$\text{Evolutionary rate} = \frac{\text{No. of identical residues}}{\text{Total number of residues}} \times 100$$

**Advantages**

- It is useful to construct phlogenic tree of taxa with the relatively constant rate of evolution.
- It is a simple and fast method
- It is every common in use.

**2. Write short notes on**

**(i) Gap Penalty**
**(ii) HMM**
**(iii) Node of a tree**
**(iv) Branch length**
**(v) E-values**
**(vi) Markovian Model**

**(i) Gap penalty:**

The gap penalty is the negative score of the substitution of residue. It is used to decide whether of not to accept a gap or insertion in an alignment. It helps to achieve a good residue-to-residue alignment at some other neighbouring point in the sequence. One cannot let gaps/insertion to occur without penalty, because an unreasonable 'gappy' alignment would result.

It is easy to accept a different residue in a position but not the part of the sequence chopped away or inserted. Gaps/ insertions should therefore be rarer than point substitutions. The following are features of gap penalty:

- A single gap-open penalty will tend to stop gaps in the sequence. But once they have been introduced, they can grow unhindered.
- A gap penalty is proportional to the gap length or residue's length. This will work against any larger gap.
- A gap penalty is equal to a gap-open value plus gap-length value.

**Hidden Markov Models (HMMs)**

This is computational method to describe the subtle patterns in families of homologous sequences. It is used to identify distant homologues of the query sequence. HMMs perform fold recognition as done by CASP programs. They are ideal for detecting distance relatives and for predicting protein folding patterns. The Hidden Markov Models were discovered by A.A. Markov.

Hidden Markov Model is based on a simple mechanism as illustrated in the following figure. A start and end are connected through several intermediate steps via arrows. While moving from the start, one may take any arrow to reach the next position and any one arrow may be selected to get in the next position. At each position one may select any one route and it determine the position to be attained. The action and successive states are governed by a set of probabilities.

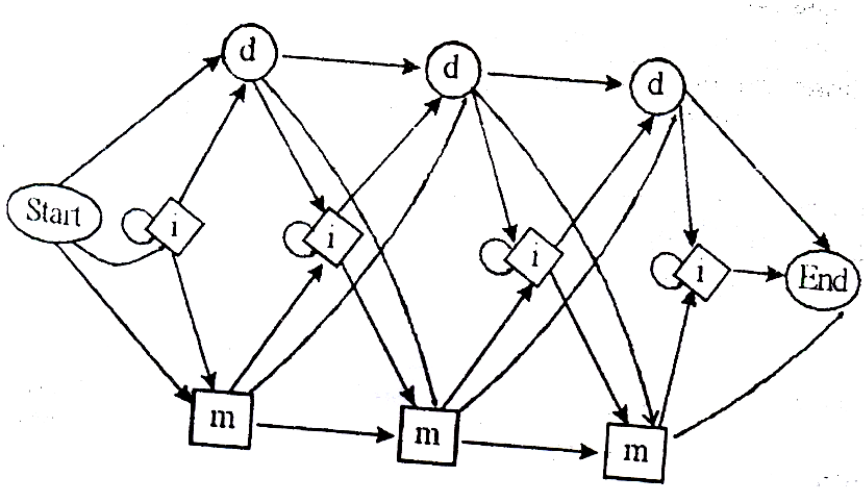



**Fig: Structure of Hidden Markov Model (HMM).**

As each state emits many probable paths, there is probability distribution for each of the 20 amino acids. A second probability distribution is associated with choice of successive state. These

two types of probability distributions give a detailed information about the family of amino acids in the sequence. This same frame-work can be adopted for any protein.

- ★ Query sequence and its homologues are aligned together by multiple sequence alignment.
- ★ Similarity regions, deletions are insertions are marked clearly.
- ★ Residues that matches well are identified and marked at the bottom.
- ★ Gaps that show position of deletions are identified and marked at the top row.
- ★ Insert state appears between the gaps row and matches row.
- ★ After analyzing the appropriate possibility of residues, the choice of the next state is calculated. It is governed by the second probability of distribution.

**Uses: By using this model,**

- Pattern of query sequence is determined
- Patterns inherent in families of proteins are discovered
- Protein truly homologous to the query is easily determined
- Identification of distant relatives
- Evolutionary changes in homologous sequences are traced well.

**(iii) Structure of Typical Phylogenetic tree**

A physlogenetic tree is a two-dimensional graphic diagram usually shown as a phenogram. It consists of a single root, many nodes and edges.

The nodes are the abstract points of divergence. Each node represents the most common ancestor of two descents. Each and every node represents a taxonomic unit. The nodes lying between the root and terminal nodes are called internal nodes. They are said to be the hypothetical taxonomic units (HTUs).

The edges are lines connecting the nodes. They are also known as branches. They denote the descendents and ancestry relationship among different nodes. The edges are parallel to one another.

The node, from which all species descended is called a root or ancestral node.

**(iv) Branch Length**

The basic unit of phylogenetic tree is a graph. The graph consist of two nodes connected by edges. Distance between the terminal node and its ancestral node is called edge length or branch length. It indicates the time of evolution, number of change or genetic distance. This is actually a measure of difference between the nodes.

**(v) Significance of the E-values:**

FASTA calculates an E-value (expectation of significance). E() values represent the number of sequences having a given z-opt score (or better) totally at random. The smaller the number, the less likely that a given alignment occurred by chance and the more likely the alignment represents some true relationship between the query and database sequences.

In the above example of FASTA search, the best hits (near the top of the list) mostly have E() values of zero (0). This can be interpreted as : "you can expect zero sequences to have the score this local alignment has, strictly by chance". Pearson has suggested that E() values of 0.02 or less indicate that the aligned proteins are homologs with fairly high confidence. E() values of 1 or greater generally indicate database sequences with some random similarity. The alignments in between require close scrutiny to separate the high scoring non-related sequences from the distantly related sequences.

E() values are calculated from the probability derived from the extreme value distribution for each z-opt score interval, and the number of sequences in the database. Therefore, as more sequences are added to a database, the E() values can change and sequences identified in one search may not be found in a later one.

The steps for calculation of extreme value distribution to calculate the significance of similarity scores found in a database search as follows.

1. The average score for sequences in the same length range is determined.
2. The average score is fitted in plotted against logarithm of average sequence I.
3. The points are fitted to a straight line by linear regression.
4. A z score, the number of standard deviations from the fitted line, is calculated for each score.
5. High scoring, presumably related or low complexity sequences, and also of very low scoring alignments that do not fit the straight line are removed from consideration.
6. Steps 1 through 5 are repeated one or more times.
7. z scores are used to calculate the probability that a score greater than z would be found between unrelated sequences, using the extreme value distribution equation:

   $P(Z > z) = 1 - \exp^{(-e-1.2825z - 0.5772 </SUP)}$

The expected value of a database search for similar sequences is this score times the number of sequences in the database: $E(Z > z) = D \times P(Z > z)$.

**(vi) Markov Models:**

The Markov model for DNA sequence is shown in figure. The arrows show the various transitions in the model.

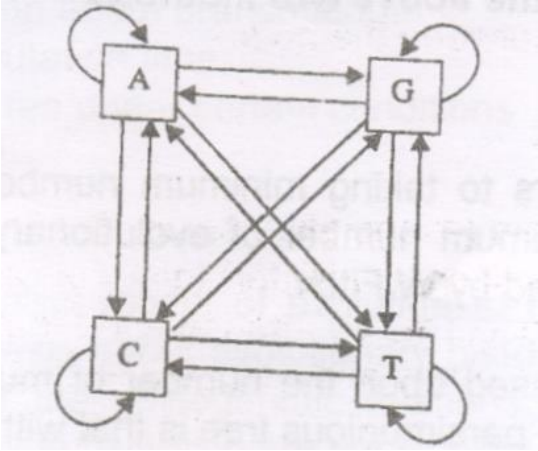



**Fig: Markov Model for DNA sequence**

In figure, the earlier figure has been modified to introduce probabilities associated with various transitions. Figure depicts a Markov Chain, with the probability of the sequence of events cggt, Pr (ccgt) =Pr(c) Pr(glc) Pr(glg) Pr(tlg).

Markov Chain Models can also have an end state. The end state can be used to represent a distribution over sequences of different lengths and also for giving preferences for ending sequences with certain symbols. This is shown in figure.

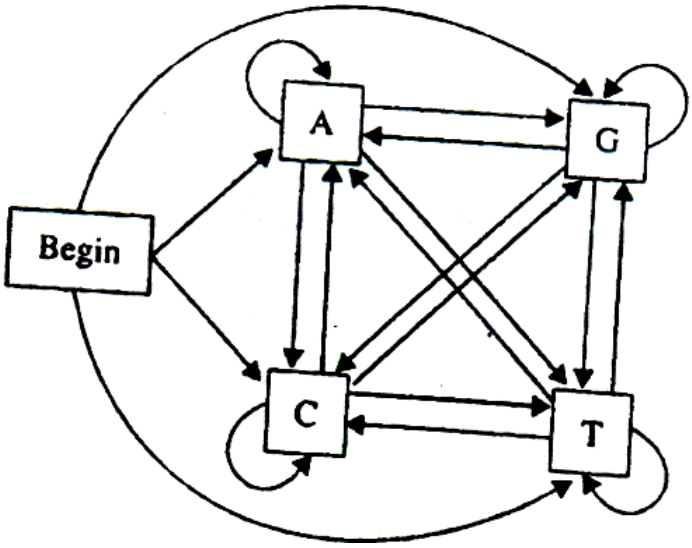



**Figure: Markov model for a sequence of events cggt.**

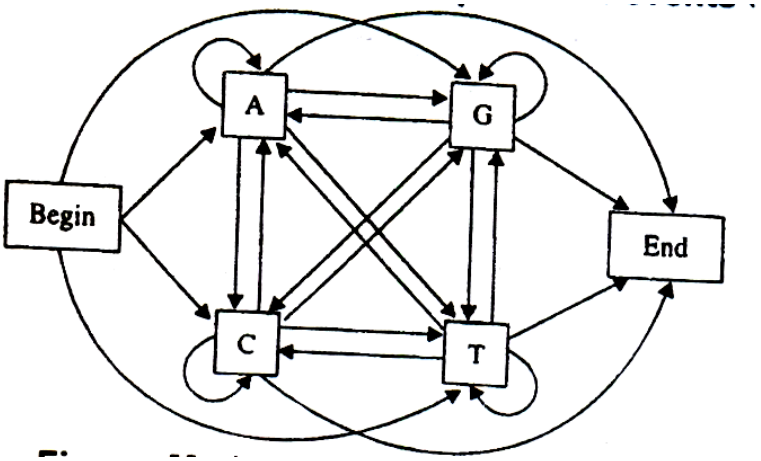



**Figure: Markov model with an end state**

**3. Explain**

**(i) Maximum Parsimony**
**(ii) Maximum likelihood**
**(iii) Distinguish between the above two methods**

**(i) Maximum parsimony**

Maximum parsimony refers to taking minimum number of steps to construct a tree. It assumes that trees with the minimum number of evolutionary changes are the most preferable trees. This method was discovered by W.Fitch.

Maximum parsimony is based upon the number of mutations in the sequences. It gives each mutation a score. The most parsimonious tree is that with fewest mutations.

**Steps for building a tree**

i. All the sequences are aligned using multiple alignments.
ii. The probability of change (mutation) at each position of the sequences is calculated using Jukes-Canton model. This model has revealed that each species will have the same frequency of mutation in the DNA sequences at the equilibrium level. According to this model, the average number of substitutions (mutations) at site K between the sequence A and B is given by the formula-

$$K_{AB} = -\frac{3}{4} \log \left[1-\frac{4}{3} d_{AB}\right]$$

Where, $d_{AB} = n_{AB} - N$

$n_{AB}$ is the number of mutations in sequence B in relations to sequence A
N is the length of the sequence
$d_{AB}$ is the average mutations (changes ) per length

iii. Probability of mutations is calculated for each residue and the data is populated into a substitution matrix.
iv. By using the value sin the substitution matrix, all possible topologies of trees are constructed by arranging the graphs one by one. Here, taxonomic units showing minimum substitution are placed close to each other.
v. All these trees are screened for one that has fewest mutations (evolutionary changes) by comparing the score values of substitution.
vi. After selecting the suitable phylogenetic tree, the evolutionary relationships among the species are interpreted on the basis of evolutionary changes occurred.

**Advantages**

- It reflects the ancestral relationship
- It uses all known evolutionary information.
- It is faster than maximum likelihood

**Disadvantages**

- It yields little information about branch length
- It require a long computation time
- It often yields biased tree under certain conditions.

**(ii)Maximum Likelihood**

Maximum likelihood use explicit model of evolution to reconstruct a phylogeny. It uses statistical tool to evaluate a hypothesis about evolutionary history from an observed data set and chooses suitable one to interpret the phylogenetic relationships between the species. The probability of mutation and pattern of substitution are considered to be important in this method.

**Steps for building a tree**

i. All the sequences are aligned by using a multiple sequence alignment method.
ii. The probability of change (mutation) at each position and the patterns of substitution are calculated by using Kimura model. This model assumes that each species will have the same frequency of mutations, if equilibrium conditions persist. Further, it states that transitions occur more frequently that transversions.

If A =1/(1-2$d_{AB\ transition}$ –$d_{AB\ transversion}$) and b =1 /(1-2$d_{AB\ transition}$ – $d_{AB\ transversion}$), the average number of substitution at site $K_{AB}$ is given by the equation.-

$K_{AB}$ =1/2 $\log_e$ (a) +1/4 $\log_e$ (b)

Thus, substitution values are calculated for each and every species and populated into a substitution matrix.

iii. From the values in the substitution matrix all possible topologies of trees are constructed by arranging the graphs one by one. Here, mutations with high frequency of transitions are brought together side-by-side.
iv. All these trees are screened for one that has minimum number of mutations and more number of transitions.
v. The phylogenetic tree that fits well with the rules, is chosen to interpret the phylogenetic relationships among the species.

**Advantages**

- It is more accurate than other methods. So, it is often used to test the existing tree.
- All the sequence information is used
- It evaluates all possible trees
- Sampling errors have least effect on the result.

**Disadvantages**

- Extremely slow
- Impractical for analyzing large data set.

**(iii) Distinguish between the above methods**

**Comparison of methods**

| Neighbour-joining | Maximum parsimony | Maximum likelihood |
|---|---|---|
| Pairwise distances are important | Shared derived characters are important | All data is important |
| Distance between nearest neighbours is minimized | Total distance is minimized | Maximizes tree likelihood given in specific parameter values |
| Very fast | Slow | Very Slow |
| Easily trapped in local optima | Assumptions fail when evolution is rapid | Highly dependent on assumed evolution model. |
| Good for generating tentative tree | Best options for character change in long sequences | Good for very small data sets and for testing trees. |

**4. Write short notes on**

**(i) Phylogenetic analysis**

The study to work out the ancestral relationship among various species or taxa is called **phylogenetic analysis** or **phylogenetics.** It demonstrates to which extent various species or taxa are related to each other and whether one of these species or taxa could be the possible ancestor for rest of the species or taxa could be he possible ancestor for rest of the species or taxa could be the possible ancestor for rest of the species. Since phylogeny is related to origin and evolution of closely related species or taxa, phylogenetic analysis helps to classify plants, animals and microbes according to their degree of relatedness with one another. From these phylogenetic systematic, we can understand all these organisms as they emerged over time. The main goal of phylogenetic analysis is he identification and estimation of relationships among taxa, species population, individual or genes.

In classical phylogenetic analysis, emphasis has been given to the morphological features of the species; this is what numerical taxonomists do for many years. Now – a- days, such studies are extended to molecular level, which leads to the emergence of **molecular phylogeny.** This method relies on the amount of information that can be obtained form DNA, RNA, proteins and immunoglobulins.

Phylogenetic relationship among various species of a known set is described in the form of a tree called **phylogenetic tree.** It is noting but a phenogram that explains the relatedness among the species. It may or may not show the ancestor (root).

Phylogenetic analysis helps us to understand-

- ★ Similarity among species or individuals
- ★ Dissimilarity between species
- ★ Close relative of a species
- ★ Whether two groups are identical in topology (evolution)
- ★ Possible ancestor of a group
- ★ Evolutionary distance between the species.
- ★ Different rate of evolutions of different species.

**(ii) Phylogenetic tree**

A tree – like graph that shows ancestral relationship among various species originated from a common ancestor is called **phylogenetic tree.** It is also known as an **evolutionary tree** or a **tree of life.** This is a two dimensional graph.

Phylogenetic trees may be shown as **phenogram or cladogram.** In the phenogram, the branches are arranged parallel to each other. On the other hand, in cladogram the branches are redialing outward from the central point. Both phenogram and cladogram explain the evolutionary origin of various species considered in this study, but phenogram alone can illustrate the extent of relationship among them. So, much importance is given for phenogram to construct a phylogenetic tree.

The idea of a "tree" originally arose from earlier ideas of life as a progression from lower to higher forms. Phylogenic tree is built from morphological data (e.g., break shades, number of legs, etc.) or molecular data (DNA and protein sequences). Modern evolutionary biologists use molecular data to construct phylogenetic trees.

**(iii) Structure of Phylogenetic tree**

A phylogenetic tree is a two – dimensional graphic diagram usually shown as a **phenogram.** It is consists of a single **root,** many **nodes and edges.**

The nodes are the abstract points of divergence. Each node represents a **taxonomic unit.** The nodes lying between the root and terminal nodes are called **internal nodes.** They are said to be the hypothetical taxonomic units (HTUs).

The edges are **lines** connecting the nodes. They are also known as **branches.** They denote the descendents and ancestry relationships among different nodes. The edges are parallel to one another.

The node, from which all species descended is called a **root** or **ancestral node.**

The root may be placed at he top or on the side. A phylogenic tree with its root is known as **rooted tree.** If the ancestral species or taxon is not known or not considered, the phylogenetic tree remains **unrooted.**

The terminal nodes represent the taxonomic units, which may be species, higher viduals. They are often known as **operational taxonomic units** (OTUs). Character data such as a position at the sequence or a residue at the position or the distance (difference) between two neighbours can be considered as OTUs.

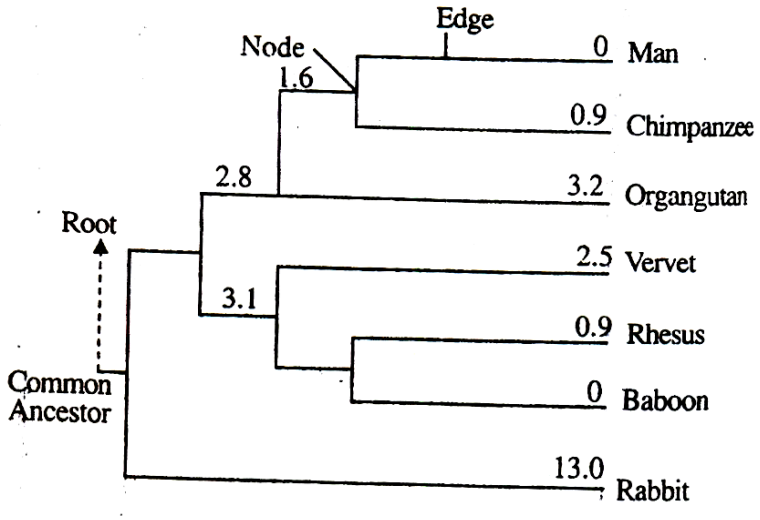
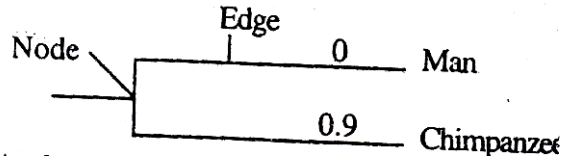
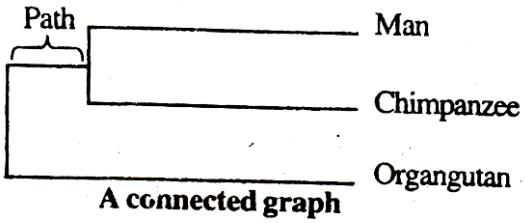



**Fig. A rooted phylogenetic tree. The phylogeny of various primates based on the sequence of 115 amino acids in carbonic anhydrase.**

The basic unit of phylogenetic tree is a **graph.** The graph consists of two nodes connected by edges. Distance between the terminal node and its ancestral node is called **edge length** or **branch length.** It indicates the time of evolution, number of changes or genetic distance. This is actually a measure of difference between the nodes.




The above segment of the phylogenetic tree represents a graph. The numbers 0 and 0.9 represent the difference between the two species in terms of carbonic anhydrase. When this graph is connected with another graph or edge, it gives a **connected graph.** The distance between the consecutive nodes is called a **path.** A connected graph has only one path.




**A connected graph**

Many such graphs are clustered together on the basis of their similarity to form a complex structure called **phylogenetic tree.** The sum of lengths of all paths in a **phylogenetic tree**. The sum

of lengths of all paths in a phylogenetic tree is the **path length.** It depicts the evolutionary distance of a species from the common ancestor.

A phylogeneitc tree with two descendents at each node is called a **binary tree.** It can b constructed by using **PERL** program.

**Unrooted tree**

The phylogenetic tree in which the possible ancestor cannot be placed is called **unrooted tree.** It may be shown as **phenogram** or **cladogram.**

**(iv) Limitation of phylogenetic tree**

Phylogenetic trees constructed from sequenced genes or genomic data do not necessarily represent actual evolutionary history. This is because such data impose the following limitations:

- The data is noisy,
- Data is based on horizontal gene transfer,
- Hybridization may have altered the original structure
- Convergent evolution could changed the actual structure
- Conserved sequences in the data

PhyloCode that is most often used to construct phylogenic trees does not assume a tree structure to overcome these limitations.

Furthermore, the tree constructed from a single gene or protein taken from a group of species may differ from that constructed from another unrelated gene or protein sequence taken from the same species. This is more evident in genetic material that underwent frequent lateral gene transfers and recombinations. Therefore, great care is needed in describing phylogenetic relationships among species.

It is quite uncertain whether extinct species should be placed at the ancestral node or at the terminal node. Since DNA had not been preserved intact in specimens older than 100,000 years, DNA sequence data cannot be obtained from them to trace their evolutionary positions. In general, extinct species are placed at the terminal nodes. This is unlikely because an extinct species could even be the common ancestor for all.

**(v) Construction of phylogenetic tree**

Phylogenetic trees are constructed using computational phylogenetics methods. Distance-matrix methods such as neighbour – joining method require multiple sequence alignments to

calculate genetic distance. They are simplest to implement. Many sequence alignment methods such as Clustal W produce both sequence alignments and phylogenetic trees.

Other methods include maximum parsimony and probabilistics inference techniques such as maximum likelihood. Bayesian inference has also been applied to phylogenetics but has been controversial.

Tree – building methods are chosen on the basis of several criteria:

- High computing efficiency using available memory.
- High power to use the available data to calculate the evolutionary distances
- Consistency in the result while repeating the program
- Rebustness
- Good falstifiability.

**5. Explain Neighbour Joining method in detail.**

**Neighbour Joining (NJ):**

This is a method to construct phylogenetic trees. It is a slight modification of UPGMA method. Neighbour joining is a special example of start decomposition method. In this method, the phylogenetic tree is constructed from a start – like tree by grouping OTUs with shortest distance of branch length together. This method is very suitable with dataset consisting descendants with largely varying rates of evolution. This method was discovered by **N. Saitou** and **M.Nei.**

**Steps for building a tree:**

The distances between the homologous sequences are calculated by residure – residue matching between the sequences. A distance is denoted by a difference in one residue. Here also the unit of distance is 1. the distances between the sequences are calculated, as has been given in the UPGMA method and populated into a **distance matrix.**

| 0 | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| A | 0 | 5 | 4 | 7 | 6 | 8 |
| B | | 0 | 7 | 10 | 9 | 11 |
| C | | | 0 | 7 | 6 | 8 |
| D | | | | 0 | 5 | 9 |
| E | | | | | 0 | 8 |

**Distance matrix**

From this distance matrix, net divergence (r) is calculated for each and every taxonomic unit. It is calculated in the following way:

$$r_{(A)} = d(A-A)+d(A-B)+d(A+C)+d(A+D)+d(A+E)+d(A+F)$$

where, d is distance. Therefore,

$r_{(A)} = 0+5+4+7+6+8=30$

similarly, $r_{(B)}$ , $r_{(C)}$, $r_{(D)}$, $r_{(E)}$ and $r_{(F)}$ are calculated. In this example –

BI – MSU – 8
$r_{(B)} = 42$
$r_{(C)} =32$
$r_{(D)} = 38$
$r_{(E)} = 34$
$r_{(F)} = 44$

A refined distance matrix is calculated from the net divergence values of the taxonomic units. It is calculated by using the formula –

$M_{ij} = d_{ij} – [r_i – r_j] / N – 2$

Where,

N is the number of taxonomic units considered
$d_{ij}$ is the distance matrix
$r_i$ is the divergence of insertion
$r_j$ is the divergence of extension
$M_{ij}$ is the refinrd matrix

| 0 | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| **A** | **0** | **-13** | **-11.5** | **-11** | **-10** | **-10.5** |
| **B** | | **0** | **-13** | **-10.5** | **-10** | **-10.5** |
| **C** | | | **0** | **-10.5** | **-10** | **-11** |
| **D** | | | | **0** | **-11.5** | **-11.5** |
| **E** | | | | | **0** | **-11.5** |

**Refined Distance matrix**

All these taxonomic units are arranged radially on a central point to form a **star – shaped tree. In this tree,** the central is called **U node.** Taxonomic units with lowest $M_{ij}$ are placed close to each other. No importance is given for branch length in the start tree.

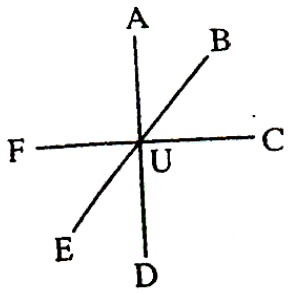



**Figure: Start – tree**

The branch length from the central node is calculated for each and every taxonomic unit. The branch lengths for A and B are calculated from the equations:

$$S_{AU} = \frac{d_{AB}}{2} + \frac{[r_A - r_B]}{2(N-2)} = 1$$

$$S_{BU} - d_{AB} - S_{AU} = 4$$

By using the values of $S_{AU}$ and $d_{AB}$, a new matrix is calculated with reference to position of the taxonomic units in relation to U node. Branch lengths of A and B are already known. The rest of the branch lengths are calculated in the following way:

$$d_{CU} = d_{AC} + d_{BC} - d_{AB}/2 = 3$$
$$d_{DU} = d_{AD} + d_{BD} - d_{AB}/2 = 6$$
$$d_{EU} = d_{AE} + d_{BE} - d_{AB}/2 = 5$$
$$d_{FU} = d_{AF} + d_{BF} - d_{AB}/2 = 7$$

The calculated branch length are populated into a fine distance matrix. It is given below:

| | C | D | E | F |
|---|---|---|---|---|
| U | 3 | 6 | 5 | 7 |
| C | | 5 | 6 | 8 |
| D | | | 7 | 9 |
| E | | | | 8 |

**Fine distance matrix**

The branch length of the taxonomic units are applied to respective units of the star tree. As a result, the star tree becomes irregular and shows some relationship between the taxonomic units.

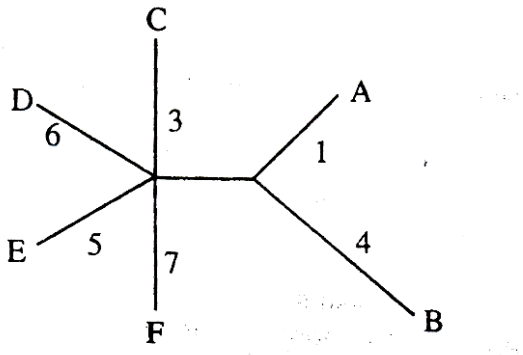



**Figure: Extended tree**

The taxonomic units that have low lowest distance are placed adjacent to each other. Then unit with the next higher distance is placed next to it. This step is repeated several times until a fine phylogenetic tree is formed.

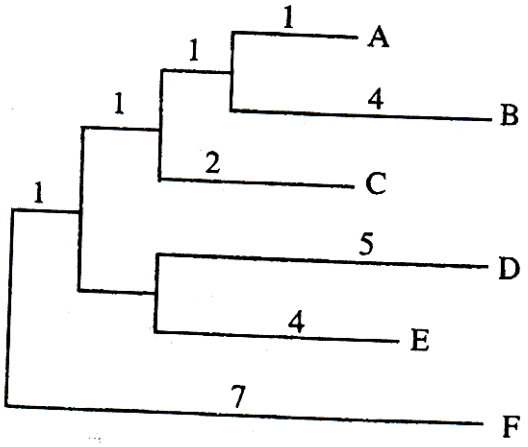



**Figure: Phylogenetic tree**

**Advantages:**

- It is relatively rapid, so it is suitable for analyzing a large dataset.
- It can calculate the branch length and give good result.
- It allows to correct multiple sequences.

**Disadvantages:**

- It constructs only one possible tree
- It often yields a biased tree under some condition.
- It compress the sequence information.

**6. Explain Cladistic methods in detail.**

**Cladistic Methods:**

The cladistic methods are character – based methods. In these methods, all possible topologies are evaluated and one that optimizes the evolution is chosen as the correct tree.

The principle behind cladistic method is parsimony. Cladistic methods group organisms that share derived characteristics in a branching hierarchy tree called a cladogram.

Cladogram is a branching, tree – like diagram in which the endpoints of the branches represent specific species of organisms. Here, the branches are not all parallel to each other. They are radiating outward from the nodes. It is used to illustrate phylogenetic relationships and show points at which various species have diverged from the common ancestral form.

In the cladogram, taxa sharing more derived characters are kept more closely than those having fewer derived characters. This means cladogram is built such that number of changes from one character state to the next is the smallest.

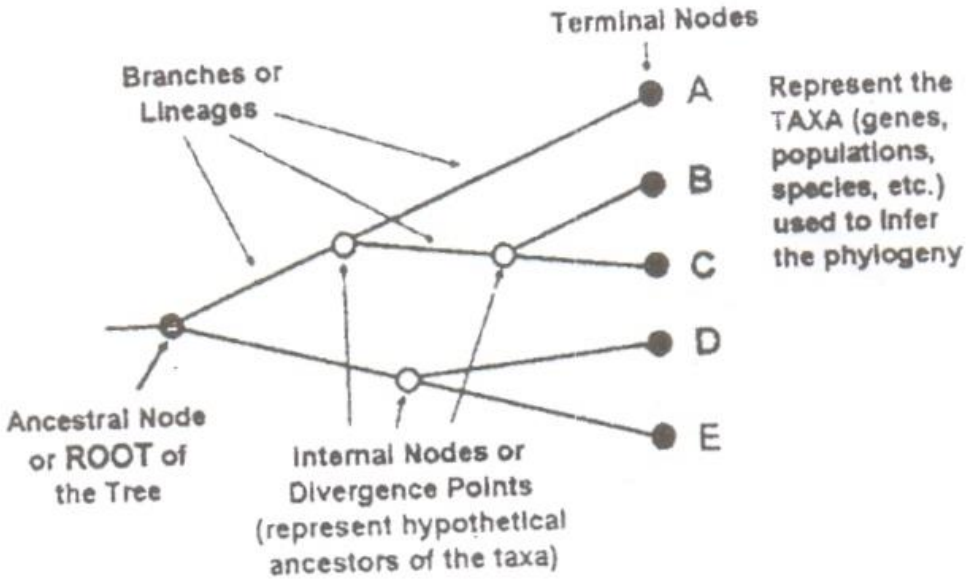



**Figure: Typical phylogenetic tree**

Cladistic methods emphasize more on the evolutionary origin of species than the relationships. It assumes that a set of sequences descended from a common ancestor by mutation and selective processes without hybridization or other by horizontal gene transfers. These methods are best in comparing trees under study with other tree to determine different line of evolution.

There are two cladistic methods to construct phylogenetic trees. They are-

1. Maximum parsimony
2. Maximum likelihood method

**1. Maximum parsimony:**

Maximum parsimony refers to taking minimum number of steps to construct a tree. It assumes that trees with the minimum number of evolutionary changes are the most preference able trees. This method was discovered by **W. Fitch.**

Maximum parsimony is based upon the number of mutations in the sequences. It gives each mutation a score. The most parsimonious tree is that with fewest mutations.

**Advantage:**

- It reflects the ancestral relationship
- It uses all known evolutionary information
- It is faster than maximum likelihood

**Disadvantages:**

- It yields little information about branch length
- It require a long computation time
- If often yields biased tree under certain conditions

**2. Maximum likelihood:**

Maximum likelihood use explicit model of evolution to reconstruct a phylogeny. It uses statistical tool to evaluate a hypothesis about evolutionary history. It constructs all possible trees of evolutionary history from an observed data set and chooses a suitable one to interpret the phylogenetic relationship the species. The probability of mutation and pattern of substitution are considered to be important in his method.

**Advantages:**

- It is more accurate than other methods. So, it is often used to test the existing tree.
- All the sequence information is used.
- In evaluates all possible trees.
- Sampling errors have least effect on the result.

**Disadvantages:**

- Extremely slow
- Impractical for analyzing large data set.

**7. Write short notes on**
**(i) OTU**
**(ii) Rooted Vs un rooted tree**
**(iii) Cladogram Vs phenogram**

The node, from which all species descended is called a **root** or **ancestral node.**

The root may be placed at the top or on the side. A phylogenic tree with its root is known as **rooted tree.** If the ancestral species or taxon is not known or not considered, the phylogenetic tree remains **un rooted.**

The terminal nodes represent the taxonomic units, which may be species, higher taxa, populations or individuals. They are often known as **operational taxonomic units** (OTUs). Character data such as a position at the sequence or a residue at the position or the distance (difference) between two neigh bours can be considered as OTUs.

**Unrooted Tree:**

The phylogenetic tree in which the possible ancestor cannot be placed is called **unrooted tree.** It may be shown as **phenogram** or **cladogram.** It explains the relatedness of different taxa, but not the ancestral relationship. It is shown as a phenogram; it shows the distance between the taxonomic units. If it is drawn as a cladogram, it explains only the evolutionary relationship among the species.

An unrooted tree is also a two – dimensional graph shown as a phenogram. It consists of several **nodes** and **edges,** but **no root.**

The terminal nodes represents the taxonomic units called **operational taxonomical units (OTUs).** The OUT may be a character of the species or difference between the taxonomic units.

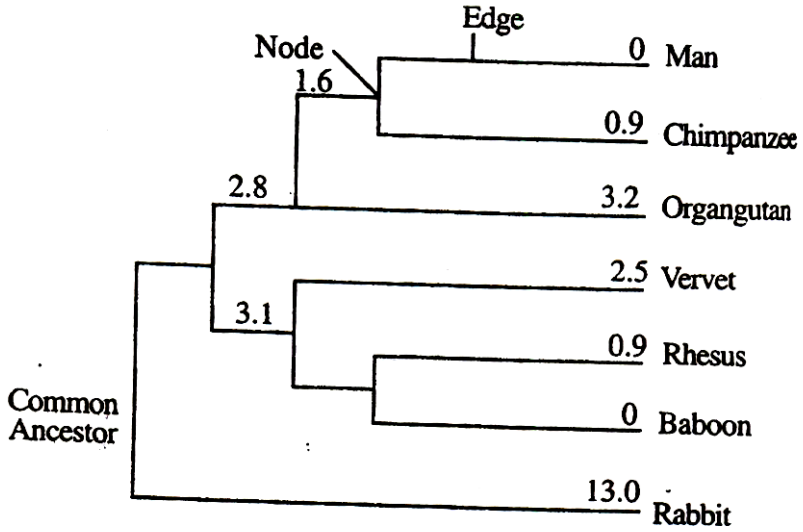



**Figure: Unrooted phylogenetic tree.**

A root cannot be inferred from an unrooted tree without some means of identifying ancestry. This is normally done by including an out group in the input data or by introducing additional assumptions such as molecular clock hypothesis.

**Phylogenetic Tree:**

A tree – like graph that shows ancestral relationship among various species originated from a common ancestor is called **phylogenetic tree.** It is also known as an **evolutionary tree** or a **tree of life.** This is a two dimensional graph.

Phylogenetic trees may be shown as **phenogram** or **cladogram.** In the phenogram, the branches are arranged parallel to each other. On the other hand, in cladogram the branches are radiating outward from the central point. Both phenogram and cladogram explain the evolutionary origin of various species considered in this study, but phenogram alone can illustrate the extent of relationship among them. So, much importance is given for phenogram to construct a phylogenetic tree.

## UNIT – V

## PART – A

**1. What is Gene prediction?**

Gene prediction is the process by which genomic DNA that encode proteins on statistical analysis of sequence bias in genome coding regions. Gene prediction programs are used to shift through new sequences & then annotate the sequence datab are entry with this information.

**2. What is celera's approach for human genome sequencing?**

Celera Genomics is a US Biotechnology company that takes the commercial potential of human genome. It took effort to sequence the human genome in 1998. It was aimed at sequencing commercially through gene identification for discovery of new drugs. No importance was given for total genome sequencing and gene mapping. Celera used maps and sequences of HGP hosted in Internet and complete its draft sequence.

**3. Define HGP.**

HGP refers to human genome project. It is the study of the actual sequences of genes in human chromosomes and their functions. It is the project to map and sequence the 3 billion nucleotides contained in the human genome and to identify all the genes present in it. It is under the control of an international body, "Human Genome Organization" (HUGO). It was started in 1990 and completed in 2003.

**4. What are the 2 common sequencing techniques used in HGP?**

1. Maxam – Gilbert Technique
2. Sanger Technique

**5. Write short notes on Maxam – Gilbert Technique.**

It is also called the chemical degradation method. This method uses chemicals to cut DNA at specific bases and hence small fragments of different lengths are formed.

**6. Write short notes on Sanger technique.**

Sanger sequence is also known as chain termination method. Here, DNA replication is stopped at position occupied by one of the 4 bases and then the resulting fragment lengths are

analyzed. This process involves using an enzymatic procedure to synthesize DNA chains of varying length in 4 different reactions.

**7. Define functional genomics.**

Functional genomics deals with pattern of expression of different genes in the genome at different stages and under different conditions. Functional genomics uses high – through out techniques like DNA micro arrays, proteomics, metabolites and mutation analysis to describe the function and interaction of genes.

**8. What are the 2 methods used in genome sequencing?**

1. Clone – based sequencing
2. Short – gun method

**9. What are the methods used in Gene prediction?**

1. Laboratory – based approaches
2. Feature – based approaches
3. Homology – based approaches
4. Statistical and HMM – based approaches

**10. Give 2 sequence assembly tools and explain how they operate.**

**1. Finch – suite**

Finch sequencing systems has a complete suite for genomic sequencing

**2. Phrap**

It is used to locate overlapping regions with in individual sequences and assemble them in to longer contigs.

**11. What is the cluster analysis methods used in micro array technology?**

1. Agglomerative clustering
2. Hierarchical clustering
3. Nearest neighbour clustering
4. Complete linkage clustering
5. Average – linkage clustering
6. K – means clustering

**12. What is DNA Micro array technology?**

DNA Micro arrays also known as DNA chips (or) gene chips enable us to measure this for thousands of genes simultaneously. Micro arrays can help in estimating the amount of protein is the cell and a lot of information can be derived from this technology.

It is used for 2 major applications:

1. To identify the sequence
2. To determine the expression level of genes.

**13. What is systems biology?**

It is the study of the interactions between the components of a biological system. It describes how there interactions give rise to the function and behaviour of that system. For eg: the enzymes and metabolites in a metabolic pathway co – ordinate in a suitable way to maintain the stability of cell (system).

**14. Define MADAM.**

MADAM – Micro array Data Manager. It is an application to load and retrieve micro array data to and from a database. The program provides data entry forms, data report form and additional applications are to maintain micro array data for further analysis.

## PART – B

**1. Explain in detail about Human Genome Project.**

**Human genome project** is the study of the actual sequences of genes in human chromosomes and their functions.

**Human Genome Project:**

The Human Genome project (HGP) is a project to and sequence the 3 billion nucleotides contained in the man genome and to identify all the genes present in it. Is under the control of an international body, **Human Genome Organization (HUGO).** It was started in 1990 and complete in 2003.

This project involves the discovery and sequencing of the full DNA complement in a single human **somatic cell.** Its primary goal is listing and locating all our **genes.**

The **"genome"** of any given individual (except for identical twins) is unique; mapping "the human genome" involves sequencing multiple variations of each gene.

The HGP has served to explore our genetic environment to make us aware of the beneficial resources that might contribute to understand and improve our lives. From the results of the HGP, we can understand how we develop from conception, how we grow and mature, how we live and how we die.

There are currently two human genome projects:

i. The international HGP governed by the international body **Human Genome Organization (HUGO)** and
ii. Celera Genomics

**International HGP:**

This project was planned in 1986 by **Charles DeLisi,** who was the Director of the US Department of Energy (DOE). The DOE and National Institute of Health (NIH) hand in hand started this project in 1990.

The 1987 report stated boldly, "The ultimate goal of this initiative is to understand the human genome" and "Knowledge of the human genome is necessary for the progress of medicine and other health sciences as knowledge of human anatomy."

Atleast 18 countries have participated in the human genome research programs. They are **Australis, Brazil, Canada, China, Denmark, European Union, France, Germany, Israel, Italy, Japan, Korea, Mexico, Netherlands, Russia, Sweden, United Kingdom and the United States.**

The Human Genome Organization (HUGO) helped to co – ordinate the international collaboration in the genome project.

**Celera Genomics HGP:**

**Celera genomics** is a US Biotechnology company that takes the commercial potential of human genome. This company took effort to sequence the human genome in 1998. it was a privately funded project co – ordinated by **Craig Venter.** It was aimed at sequencing commercially through gene identification for discovery of new drugs. No importance was given for total genome sequencing and gene mapping. Celera used maps and sequences of HGP hosted in Internet.

**Goals of HGP:**

The goals of the HGP were –

- To **identify** all the approximately 20,000 – 25,000 genes in human DNA,
- To **determine** the sequences of the 3 billion chemical base pairs that make up human DNA,
- To **store** this information in databases,
- To **improve** tools for data analysis,
- To **transfer** related technologies to the private sector and
- To **address** the **ethical, legal** and **social issues (ELSI)** that may arise from the project.

**Techniques of HGP:**

There are many techniques for sequencing DNA and mapping the human genome. The two most common sequencing techniques are:

1. Maxam – Gilbert Technique and
2. Sanger Technique

**Maxam – Gilbert sequencing** is also called the **chemical degradation method.** This method uses chemicals to cut DNA at specific bases and hence small fragments of different lengths are formed.

**Sanger sequencing** is also known as **chain termination methods.** Here, DNA replication is stopped at positions occupied by one of the four bases and then the resulting fragment lengths are

analyzed. This process involves using an enzymatic procedure to synthesize DNA chains of varying length in four different reactions.

The specific base at the end of each successive fragment is detectable after the fragments are separated by gel electro – phoresis followed by auto radiographic imaging.

Both **Maxam – Gilbert** and **Sanger** techniques are first generation sequencing techniques.

The second – generation of interim sequencing technologies enable the process to increase in speed and accuracy while decreasing in price. The processes involved in the second generation technologies are,

1. high voltage capillary and
2. Ultra thin electrophoresis

There are also third – generation sequencing technologies that proceed without the use of gel electrophoresis. These include

1. Enhanced fluorescence detection of individual bases in flow cytometry.
2. Direct reading of the base sequence of a DNA strand
3. Enhanced mass spectrometric analysis of DNA sequence

**Human Genome Project Goals and Completion Dates**

| Area | HGP Goal | Standard achieved | Date of achievement |
|---|---|---|---|
| Genetic map | 2 to 5 – cM resolution map (600 – 1,500 markers) | 1 – cM resolution map (3,000 markers) | September 1994 |
| Physical map | 30,000 STSs | 52,000 STSs | October |
| DNA sequence | 95% of gene containing part of human sequence finished to 99.99% accuracy | 99% of genecontaining part of human sequence finished to 99.99% accuracy | April 2003 |
| Capacity and cost of finished sequence | Sequence 500 Mb/year at < $0.25 per finished base | Sequence > 1,400 Mb / year at < $0.09 per finished base | November 2002 |
| Human sequence variation | 100, 000 mapped human SNPs | 3.7 million mapped human SNPs | February |
| Gene identification | Full – length human cDNAs | 15,000 full – length human cDNAs | March 2003 |
| Model organisms | Complete genome sequence of<br>E. coli,<br>S. cerevisiae, | Finished genome sequences of<br>E. coli,<br>S. cerevisiae, | April 2003 |

| | | | |
|---|---|---|---|
| | C. elegans,<br>D. melanogaster | C. elegans,<br>D. melanogaster,<br>C. briggsae,<br>D. peudoobscura,<br>Mouse and rat | |
| Functional analysis | Development of genomic – scale technologies | High – throughput oligonucleotide synthesis | 1994 |
| | | DNA microarrays | 1996 |
| | | Eukaryotic, whole – genome knockouts (yeast) | 1999 |
| | | Scale – up of two – hybrid system for protein – protein interaction | 2002 |

**Potential Benefits of HGP**

**The Human Genome Project** illustrates all about the human genome. Therefore, we can get the maximum benefit from the results of HGP. The following are the most like benefits we can get from HGP for humankind:

- DNA sequence information helps to understand the **structure, organization and function of DNA** is chromosomes.
- Genome maps of other organisms will provide the base for comparative studies to understand more complete biological systems.
- Information generated and technologies developed and revolutionizing future biological explorations.
- Detailed knowledge of the human genome will provide new avenues for advances in medicine and biotechnology.
- Companies, such as Myriad Genetics, started offering easy ways to administer genetic tests to a variety of illnesses:
    - ❖ Breast cancer
    - ❖ Disorders of homeostasis
    - ❖ Cystic fibrosis,
    - ❖ Liver diseases and
    - ❖ Many others.

- Etiologies for cancers, **Alzheimer's** disease and others of clinical interest are considered likely to benefit from genome information.

    - ❖ May lead in the long term to significant advances in their management
    - ❖ Easy management of these severe diseases.

**2. Explain briefly the following:**

**(i) Gene Prediction by HMM and Neural Network Methods**
**(ii) Shotgun strategy for complete genome sequencing and bioinformatics tools to assemble the data.**

**Statistical and HMM approaches:**

HMMs for gene prediction can be developed by using simplified gene grammar rules like start – codon, end – codon, length is divisible by 3 and no stop – codons in the reading frame. The language may also consider dinucleotide preferences (e.g.) AG is more common than AC and that nucleotides are not necessarily independent.

HMM gene (http://www.cbs.dtu.dk/services/HMMgene/) is a program for prediction of genes in anonymous DNA. The program predicts whole genes, and so the predicted exons always splice correctly. It can predict several whole or partial genes in one sequence, and can be used on whole cosmids or even longer sequences. HMM gene can also be used to predict splice sites and start / stop codons. If some features of a sequence are known, such as hits to ESTs, proteins, or repeat elements, these regions can be locked as coding or non – coding and then the program will find the best gene structure under these constraints. Apart from reporting the best prediction, HMM gene can also report the N best gene predictions for a sequence. This is useful if there are several equally likely gene structures and may even indicate alternative splicing.

**Neural Networks:**

Most of the effective structure prediction models extract patterns from databases of known protein structures. Neural networks comprise a particular tool for pattern recognition and classification.

The simplest layered feed – forward neural network consists of a layer of input units and a layer of output units. Signals are transmitted from input to output layer (feed – forward) via the connections. In figure a simple neural network is shown. There are two input units (J's) and one output unit.

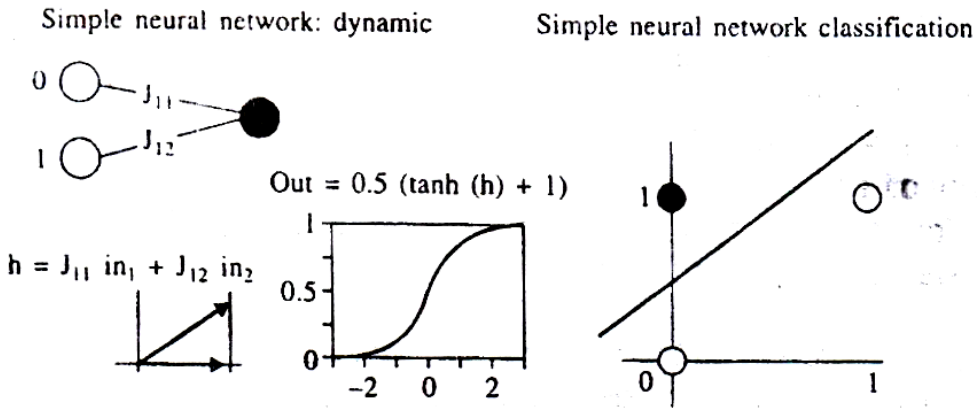



**Figure: A simple neural network example**

The value of each input unit (example: 0 for unit 1; 1 for unit 2) is multiplied with the strength of the connection; the products sum to a local field (h) representing the signal that arrives at the output unit. The multiplication represents a projection of the input vector onto the vector of the connections. (2) The final output is determined by applying a sigmoid function (shown in the hyperbolic tangent) to the local field. The result is that the output is constrained to values between 0 and 1. On the right hand side the potential of such a network is illustrated: a line separates the open, and the dark circles.

Neural networks can be used for protein prediction. The protein sequence is translated into patterns by shifting a window of n adjacent residues (typical values of n = 13 – 21) through the protein. The output of the network is uniquely determined. The only free variables are the connections.

Training or learning a neural network implies changing the connections so that the error decreases for the given examples. A training set can comprises about, 30,000 examples. If training is successful, the patterns are correctly classified. The network can succeed in extracting general rules by the classification of the training patterns. The generalization ability is checked by another set of test samples for which the mapping of sequence window to secondary structure is known as well. Sufficient testing is crucial and has to meet two requirements. First, any significant sequence similarity between test and training set has to be removed. Second, evaluations of expected prediction accuracy have to be based on a sufficient number of test proteins (> 100).

**Genome sequencing:**

When the contigs are completed, the next step is to generate sub clones that are small enough for sequencing and then do the actual sequencing. Highly automated sequencing procedures have been developed in the recent years. The sequence can very rapidly be read and the size of the DNA segments can be easily sequenced.

Sequencing of DNA involves the determination of precise sequence of nucleotides in a sample. Dideoxy method (or chain termination method) is the most popular; it gets its name from the key role played by synthetic nucleotides that lack OH at the 3' terminal.

The DNA fragment to be sequenced must first be cloned into a vector (such as a plasmid or a cosmid) to obtain sufficient quantity of the fragments to be sequenced. Another limitation imposed by the technology is that sequences can only be determined in approximately 500 base pair (bp) chunks known as reads. This is due to both the biochemistry of the DNA polymerase reaction and the resolution of polyacrylamide gel electrophoresis (PAGE). As most genes contain many thousands of bp, all sequencing efforts must involve that division of the target DNA into a set of overlapping 500 bp fragments. Once the sequences of these fragments are determined, they must be pieced back together into contigs.

There are two basic methods of DNA sequencing methodologies: Clone – based sequencing and the Shot – gun sequencing.

**Shot – gun Method:**

For this method, you need to obtain a pure sample of the entire genome of the organism that you want to study. The DNA is sheared physically into small fragments to make random inserts of size 1 to 2 kb. These are then cloned into M 13 or plasmids. These fragments are cloned creating a genomic library. You need to sequence as many of the clones as necessary. These sub clones are then sequenced using automated DNA sequencing.

The automated DNA sequencing needs to go through several steps. You need to reassemble the genome by computational analysis of the sequence fragments. This needs an oversampling of the genome so that the residual number of unsealed gaps is as low as possible. After the assembly is done, there may be gaps in the sequence. the gaps are basically the segments where "reads" did not cover the clone, or where all reads were of low quality. As a result, you would get two or more contigs.

In the final phase, additional sub clones spanning the gaps are obtained and sequenced. The goal is to allow all data to be joined into a single contig with an error rate of 10 or better. Once the sequence of piece of DNA is known, you can make a comparison with other sequences in the database to identify it. You can find genes, regulatory elements and the like using various tools to understand the sequence.

**3. Explain Gene Prediction methods in detail.**

**Gene prediction methods:**

There are several methods of gene prediction, differing in the approach, algorithm, and the efficacy of prediction methods. Important methods of gene prediction are as follows:

- Laboratory – based approaches
- Feature – based approaches
- Homology – based approaches
- Statistical and HMM – based approaches

**Laboratory - based approaches:**

This is the traditional method to find a gene, which includes experimental procedures for locating genes in a sample of DNA. These can now be discussed.

**Identification using blotting methods:**

Blotting is a technique used for detection of nucleic acids and proteins. The technique employs the transfer of bio molecules on to a membrane support that usually accomplishes blotting.
The entire procedure involves the following steps:

1. Preparing a cell – free extract containing the bio molecule(s) of interest
2. Resolving the mixture by gel electrophoresis
3. Transferring the resolved mixture onto a membrane support such as a nitrocellulose paper
4. Incubating the paper with a detection system that specifically hybridizes to the molecule of interest.

When DNA is blotted, it is termed as southern blotting, whereas Northern and Western blotting techniques are coined for RNA and protein, respectively.

**Southern blotting:** Suppose, one of the cDNA clones you isolate and sequence represents a new gene. If you are interested in studying this gene further, you might want to determine the structure of the gene by identifying introns, exons, and regulatory elements.

An essential step for such analysis is Southern blotting, which is a very popular technique used for a variety of purposes. It is used to determine the size and arrangement of the genomic copy of a gene, to determine the number of genes related to a clone of interest and to investigate

the evolutionary conservation of a gene. The southern blotting technique has been used for understanding a variety to biological processes such as RNA splicing and genomic rearrangements to form antibodies and T cell receptors. This technique has also played a major role in the identification of numerous rearranged genes that are associated with a variety of human genetic disorders and cancers. With the introduction of highly resolving gel systems, it is now possible to use Southern blotting to detect gene mutations involving single base – pair changes. This has led to the early diagnosis and prevention of potentially harmful diseases.

**Northern blotting:** Northern blotting is a technique used to examine the size, the temporal and spatial expression pattern of specific RNAs. Usually, total cellular RNA, or poly(A) RNA is isolated and separated by size on an agarose gel. The RNA molecules in the gel are transferred to nitrocellulose paper or nylon as described above and detected using an appropriate DNA or RNA probe.

Although Southern and Northern blotting techniques exhibit a number of similarities, there are several important differences also. The major difference is in the extreme care required to isolate non – degraded RNA. Full – length mRNA isolation is an important goal in generating high quality cDNA libraries. The difficulty in RNA isolation is that most ribonucleases are very stable and active and do not require activators or cofactors to function. As a precaution, the first step in all RNA purification procedures is to lyse the cell in a solution that denatures, thus inactivating ribonucleases. Another difference in the two procedures is in the type of gel used to resolve RNA. Unlike DNA that is only found as a double – stranded version, RNA migrates as a function of hybrid length, RNA can engage in non – uniform amount of intra – molecular base pairing. Therefore, RNA must undergo electrophoresis under denatured conditions if it is to migrate as a function of nucleotide length.

**Feature – based Approach:**

Feature – based approaches are based on pattern recognition, treating DNA fragments as sequences.

**Gene finding by ORF prediction:**

ORFs without stop codons are strongly suggestive of genes. ORF has the presence of a long series of codons in a DNA sequence without the series being interrupted by a termination codon. An ORF signal is enhanced even further by the presence of sequence patterns for starting and stopping transcription before and after the ORF. Dynamic programming can be used to identify the highest scoring regions. The best gene recognition systems tend to be species – specific, trained on examples of known genes in the given organism. The initiation site of box is always an ATG codon and it is always about 30 base pairs down stream from a TAATAA sequence.

GRAIL (Gene Recognition and Analysis Internet Link) (http://compbio,ornl.gov/Grail-1.3/) is perhaps the most widely used ORF identification tool. (It was also one of the first to be made available). It provides analysis of protein coding potential of a DNA sequence. GRAIL uses variable – length windows tailored to each potential exon candidate defined as an open reading frame bounded by a pair of start /donor, acceptor /donor or acceptor / stop sites. This scheme facilitates the use of more genomic context information (splice junctions, translation starts, non – coding scores of 60 – base regions on either side of a putative exon) in the exon recognition process. GRAIL finds about 91% of all coding regions with an apparent false positive rate of 8.6%.

Grail II helps in analyzing protein – coding regions, poly (A) sites, and promoters, enables to construct gene models, predicts encoded protein sequences. And provides database searching capabilities. A list of most likely exon candidates is first established, and these are evaluated further using a neural network approach. The algorithm makes it final prediction by selecting the best candidates. A DP approach is then used to define the most probable gene models.

There are various tests to verify that a predicted ORF is in fact likely to encode a protein. Some of these are as follows:

1. The method is based on an unusual type of sequence variation that is found in ORFs – every third base tends to be the same one much more often than by change alone. This property is due to non – random use of codons in ORFs and is true for any ORF, independent of the species. The program Test Code (http://www.accelrys.com/products/gcg_wisconsin_package/) provides a plot of the non – randomness of every third base in the sequence.
2. This method is based on the analysis to determine whether the codons in the ORF correspond to those used in other genes of the same organism. For this test, information on codon use for an organism is necessary, averaged overall genes.
3. The ORF may be translated into an amino acid sequence and the resulting sequence then compared to the databases of existing sequences. If one or more sequences of significant similarity are founds, there will be much more confidence in the predicted ORFs.

**Homology – based approaches:**

Eukaryotic genes are more difficult to identify than those of the prokaryotes. Searching for known homologues is the most widely used method for identifying genes. Homology search depends only on evolutionary relatedness, and so are widely applicable. A major advantage of finding homologous product is that some of the information about the gene may be already known.

Search for genes can include matches to one of the following:

- Known proteins
- Protein motifs (e.g. zinc finger, ATP and GTP – binding motifs, etc.)
- ESTs (Expressed Sequence Tags) and ACRs (Ancient Conserved Regions)

Homology – based gene prediction systems can find similarities to previously identified coding regions. Alternatively, a different homology – based approach is to identify totally unknown genes to compare two whole genomes and look for conserved regions on the theory that sequence is only conserved if it is important.

Procrustes (http://hto-13.usc.edu/software/procrustes/) is a homology – based program (currently not fully functional), which accepts as input one genomic DNA sequence, and one or several protein sequences. The proteins (targets) are assumed to be similar to the protein encoded in the genomic fragment. Procrustes finds the chain of exons with the best fit to the target proteins; if several targets are specified, it makes one gene prediction per target. Procrustes also outputs the amino acid sequence of the predicted protein and the alignment between the predicted and target proteins.

Finding coding regions can also be done by similarity searching using TBLASTX for finding exons. The approach to this problem is to translate the sequence in all six reading frames (3 forward and three reverse) and do a similarity search against the protein databanks. TBLASTX translates a DNA query sequence and performs a similarity search against protein databanks. If a protein sequence matches, get its DNA sequence and align it with your unknown sequence. The start and stop codons would get identified. If the query sequence were genomic, then the introns would also be identified.

**Statistical and HMM approaches:**

In chapter you have learned that one of the important applications of HMMs is in gene identification. One of the programs discussed there was GeneMark (http://www.ebi.ac.uk/genemark/) that used HMMs for gene identification. There are several others and some can now be discussed here.

HMMs for gene prediction can be developed by using simplified gene grammar rules like start – codon, end – codon, length is divisible by 3 and no stop – codons in the reading frame. The language may also consider dinucleotide preferences (e.g.) AG is more common than AC and that nucleotides are not necessarily independent.

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**4. What is DNA micro array technology? Gene expression data can be analyzed by hierarchical cluster method describe the process.**

Micro arrays can help in estimating the amount of protein in the cell and a lot of information can be derived from this technology. Hence, micro arrays provide a tool for answering a wide range of questions about the dynamics of cells.

DNA micro array technology is used for two major applications: one, to identify the sequence (of gene or the mutated gene) and two, to determine the expression level of genes. The micro array technology is a combination of wet – lab techniques, statistical analysis and application of informatics to the data.

The following are the basic steps for gene expression study using micro arrays :

1. Print and cross – link DNA Clones (probes) onto a glass slide.
2. Reverse transcribe mRNAs from sample tissues into cDNAs (targets) and label with different fluorescence dyes (Cy3 and Cy5).
3. Hybridize targets to probes. The amount of target hybridized to a probe is measured by the intensity of fluorescence emission from that probe.
4. Images of fluorescence emission are compared to find out differentially expressed genes. Those genes are annotated and further studied.

**Clustering gene expression profiles:**

Clustering involves organization of these profiles into clusters so that either instances in the same cluster are highly similar to each other or instances from different clusters have low similarity to each other. Clustering is an exploratory data analysis tool that can help to understand the general characteristics of data and to help visualize the data.

Various clustering techniques are used to identify the patterns in gene – expression data. If you use hierarchical techniques, the resultant classification has an increasing number of nested classes and the result can be represented as a dendrogram. If you use non – hierarchical techniques, you basically partition objects into different clusters without specifying the relationship between individual elements.

Clustering techniques can also be classified as divisive or agglomerative. While agglomerative techniques start with single – member clusters and gradually joint these individual cluster, a divisive method is a method to break one cluster into smaller and smaller clusters.

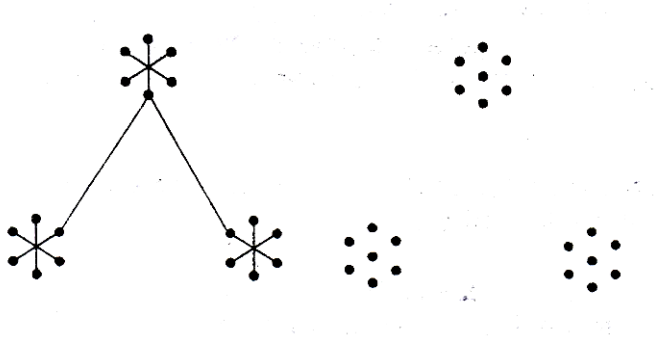


**Figure: Agglomerative clustering**

Another clustering classification is based on supervised or unsupervised methods. Supervised methods use biological information about specific genes that are functionally related to define the clustering algorithm. Application of clustering techniques can result in cluster, but it does not imply that all the clusters are biologically meaningful. Hence, biological knowledge needs to be applied to interpret the clusters.

**Hierarchical Clustering:**

Hierarchical clustering is one of the most widely used techniques for the analysis of gene expression data. Hierarchical clustering in a bottom – up approach is an agglomerative approach in which single expression profiles are joined to form groups, which are further joined until the process has been carried to completion. This forms a single hierarchical tree.

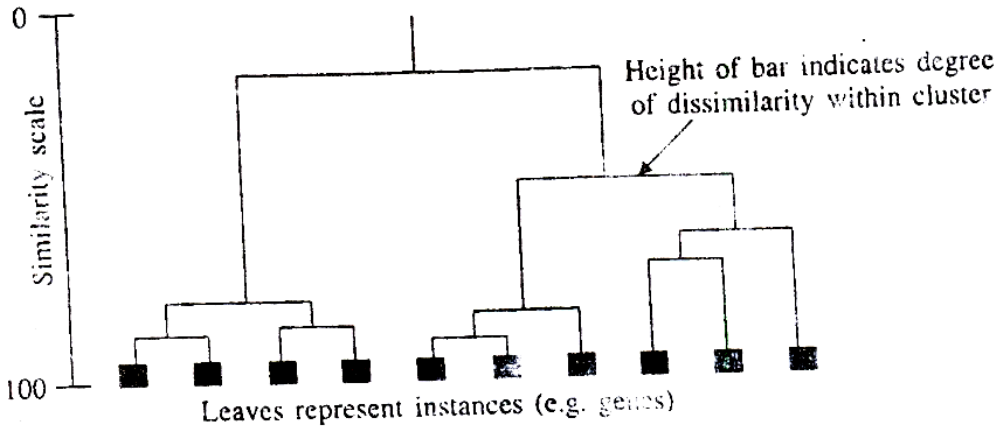



**Figure: Hierarchical clustering**

The process of hierarchical clustering is as follows:

1. The pair – wise distance matrix is calculated for all of the genes to be clustered.
2. The distance matrix is searched for the two most similar genes or clusters. Initially each cluster consists of a single gene. If several pairs have the same separation distance, a heuristic is used to decide between alternatives.
3. The tow selected clusters are merged to produce a new cluster that would now contain at least two objects.
4. The distances are calculated between this cluster and all other clusters.
5. Steps 2 – 4 are repeated until all objects are in one cluster.

There are several variations of hierarchical clustering

**Nearest neighbour (single – linkage) clustering:**

The distance between two clusters is calculated as the minimum distance between a member of each cluster. This technique is referred to as the minimum or nearest – neighbour method. The clusters produced are 'loose' because they can be jointed if any two members are close together. The method produces trees with may long, single addition branches representing clusters that have grown by agglomeration.

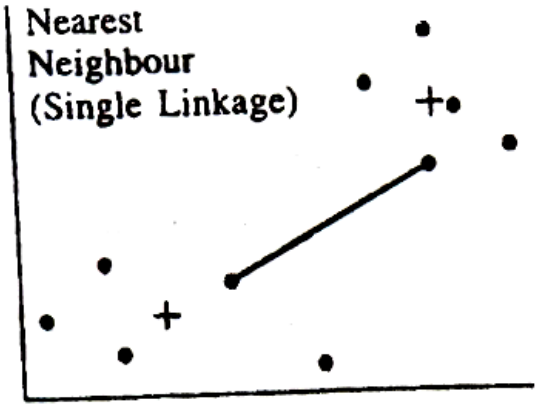



**Figure: Nearest neighbour method**

**Complete – linkage clustering:**

Complete – linkage clustering is also known as the maximum or furthest – neighbour method. The distance between two clusters is calculated as the greatest distance between members of the relevant clusters. This method produces compact clusters of elements and these are very similar in size.

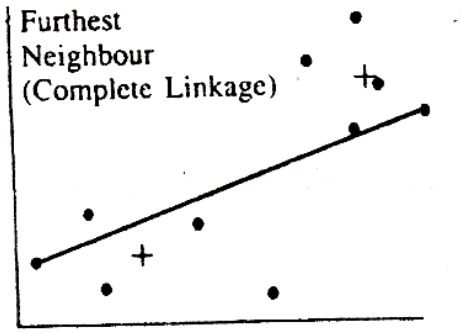



**Figure: Complete – linkage clustering**

**Average – linkage clustering:**

This method uses calculation of using average values. There are various methods for calculating averages like unweighed pair – group method average (UPGMA). The two clusters with the lowest average distance are jointed together to form a new cluster. There are related methods that substitute the centroid or the median for the average.

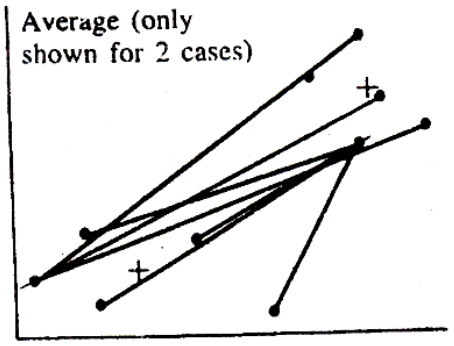



**Figure: Average – linkage clustering**

**Weighed pair – group average:**

This method is similar to UPGMA – the size of the respective clusters (that is, the number of objects contained in them) is used as a weight. This method should be used when the cluster sizes are suspected to be greatly uneven.

**Within – groups clustering:**

This is also similar to UPGMA except that clusters are merged and a cluster average is used for further calculations rather than the individual cluster elements. This produced tighter clusters than UPGMA.

**Ward's method:**

In this method, cluster membership is determined by calculating the total sum of squared deviations from the mean of a cluster. The clusters are jointed in a manner that it produces the smallest possible increase in the sum of squared errors.

**K – means Clustering:**

K – means clustering is an alternative method to the hierarchical methods. It is a top – down approach and is useful if there is prior knowledge about the number of clusters that should be represented in the data. In k – means clustering, objects are partitioned into a fixed number (k) of clusters, such that the clusters are internally similar but externally dissimilar. You can use hierarchical techniques on each of the data partitions after they are constructed. You can always generated a partition clustering from a hierarchical clustering by "cutting" the tree at some level. This is shown in figure.

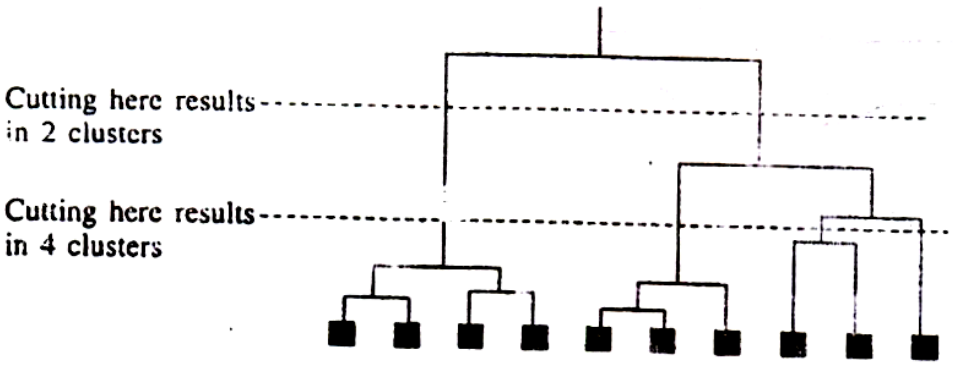



**Figure: k – means clustering**

The process involved in k – means clustering is as follows:

1. All initial objects are randomly assigned to one of k cluster (where k is pre-specified). By using k – means clustering on experiments with k = 2, the data will be partitioned into two groups.
2. An average expression vector is calculated for each cluster and this is used to compute the distances between clusters.
3. An iterative method is used and objects are moved between clusters and intra – and inter – cluster distances are measured with each move.
4. The expression vectors for each cluster are recalculated.
5. The shuffling of objects proceeds until moving any more objects would make the clusters more variable, increasing intra – cluster distance and decreasing inter – cluster dissimilarity

**5. Explain in detail the application of micro array technology.**

**Applications of Micro array technology:**

Micro array technology is now being used widely and some of its applications are described in the following sections:

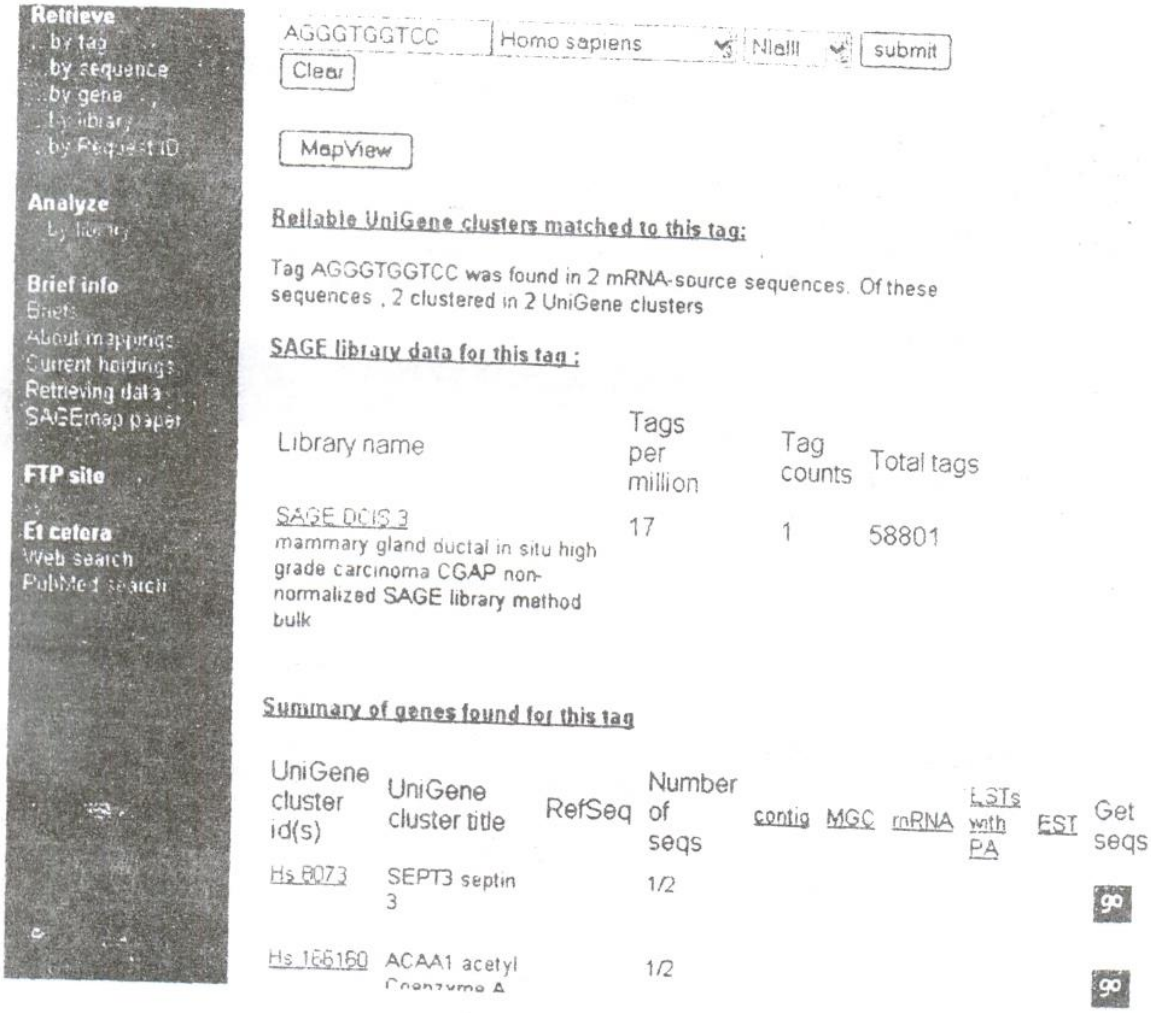



**Figure: Search for SAGE tag from NCBI database.**

**Functional Genomics:**

Micro array technology is used in the area of functional genomics and gene annotation, i.e. in determination of the function of large number of genes discovered in the sequenced genomes. The inference of gene functions is done from sequence similarity to genes with known functions.

There is a correlation between expression pattern and function and hence the expression pattern of gene can be used to hypothesize about its possible functions. Genes with similar functions may have similar expression profiles, implying that their transcription is co-regulated. You can use clustering algorithms to group genes with similar expression profiles. In the function of at least one of the genes in a cluster is known, the other genes in the same cluster may also participate in the same process. However, there are many imponderables and you need to undertake wet laboratory experiments to confirm the function.

**Comparative Genomics:**

Micro arrays data from different species can be used to study genomic differences between the species. This can be done by hybridization of genomic DNA from different strains of a species, or from different closely related species that can reveal some of the differences between the species. This method can be used to get information about gene content in organisms that are closely without having to get into the tedious process of sequencing each organism.

**Medical applications:**

Another important application of micro array technology is to identify genes involved in diseases by comparing gene expression between tissues from healthy and diseased individuals. It is likely that the genes which are differentially up – or down – regulated in diseased tissue are potential targets for drug development. Data from micro arrays experiments can also demonstrate the genes that are specifically induced in specific tissues. Cells from two different tissues, e.g. cardiac muscles and prostate epithelium are specialized to perform different function in an organism. Although we can recognize cells from different tissues by their phenotypes, a cell's role at a basic level determined by the proteins it produces, which in turn depends on its expressed genes. Comparative hybridization experiments can help you understand the genes that are preferentially expressed in specific tissues. This method can be useful in drug design also.

**Protein and Peptide Micro arrays:**

DNA arrays are limited to providing information on the identity or quantity of DNA or RNA that is present in a sample. Translational products of genes cannot be analyzed on such arrays and require the use of peptide – based arrays. Use of peptide – based arrays is particularly important in drug discovery applications, as most of the drug targets are proteins. However, the protein micro array technology is still in the evolutionary phase. The challenge in using protein micro arrays is to maintain functionality in post – translational modifications.

Protein micro arrays can be used to identify protein – protein, enzyme – substrate and protein – small molecule interactions. The advantages of protein micro arrays in drug discovery over conventional methods like Yeast – 2 hybrid systems, ELISA and western blotting, are that they are quick and can be used effectively even when the sample quantity is small.

**Glycomics:**

Glycomics is the study of structure and functions of oligosaccharides. The glocome is referred to the complete set of sugars an organism or cell makes, analogous to the protecome or the genome. As mentioned above, current protein micro arrays have a limitation that they cannot

address the problem of pos – translational modification. There are many proteins and bio molecules that are modified by the covalent attachment of sugar residues (glycans). Studies on glycans are important to define complex life systems and cell communities because all living organisms consists of diverse cells that have numerous heterogeneous carbohydrates. Glycans are difficult to study because of the low evolution of basic micro array technologies ( that are well – developed in case of DNAs and proteins). The new developments in glycotechnologies are in the form of 'bio – chips', which include 'oligosac – charide arrays' or 'glyco – chips'.

**cDNA Micro arrays:**

cDNA micro array is a powerful expression profiling technique to evaluate changes in gene expression in organisms and tissues to study specific bio chemical pathways or diseases. cDNA micro arrays are complex, involving multi steps that include array fabrication, fluorescent – probe labeling, hybridization and analysis of data. Since various laboratories have developed their own protocols, systematic variations can occur at various steps which may affect variations in gene expression levels. Such variations can be, however, taken care of by a large number of repeat experiments and independent validation of results. In order to achieve this, the datasets must be made public in public repositories (e.g. Array Express:

http://www.ebi.ac.uk/microarray/ArrayExpress and NCBI Gene Expression Omnibus:

http://www.ncbi.nlm.nith.gov/geo). The micro array for genome – wide gene expression libraries or DNA arrayed on a coated glass slide or nitrocellulose membrane. The cDNA micro array experiment is so designed as to allow a comparison with a reference RNA sample and the data is subsequently integrated and normalized. In order to design micro array platform, it is essential to consider a few points such as synthesis and purification of gene fragments, extraction of high quality and purified RNA, use of a good fluorescent cDNA labeling method and controls.

**Tissue and Cell Micro arrays:**

Micro array screening is useful in providing information about the biological function of the gene, its clinical impact or its suitability as a drug target. Functional genomics enables validation of the target. The conventional histological analysis of tissues is a labour and time intensive process. Also, the number of investigations that can be performed on a tissue sample is limited to a few hundred only and is restricted to the availability of the sample size. The tissue samples are initially preserved in formation, then embedded in paraffin to prepare block. The paraffin blocks are sectioned, stained, and spread on glass slides for microscopic examination.

**Antibody Micro arrays:**

Antibody micro arrays hold a great promise as diagnostic tools for protein profiling by using a small number of antibodies. The technique is highly selective and sensitive to detect proteins and other analytes in multiple samples. Antibody arrays are solid phase assays which use immobilized antibodies or other ligand – binding molecules. These molecules are attached to glass slides or membranes and used to measure concentrations of proteins and other analytes in plasma or tissue extracts. These are miniaturized assays that can perform multiple immunoassarys simultaneously, hence important in the field of diagnostics. Antibody arrays can be used in proteomics to compare quantitatively protein levels in diseases involving protein expression profiling and transcriptional profiling.

**6. Write short notes on:**
**(i) Functional genomics**
**(ii) Bio molecular and cellular computing**

**Functional Genomics: Functional genomics** deals with pattern of expression of different genes in the genome at different stages and under different conditions. Functional genomics uses high – through techniques like DNA micro arrays, proteomics, metabolomics and mutation analysis to describe the function and interactions of genes.

**Functional Genomics:**

Micro array technology is used in the area of functional genomics and gene annotation, i.e. in determination of the function of large number of genes discovered in the sequenced genomes. The inference of gene functions is done from sequence similarity to genes with known functions.

There is a correlation between expression pattern and function and hence the expression pattern of gene can be used to hypothesize about its possible functions. Genes with similar functions may have similar expression profiles, implying that their transcription is co-regulated. You can use clustering algorithms to group genes with similar expression profiles. In the function of at least one of the genes in a cluster is known, the other genes in the same cluster may also participate in the same process. However, there are many imponderables and you need to undertake wet laboratory experiments to confirm the function.

**Bio molecular & cellular computing:**

Bio molecular computing, 'computations performed by bio molecules', is challenging traditional approaches to computation both theoretically and technologically. Often placed within the wider context of 'natural' or even 'unconventional' computing the study of natural and

artificial molecular computations is adding to our understanding both of biology and computer science well beyond the framework of neuroscience.

Molecular computing is a discipline is a discipline that aims at harnessing individual molecules at nanoscales for computational purposes. The best – studied molecules for this purpose to data have been DNA and bacteriorhodopsin. Bio molecular computing allows one to realistically entertain, for the first time in history, the possibility of exploiting the massive parallelism at nanoscales inherent in natural phenomena to solve computational problems. The implementation of evolutionary algorithms in bio molecules would bring full circle the biological analogy and present an attractive alternative meet large demands for computational power.

**The Origins of Molecular Computing:**

Lately, advances in computer science have been characterized by the computational implementation of well – established biological paradigms. Notable advances are articial neural nets inspired by the brain and its obvious connection to natural intelligence, and evolutionary computation, inspired by the Darwinian paradigm of natural selection. Early ideas of molecular computing attempted to emulate conventional electronic implementations in other media, e.g., implementing Boolean gates in a variety of ways. A fundamental breakthrough characteristic of a new era was made by Adleman's 1994 paper [1], where he reports an experiment performed with molecules of fundamental importance for life, DNA (deoxyribonucleic acid) molecules, to solve a computational problem known to be difficult for ordinary computers, namely the Hamiltonian path problem (happ). This problem is typical of an elite set of problems in the well – known complexity class NP that exemplify the computational difficulty of search procedures that plague a number of very important applications in combinatorial optimization, operations research, and numerical computation. Adleman's experiment ushered in a new computational paradigm in molecular computing for several reasons. First, it showed that it is indeed possible to orchestrate individual molecules to perform computational tasks. Second it showed the enormous potential of DNA molecular for solving problems beyond the reach of conventional computers that have been ormay be developed in the further based on solid – state electronics. Shortly after, the rst conference on DNA – based computing was organized at Princeton University in 1995, and several events have been held since annually.

**7. Explain in detail about systems Biology.**

**System Biology:**

System biology is the study of the interactions between the components of a biological system. It describes how these interactions give rise to the function and behaviour of that system. For example, the enzymes and metabolites in a metabolic pathway co – ordinate in a suitable way to maintain the stability of cell (system).

The systems biology approach involves the development of mechanistic models, such as the reconstruction of dynamic systems from the quantitative properties of their elementary building blocks. For example, a cellular network can be modeled mathematically using methods coming from chemical kinetics and control theory. Due to the large number of parameters, variables and constraints in cellular networks, numerical and computational techniques are often used.

Other aspects of computer science are also used in systems biology. They include:

- Text mining to find parameter data from literature, online databases and repositories.
- Sharing data and models (from Bio Models Database)
- Development of the Systems Biology Markup Language.

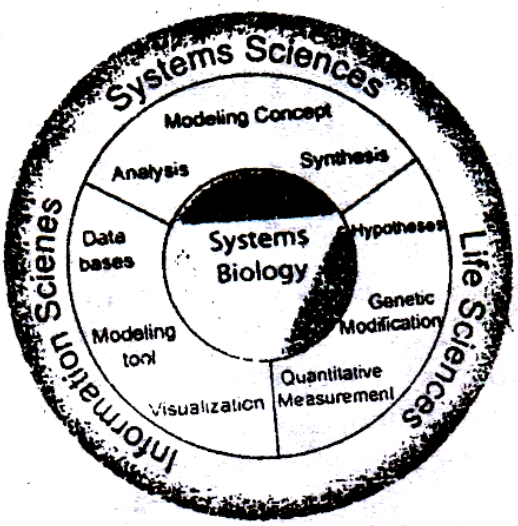



**Figure: Systems Biology**

The systems biology approach is characterized by a cycle of theory, computational modeling and experiment to quantitatively describe cells or cell processes.

**Techniques used in systems biology:**

System Biology helps to ***obtain, integrate and analyze complex data*** from multiple experimental sources using interdisciplinary tools. Some typical technology platforms are:

- Gene expression measurement through DNA micro arrays and SAGE.
- Protein levels through two – dimensional gel electrophoresis and mass spectrometry.
- Phosphoproteomics and other methods to detect chemically modified proteins.
- Metabolomics for small – molecule metabolites.
- Glycomics for sugars.
- Interactomics for interactomes.

**Applications of systems Biology:**

Many predictions concerning the impact of genomics on health care have been proposed:

- The development of novel therapeutics and the introduction of personalized treatments are combined to treat diseases.
- Biotechnology companies are using this cell – biology driven approach to develop novel therapeutics.
- Predictions upon the roles of specific genes in human and pathogen physiologies have been made.

The ultimate goal of systems biology is to derive the prerequisite knowledge and tools.

**********

# UNIT – I

## PART – A

**1. What is Bioprocess Engineering?**

Bioprocess engineering is the application of engineering principles to design, develop, and analyze processes using biocatalyst. These processes may result in the formation of desirable compounds or in the distraction of unwanted or hazardous substances.

**2. What is GMP?**

Drugs sold on the market or used in clinical trials must come from facilities that are certified as GMP. GMP stands for good manufacturing practice. GMP concerns the actual manufacturing facility design and lay out, the equipment and procedures, training of production personnel, control of process in put 3, and handling of product.

**3. What is enzymes?**

Enzymes are usually proteins of high molecular weight (15,000<MW< several million Daltons) that act as catalysts. Enzymes are specific, versatile, and very effective biological catalysts, resulting in much higher reaction rates as compares to chemically catalyzed reactions under ambient conditions.

**4. What are the major classification of Enzymes and their functions?**

| Enzymes | Functions |
|---|---|
| 1. Oxidoreductases | Oxidation and reduction reactions |
| 2. Transferases | Transfer of functional groups |
| 3. Hydrolases | Hydrolysis reaction |
| 4. Lyases | Addition to double bonds |
| 5. Isomerases | Isomerization reactions |
| 6. Ligaseg | Formation of bonds with ATP cleavage. |

**5. Draw the lock and key model of enzyme catalysis.**

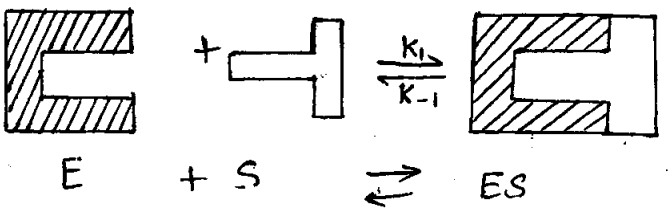


**Fig:**

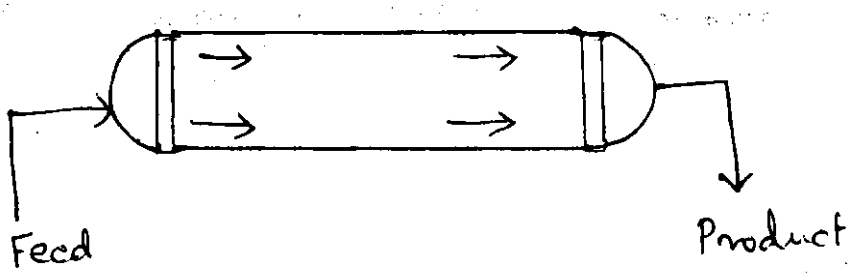
E - Enzyme
S – Substrate

The substrate is a relatively small molecule and bits in to a certain region on the enzyme molecule, which is a much larger molecule. The simplest model describing the interaction is the lock-and-key model, in which the enzyme represents the lock and the substrate represents the key.

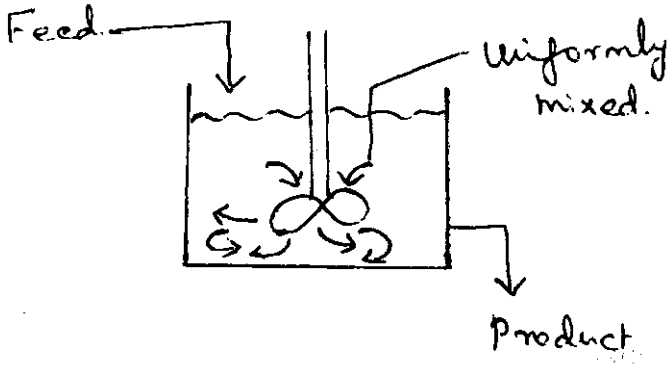
**6. What are the two types of flow patterns in a reactor?**

There are two types of flow patterns in a reactor namely plug flow and mixed flow patterns.

a) Plug Flow reactor




b) Mixed flow reactor




**7. What is a plug flow reactor?**

Plug flow reactor is referred as PFR and the pattern of flow as plug flow. It is characterized by the fact that the flow of fluid through the reactor is orderly with no element of fluid over taking or mixing with any other element a head or behind.

**8. What is the necessary and sufficient conditions for a plug flow reactor?**

1. There must be no mixing or diffusion along the flow path.
2. Residence time in the reactor to be the same for all elements of fluid.

**9. What are the two ideal reactors?**

There are two ideal steady state reactors namely,

1) Plug flow reactor – Nomixing or diffusion along the flow path
2) Mixed flow reactor – uniform mixing takes place.

(Juclude PFR, MFR diagram)
(Q 6 a, b diagram)

**10. What is a mixed flow reactor?**

MFR is a ideal stready-state flow reactor. It is a reactor in which the contents are well stirred and uniform throughout. Thus, the exit stream from this reactor has the same composition as the fluid with in the reactor.

**11. What are the different names for a mixed flow reactor?**

MFR as following names namely.

1) Ideal steady-state flow reactor
2) The back mix reactor
3) The ideal stirred tank reactor
4) CSTR or the CFSTR (constant flow stirred tank reactor)

**12. What is i) reactor volume? Ii) Internal volume of a reactor?**

The reactor volume (v) refers to the volume of fluid in the reactor. When the reactor volume differs from the internal volume of the reactor, then $V_r$ designates the internal volume of reactor. 'V' is the volume of reacting fluid.

Equation $V = \varepsilon V_r$.

V = volume of reacting fluid
$V_r$ = Internal volume
$\varepsilon$ = voidage in solid catalyzed reactors

**13. Write the rate equation for a second order reaction.**

For a second order reaction,

$A \rightarrow$ Products

The rate equation can be written as,

$$-r_A = K\, C_A^2$$

where, $r_A \rightarrow$ rate of the reaction

$K \rightarrow$ Equilibrium constant

$C_A \rightarrow$ Count ration of the reactant A'

**14. What are the interrelated factors responsible for a flow pattern?**

There are three interrelated factors that make up the contacting or flow pattern,

1. The RTD or residence time distribution of material which is flowing through the ressel.
2. The state of aggregation of the flowing material, its tendency to clump and for a group of molecules to more about together.
3. The earliness and lateness of mixing of material in the vessel.

**15. What is residence time distribution?**

A fluid following in a reactor takes different times to pass through the reactor. The distribution for these streams of liquid learning the vessel is called the residence time distribution (RTD) of fluid.

**16. Draw any two diagrams showing nonodeal flow patterns.**

**Or**

**Draw the nonideal flow patterns in process equipment.**

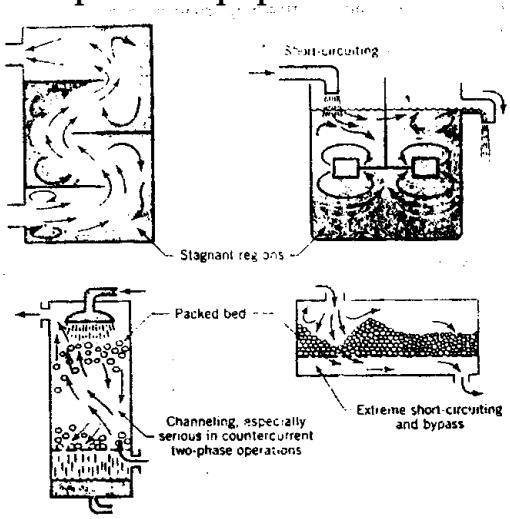



**Fig. Nonideal flow patterns which may exist in process equipment.**

Setting aside this goal of complete knowledge about the flow, let us be less ambitious and see what it is that we actually need to know. In many cases we really do not need to know very much, simply how long the individual molecules stay in the vessel, or more precisely, the distribution of residence times of the flowing fluid. This information can be determined easily and directly by a widely used method of inquiry, the stimulus-response experiment.

This chapter deals in large part with the residence time distribution (or RTD) approach to nonideal flow. We show when it may legitimately be used, how to use it, and when it is not applicable what alternatives to turn to.

In developing the "language" four this treatment of nonideal flow (see Danck-waters, 1953), we will only consider the steady-state flow, without reaction and without density change, of a single fluid through a vessel.

**17. Write the formula to calculate residencetime of a batch reactor.**

Average time or residence time can be calculated using the formula,

$$T_m = \int E(+)dt \qquad (E(t)dt = 1)$$

$dt = \text{time}$

where $T_m = \text{Residence time}$

$E(t) = \text{RTD function}$

$E(t)$ can be calculated using the formula

$$E(t) = \frac{C_{pulse}}{\left(M/V\right)}$$

'V' - volumetric flow rate 'v' in $m^3/sec$

'M'– tracer molecules in units moles or kg.

**18. What is state of aggregation?**

Flowing material due to aggregation remain in some extreme states and they can be called micro fluids and macro fluids. State of aggregation is an important factor which make up the contacting pattern Aggregation patterns differs in single phase and two phase systems.

**19. Draw the figures showing aggregation of fluid.**

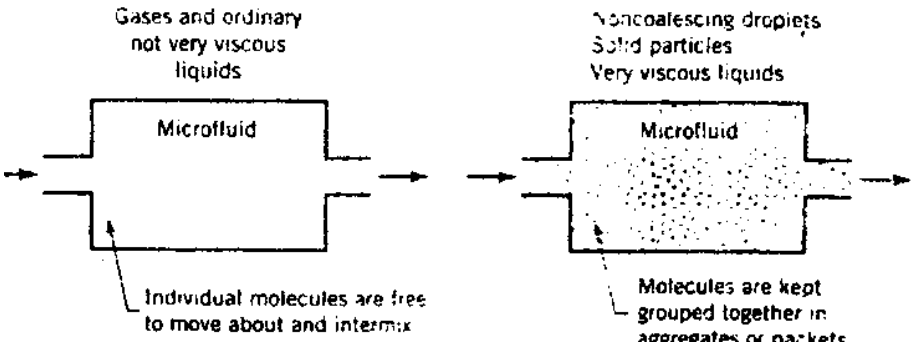



**Figure: Two extremes of aggregation of fluid.**

**20. What is single phase systems? Draw diagram for SPS.**

In nature, the flowing material remain in some particular state of aggregation. When the flowing material lie somewhere between the extremes of macrofluids and micro fluids, they remain in single phase system.

**21. Draw the figure explaining the macro fluid and micro fluid behaviour.**

In some situations one of these three factors can be ignored: in others it can become crucial. Often, much depends on the time for reaction, $\bar{t}_{rx}$, the time for

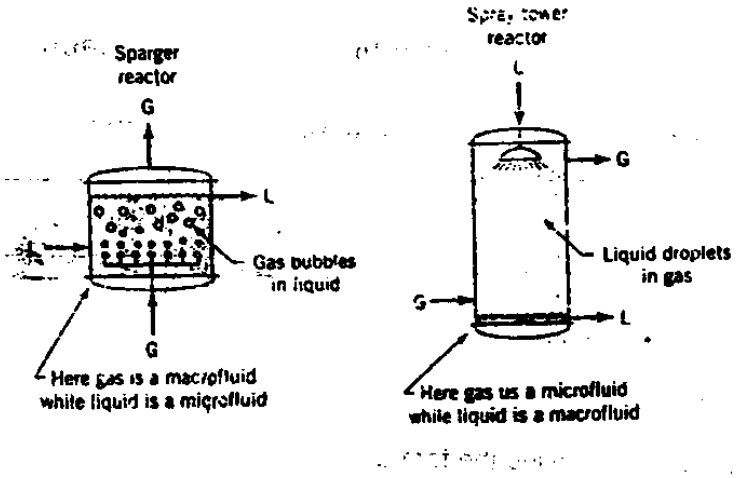



**Figure: Example of macro-and microfluid behavior.**

**22. What is Earliness of mixing?**

The fluid elements of a single flowing stream can mix with each other either early or late in their flow through the vessel. Earliness of mixing very important for a system with two reactants.

**23. Draw the diagram for early and late mixing of fluid.**

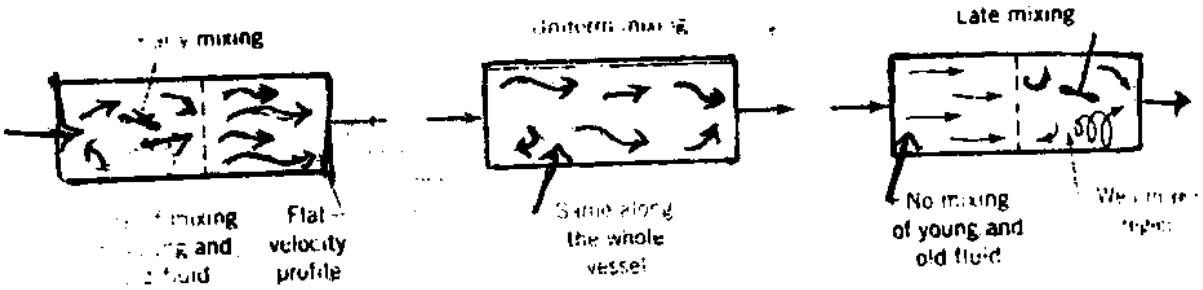



**Figure: Example of early and of late mixing of fluid.**

**24. c**

A fluid passing through the reactor may take different lengths of time to pass through the vessel. The distribution of these times for the stream of fluid leaving the vessel is called exit age distribution E.

'E' has the anits of time$^{-1}$.

**25. Draw 'E' curve.**

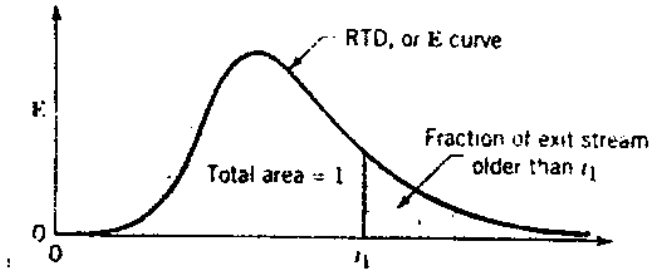



**Figure: The exit age distribution curve E for fluid flowing through a vessel: also called the residence time distribution or RTD.**

**26. Give a method to experimentally determine the residence time distribution of a CSTR?**

There are four different ways to find the 'E' or resisence time distribution, one nonchemical method is explained below.

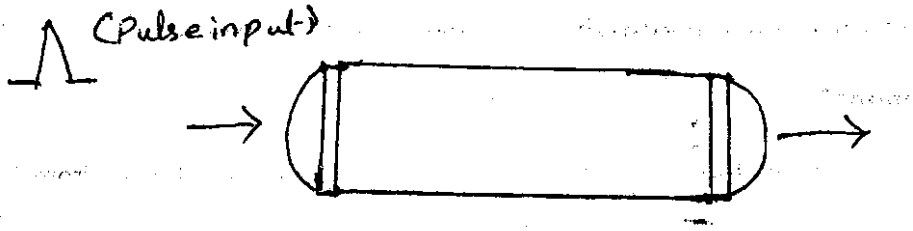



**Figure: (a)**

Let, 'V' b, the volume of the vessel ($m^3$)
'V' b, the flow rate of the fluid ($m^3/s$)
'M' units of tracer (kg or moles).

Step 1: Cpulse curve

V, V, M enters the vessel concentration time of tracer leaving the vessel. This is the $c_{pulse}$ curve.

Step 2: 'E' curve.

To find the E curve from the $C_{pulse}$ curve simply change the concentration scale such that the area under the is unity u, simply divide the concentration readings by M/v.

$$\text{So,} \qquad E = \frac{\text{Cpulse}}{M/v}$$

**27. What are the methods to determine the residence time experimental distribution of a CSTR?**

There are four methods to determine the flow pattern or residence time distribution of a CSTR namely,

1) Pulse experiment
2) Step experiment
3) Periodic experiment
4) Random experiment

**28. Draw the diagrams for studying the flow pattern in vessels.**

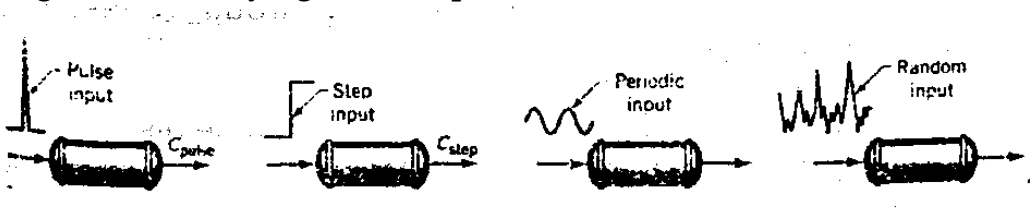



**Figure: Various ways of studying the flow pattern in vessels.**

**29. What is pulse experiment?**

Pulse experiment helps to find the 'E' curve for a vessel. 'E' curve is obtained from Cpulse curve.

**Cpulse curve**

Consider, 'V' – volume of the vessel ($Vm^3$)
'V' – Flow rate ($m^3/s$)
'M' – tracer molecules (kg or moles)

when 'v' flows through the vessel 'v' introduce 'M' units of tracer in to the fluid entering the vessel and record the concentration – time of tracer leaving the vessel. This is Cpulse curve.

$$E = \frac{Cpulse}{M/v}$$

**30. Draw the i) Cpulse ii) E curve.**

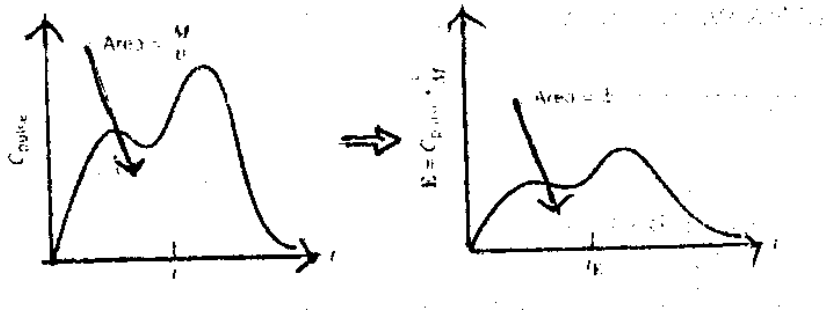


**Figure: Transforming an experimental $C_{pulse}$ curve an E curve.**

## PART – B

**1. Explain the interrelated factors that make up the flow pattern.**

The three interrelated factors which make up the flow pattern are,

The Residence time distribution of material.

1) Which is flowing through the vessel.
2) The state of aggregation of the flowing material, its tendency toclump and for a group of molecules to more about together.
3) The earliness and lateness of mixing of material in the vessel.

**The Residence Time Distribution, RTD**

Deviation from the two ideal flow patterns can be caused by channeling of fluid, by recycling of fluid, or by creation of stagnant regions in the vessel. Figure shows this behavior. In all types of process equipment, such as heat exchangers, packed column, and reactors, this type of flow should be avoided since it always lowers the performance of the unit.

If we know precisely what is happening within the vessel, thus if we have a complete velocity distribution map for the fluid in the vessel, then we should, in principle, be able to predict the behavior of a vessel as a reactor. Unfortunately, this approach is impractical, even in today's computer age.

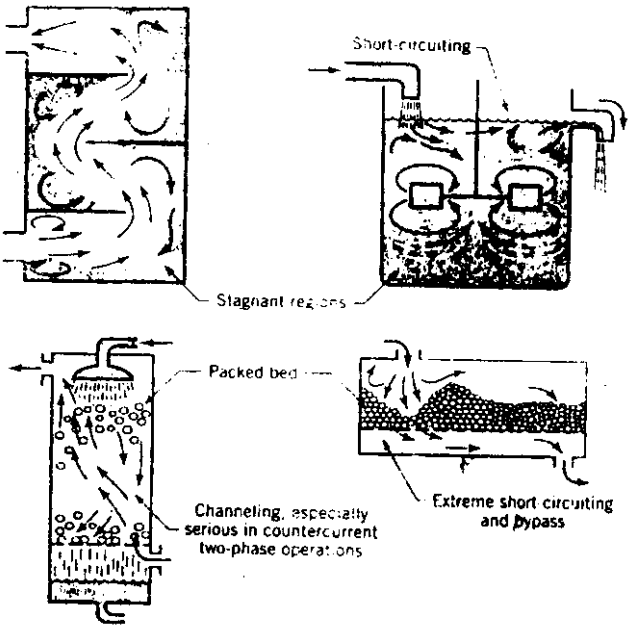



**Figure: Nonideal flow patterns which may exist in process equipment.**

Setting aside this goal of complete knowledge about the flow, let us be less ambitious and see what it is what we actually need to know. In many cases we really do not need to know very much, simply how long the individual molecules stay in the vessel, or more precisely, the distribution of residence times of the flowing fluid. This information can be determined easily and directly by a widely used method of inquiry, the stimulus-response experiment.

This chapter deals in large parts with the residence time distribution (or RTD) approach to nonideal flow. We show when alternatives to turn to.

In developing the "language" for this treatment of nonideal flow (see Danckwerts, 1953), we will only consider the steady-state flow, without reaction and without density change, of a single fluid through a vessel.

**State of Aggregation of the Flowing Stream**

Flowing material is in some particular state of aggregation, depending on its nature. In the extremes these states can be called microfluids and macrofluids, as sketch in fig.

**Single-Phase Systems.** These lie somewhere between the extremes of macro-and micro fluids.

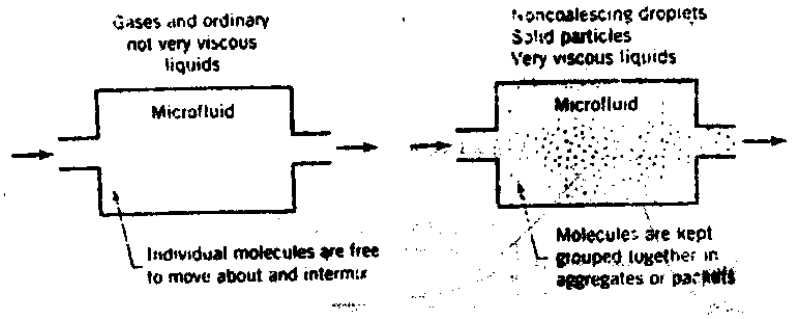



**Figure: Two extremes of aggregation of fluid.**

**Two-Phase Systems.** A stream of solids always behaves as a macrofluid, but for gas reacting with liquid, either phase can be a macro-or microfluid depending on the contacting scheme being used. The sketches of Fig. show completely opposite behavior. We treat these two phase reactors in later chapters.

**Earliness of Mixing**

The fluid elements of a single flowing stream can mix with each other either early or late in their flow through the vessel. For example, see Fig.

Usually this factor has little effect on overall behavior for a single flowing fluid.

**2. What is RTD? Explain the age distribution with figures.**

**The Age Distribution of Fluid, The RTD.**

It is evident that elements of fluid taking different routes through the reactor may take different lengths of time to pass through the vessel. The distribution of these times for the stream of fluid leaving the vessel is called the exit age distribution E, or the residence time distribution RTD of fluid. E has the units of time.

We find it convenient to represent the RTD in such a way that the area under the curve is unity, or

$$\int_0^\infty E dt = 1 \quad [-]$$

This procedure is called normalizing the distribution, and Fig. shows this we should note one restriction on the E curve-that the fluid only enters and only leaves the vessel one time This means that there should be no flow or diffusion or upflow eddies at the entrance or at the vessel exit. We call this the closed vessel boundary condition. Where elements of fluid can cross the vessel boundary more than one time we call this the open vessel boundary condition.

With this representation the fraction of exit stream of age* between t and t+ dt is

E dt [-]

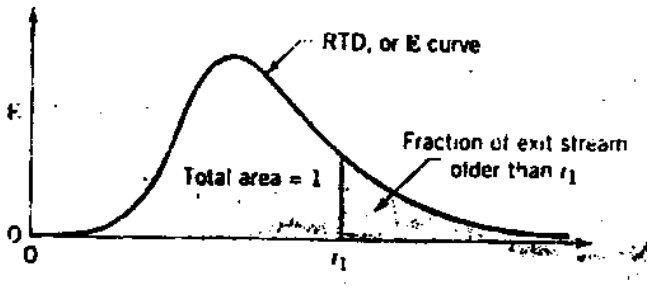



**Figure: The exit age distribution curve E for fluid flowing through a vessel: also called the residence time distribution, or RTD.**

The fraction younger than age $t_1$ is

$$\int_0^{t_1} E \, dt \ [-] \qquad \rightarrow (1)$$

whereas the fraction of material older than $t_1$, shown as the shaded area in Fig. is

$$\int_{t_1}^{\infty} E\,dt = 1 - \int_0^{t_1} E\,dt \quad [-] \qquad \rightarrow (2)$$

The E curve is the distribution needed to account for nonideal flow.

**3. What are the experimental methods for finding E?**

**Experimental Methods (Nonchemical) for Finding E**

The simplest and most direct way of finding the E curve uses a physical or nonreactive tracer. For special purposes, however, we may want to use a reactive tracer. This chapter deals in detail with the nonreactive tracer, and for this all sorts of experiments can be used. Figure. Shows some of these. Because the pulse and the step experiments are easier to interpret, the periodic and random harder, here we only consider the pulse and the step experiment.

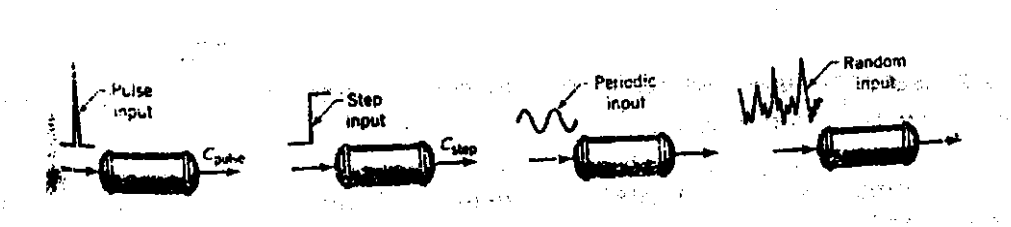



**Figure: Various ways of studying the flow pattern in vessels.**

**4. Briefly explain the pulse experiment.**

**The Pulse Experiment**

Let us find the E curve for a vessel of volume V $m^3$ through which flows v $m^{3/8}$ of fluid. For this instantaneously introduce M units of tracer (kg or moles) into the fluid entering the vessel, and record the concentration-time of tracer leaving the vessel. This is the $C_{pulse}$ curve.

$$\left(\text{Area under the } C_{pulse} \text{ curve}\right): \quad A = \int_0^{\infty} C\,dt = \sum_i C_i \Delta t_i = \frac{M}{v}\left[\frac{kg.s}{m^3}\right]$$

$$\left(\text{Mean of the } C_{pulse} \text{ curve}\right): \qquad \bar{t} = \frac{\int_0^{\infty} tC\,dt}{\int_0^{\infty} C\,dt} = \frac{\sum_i t_i C_i \Delta t_i}{\sum_i C_i \Delta t_i} = \frac{V}{v}[s]$$

All this is shown in Fig.

To find the E curve from the $C_{pulse}$ curve simply change the concentration scale such that the area under the curve is unity. Thus, simply divide the concentration readings by $M/v$ as shown in

Fig.

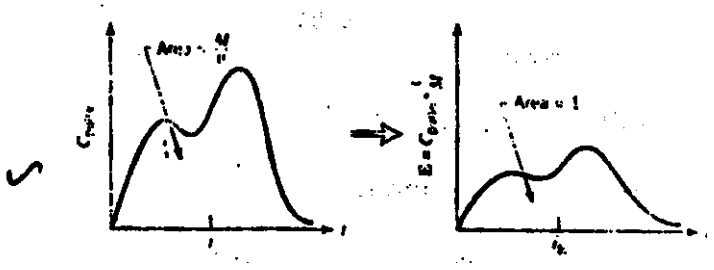


**Figure: Transforming an experimental $C_{pulse}$ curve into an E curve.**

We have another RTD function $E_\theta$. Here tune us measured in terms of mean residence time $\theta = \bar{t}t$. Thus

$$E_\theta = \bar{t}E = \frac{V}{\nu}.\frac{C_{pulse}}{M/\nu} = \frac{V}{M}C_{pulse}$$

$E_\theta$ is a useful measure when dealing with flow models which come up in Chapters and Figure shows how to transform E into $E_\theta$.

One final reminder, the relationship between $C_{pulse}$ and the E curves only holds exactly for vessels with closed boundary conditions.

**5. Briefly explain the step experiment.**

**The Step Experiment**

Consider $\nu\ m^2/s$ of fluid flowing through a vessel of volume V. Now at time t = 0 switch from ordinary fluid to fluid with tracer of concentration $C_{max} = \left[\frac{kg\ or\ mol}{m^3}\right]$, and measure the outer tracer concentration $C_{step}$ versus t, as shown in Fig.

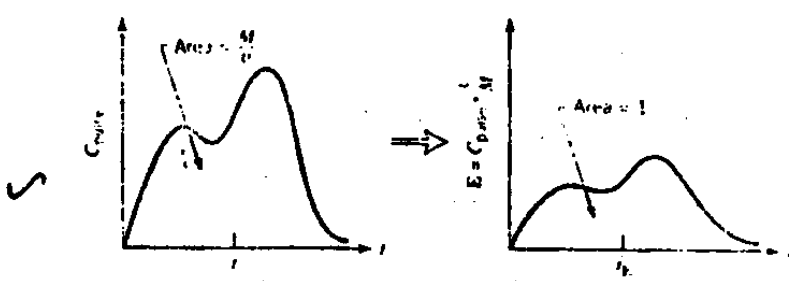


**Figure: Transforming an E curve into an $E_\theta$ curve.**

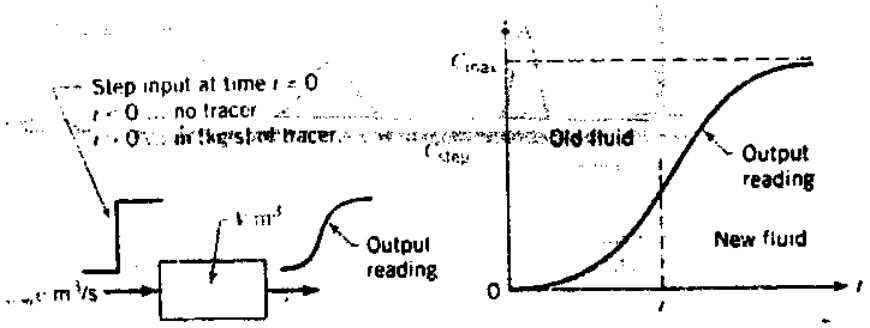



**Figure: Information obtainable from a step tracer experiment.**

A material balance relates the different measured quantities of the output curve of a step input

$$C_{max} = \frac{m}{\nu}\left[\frac{kg.s}{m^3}\right]$$

$$\text{and} \quad (\text{shaded area of fig.}) = C_{max}\bar{t} = \frac{mV}{\nu^2}\left[\frac{kg.s^2}{m^3}\right]$$

$$\bar{t} = \frac{\int_0^{C_{max}} t dC_{step}}{\int_0^{C_{max}} dC_{step}} = \frac{1}{C_{max}}\int_0^{C_{max}} t\, dC_{step}$$

where m [kg/s] is the flow rate of tracer in the entering fluid.

The dimensionless form of the $C_{step}$ curve is called the F curve. It is found by having the tracer concentration rise from zero to unity.

**6. Explain the dispersion model.**

**Axial Dispersion**

Suppose an ideal pulse of tracer is introduced into the fluid entering a vessel. The pulse spreads as it passes through the vessel, and to characterize the spreading according to this model (see Fig.), we assume a diffusion-like process superimposed on plug flow. We call this dispersion or longitudinal dispersion to distinguish it from molecular diffusion. The dispersion coefficient D ($m^2/s$) represents this spreading process. Thus

large D mean rapid spreading of the tracer curve
small D means spreading
D = 0 means no spreading, hence plug flow

Also

$\left(\frac{D}{uL}\right)$ is the dimensionless group characterizing the spread in the whole vessel.

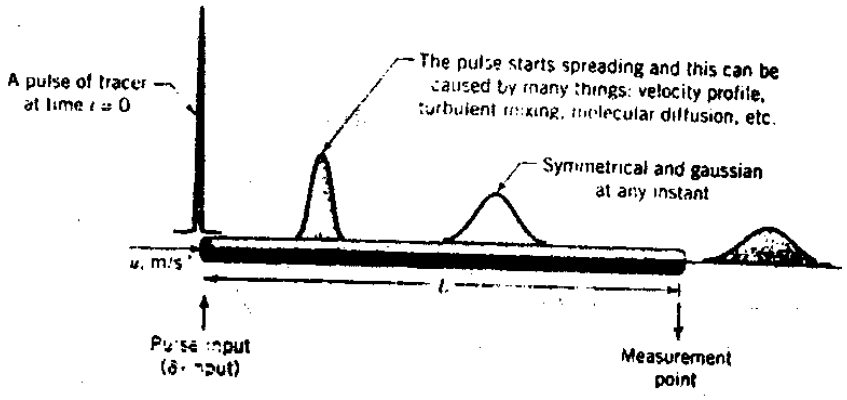



**Figure: The spreading of tracer according to the dispersion model.**

We evaluate D or D!uL by recording the shape of the tracer curve as it passes the exit of the vessel. In particular, we measure

$\bar{t}$ = mean time of passage, or when the curve passes by the exit

$\sigma^2$ = variance, or a measure of the spread of the curve

The measures. T and $\sigma^2$, are directly linked by theory to D and D/uL. The mean, for continuous or discrete data, is defined as

$$\bar{t} = \frac{\int_0^\infty tCdt}{\int_0^\infty Cdt} = \frac{\sum t_i C_i \Delta t_i}{\sum C_i \Delta t_i}$$

The variance is defined as

$$\sigma^2 = \frac{\int_0^\infty \left(t-\bar{t}\right)^2 Cdt}{\int_0^\infty Cdt} = \frac{\int_0^\infty t^2 Cdt}{\int_0^\infty Cdt} - \overline{t^2}$$

or in discrete form

$$\sigma^2 \cong \frac{\sum \left(t_i-\bar{t}\right)^2 C_i \Delta t_i}{\sum C_i \Delta t_i} = \frac{\sum t_i^2 C_i \Delta t_i}{\sum C_i \Delta t_i} - \overline{t^2}$$

The variance represents the square of the spread of the distribution as it passes the vessel exit and has units of (time)$^2$. It is particularly useful for matching experimental curves to one of a family of theoretical curves. Figure illustrates these terms.

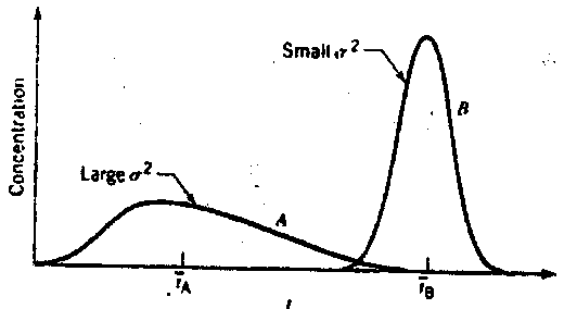



**Figure:**

Consider plug flow of a fluid, on top of which is superimposed some degree of back mixing, the magnitude of which is independent of position within the vessel. This condition implies that there exist no stagnant pockets and no gross bypassing or short-circuiting of fluid in the vessel. This is called the dispersed pug flow model, or simply the dispersion model. Figure shows the conditions visualized. Note that with varying intensities of turbulence or intermixing the predictions of this model should range from plug flow at one extreme to mixed flow at the other. As a result the reactor volume for this model will lie between those calculated for plug and mixed flow.

Since the mixing process involves a shuffling or redistribution of material either by slippage or eddies, and since this is repeated many, many times during the flow of fluid through the vessel we can consider these disturbances to be statistical in nature, somewhat as in molecular diffusion. For molecular diffusion in the x-direction the governing differential equation is given by Fick's law:

$$\frac{\partial C}{\partial t} = \wp \frac{\partial^2 C}{\partial x^2}$$

where $\wp$, the coefficient of molecular diffusion, is a parameter which uniquely characterizes the process. In an analogous manner we may consider all the contributions to intermixing of fluid flowing in the x-direction to be described

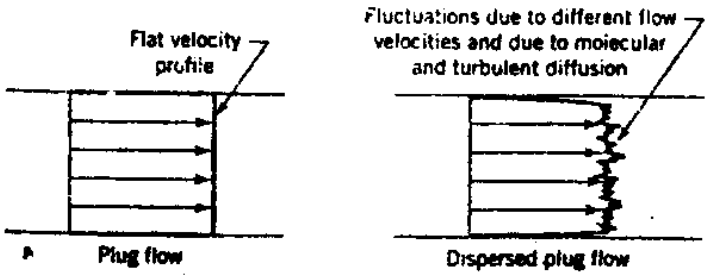



**Figure: Representation of the dispersion (dispersed plug flow) model.**

by a similar from of expression, or

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}$$

where the parameter D, which we call the longitudinal or axial dispersion coefficient, uniquely characterizes the degree of backmixing during flow. We use the terms longitudinal and axial because we wish to distinguish mixing in the direction of flow from mixing in the lateral or radial direction, which is no our primary concern. These two quantities may be quite different in magnitude. For example in streamline flow of fluids through pipes, axial mixing is mainly due to fluid velocity gradients, whereas radial mixing is due to molecular diffusion alone.

In dimensionless form where z = (ut + x)/L $\theta = t\bar{t} = tu/L$, the basic differential equation representing this dispersion model becomes.

$$\frac{\partial C}{\partial \theta} = \left(\frac{D}{uL}\right)\frac{\partial^2 C}{\partial z^2} - \frac{\partial C}{\partial z}$$

where the dimensionless group $\left(\frac{D}{uL}\right)$, called the vessel dispersion number, is the parameter that measures the extent of axial dispersion. Thus

$\frac{D}{uL} \to 0$ negligible disperison, hence plug flow

$\frac{D}{uL} \to \infty$ large dispersion, hence mixed flow

This model usually represents quite satisfactorily flow that deviates not too greatly from plug flow, thus real packed beds and tubes (long ones if flow is streamline).

**7. Explain the large deviation from plug flow $D/uL > 0.01$ in a dispersion model.**

**Large Deviation from Plug Flow, $\frac{D}{uL} > 0.01$.**

Here the pulse response is broad and it passes the measurement point slowly enough that it changes shape-it spreads-as it is being measured. This gives a nonsymmetrical E curve.

An additional complication enters the picture for large D/uL. What happens right at the entrance and exit o the vessel strongly affects the shape of the tracer curve as well as the relationship between the parameters of the curve and D/uL.

Let us consider two types of boundary conditions: either the flow is undisturbed as it passes the entrance and exit boundaries (we call this the open b.c.). or you have plug flow outside the vessel up to the boundaries (we call this the closed b.c.). This leads to four combinations of boundary conditions, closed-closed, open-open, and mixed. Figure illustrates the closed and open extremes. Whose RTD curves are designated as $E_{cc}$ and $E_{00}$.

Now only one boundary condition gives a tracer curve which is identical to the E function and which fits all the mathematics of Chapter 11, and that is the closed vessel. For all other boundary conditions you do not get a proper RTD.

In all cases you can evaluate D/uL from the parameters of the tracer curves; however, each curve has its own mathematics. Let us look at the tracer curves for closed and for the open boundary conditions.

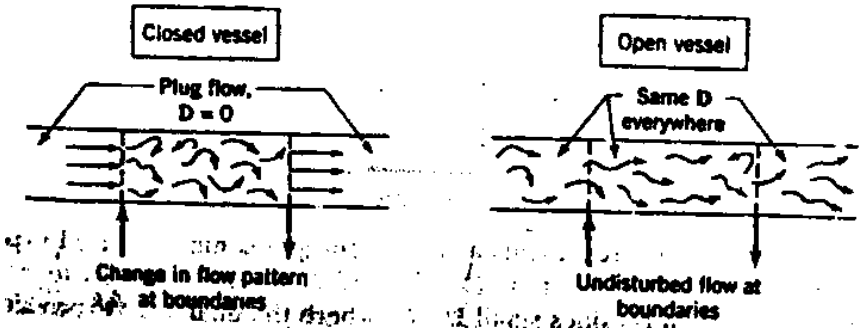



**Figure: Various boundary conditions used with the dispersion model.**

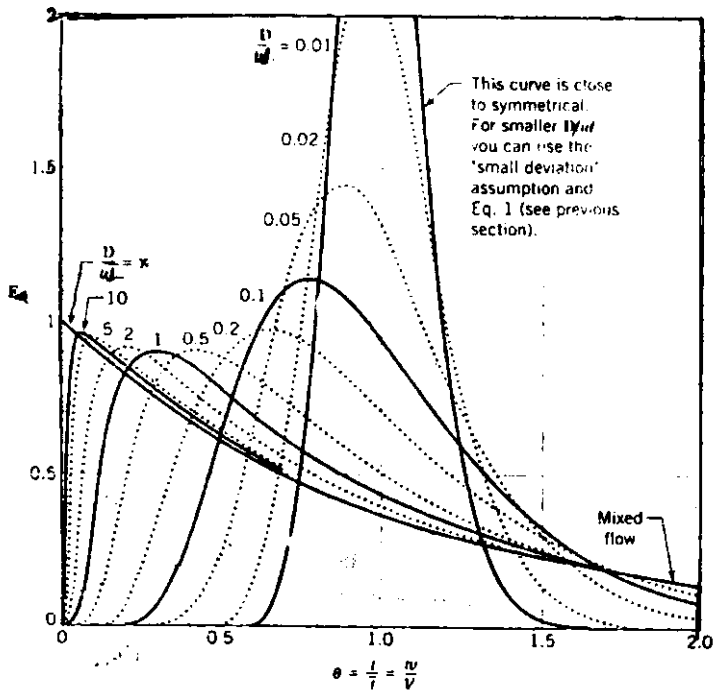



**Fig. Tracer response curves for closed vessels and large deviations from plug flow.**

**Closed Vessel.** Here an analytic expression for the E curve is not available However, we can construct the curve by numerical methods. See Fig. or evaluate its mean and variance exactly, as was first done by van der Lann (1958). Thus

$$\overline{t_E} = \bar{t} = \frac{V}{\nu} \text{.....or.....} \overline{\theta_E} = \frac{\overline{t_E}}{t} = \frac{\overline{t_E}\nu}{V} = 1$$

$$\sigma_\theta^2 = \frac{\sigma_t^2}{\bar{t}^2} = 2\left(\frac{D}{uL}\right) - 2\left(\frac{D}{uL}\right)^2 \left[1 - e^{-uL/D}\right]$$

**Open Vessel.** This represents a convenient and commonly used experimental device, a section of long pipe (see Fig). It also happens to be the only physical situation (besides small D/uL) where the analytical expression for the E curve is not too complex.

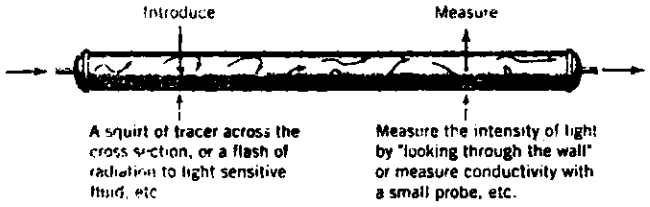



**Figure: The open-open vessel boundary condition.**

In Fig. and by the following equations, first derived by Levenspiel and Smith (1957).

$$E_{\theta.00} = \frac{1}{\sqrt{4\pi(D/uL)}} \exp\left[\frac{(1-\theta)^2}{4\theta(D/uL)}\right]$$

$$E_{t.00} = \frac{u}{\sqrt{4\pi Dt}} \exp\left[\frac{(L-ut)^2}{4Dt}\right]$$

$$\bar{\theta}_{E.00} = \frac{\bar{t}_{E.00}}{\bar{t}} 1 + 2\left(\frac{D}{uL}\right) \quad \text{...or...} \quad t_{E00} = \frac{V}{\nu}\left(1 + 2\frac{D}{uL}\right)$$

$$\text{open} - \text{openvessel}$$

$$\sigma_{\theta.00}^2 = \frac{\sigma_{t.00}^2}{\bar{t}^2} = 2\frac{D}{uL} + 8\left(\frac{D}{uL}\right)^2$$

To evaluate D/uL either match the measured tracer curve or the measured $\sigma^2$ to theory. Matching $\sigma^2$ is simplest, though not necessarily best; however, it is often used. But be sure to use the right boundary conditions.

**8. Explain the tank in series model.**

**The Tank-In-Series Model**

Can be used whenever the dispersion model is used; and for not too large a deviation from plug flow both models give identical results, for all practical purposes.

The dispersion model has the advantage in that all correlations for flow in real reactors invariably use that model. On the other hand the tanks-in-series model is simple, can be sued with any kinetics, and it can be extended without too much difficulty to any arrangement of compartment, with or without recycle.

**Pulse response experiments and the RTD**

Figure: shows the system we are considering. We also define

$\theta_i = \dfrac{t}{t_i}$ = dimensionless time based on the mean residence time per tank $\bar{t}_i$

$\theta = \dfrac{t}{t}$ = dimensionless time based on the mean residence time in all N tanks. $\bar{t}$.

Then

$$\theta_i = N\theta \quad \text{... and ....} \quad \bar{\theta}_i = 1, \ \bar{\theta} = 1$$

and at any particular time, from Eq. 11 in Chapter 11

**For the first tank.** Consider a steady flow $\nu\ m^3/s$ of fluid into and out of the first of these ideal mixed flow units of volume $V_1$. At time t = 0 inject a pulse of tracer into the vessel which when evenly distributed in the vessel and it is) has a concentration $C_0$.

At any time t after the tracer is introduced make a material balance, thus

$$(\text{rate of disappearance of tracer}) = (\text{input rate}) - (\text{output rate})$$

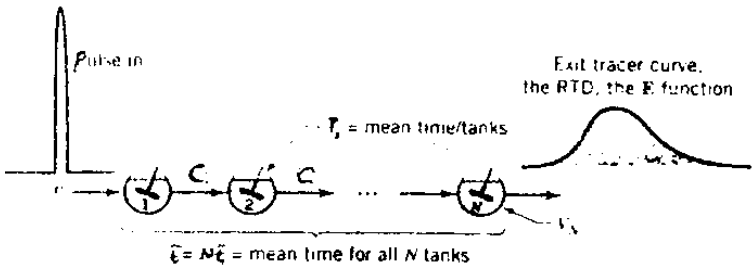



**Figure: The tanks-in-series model.**

In symbols this expression becomes

$$V_1 \frac{dC_1}{dt} = 0\nu C_1 \left[\frac{\text{mol tracer}}{s}\right]$$

where $C_1$ is the concentration of tracer in tank …1. "Separating and integrating then gives

$$\int_{C_0}^{C_1} \frac{dC_1}{C_1} = -\frac{1}{\bar{t}_1}\int_0^t dt$$

or

$$\frac{C_1}{C_0} = e^{-t/\bar{t}_1}$$

Since the area under this $C/C_0$ versus t curve is $\bar{t}_1$ (check this if you wish) it allows you to find the E curve: so one may write

$$\bar{t}_1 E_1 = e^{-t/\bar{t}_1} \quad [-] \; N=1$$

For the second tank where $C_1$ enters, $C_2$ leaves, a material balance gives

$$V_2 \frac{dC_2}{dt} = \nu.\frac{C_0}{\bar{t}_1} e^{-t/\bar{t}_{1-\nu}} C_2 \left[\frac{\text{mole tracer}}{s}\right]$$

$$----$$

$$C_1$$

Separating gives a first-order differential equation, which when integrated gives

$$\bar{t}_2 E_2 = \frac{t}{t_2} = e^{-t/\bar{t}_2} \quad [-] \; N=2$$

For the Nth tank. Integration for the 3rd, 4th,…..Nth tank becomes more complicated so it is simpler to do all of this by Laplace transforms.

The RTD's means and variances, both in time and dimensinless time were first derived by MacMullin and Weber (1935) and are summarized by Eq.3.

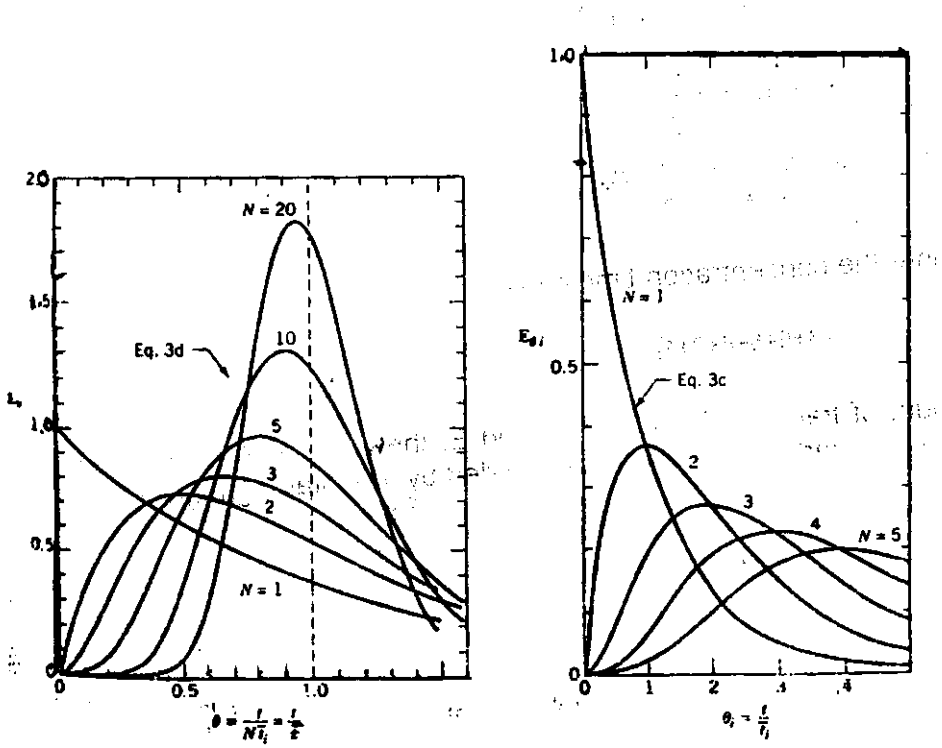
$$\bar{t}E = \left(\frac{t}{\bar{t}}\right)^{N-1} \frac{N^N}{(N-1)!} e^{-tN/\bar{t}} \;\; .....\bar{t} = N\bar{t}_i .....\sigma^2 = \frac{\bar{t}^2}{N}$$

$$\bar{t}_i \; E = \left(\frac{t}{\bar{t}_i}\right)^{N-1} \frac{1}{(N-1)!} e^{-t/\bar{t}_i} \;\; .....\bar{t}_i = \frac{\bar{t}}{N} ....\sigma^2 = N\bar{t}_1^{-2}$$

$$E_{\theta i} = \bar{t}_i E = \frac{\theta_i^{N-1}}{(N-1)!} e^{-\theta_i} \qquad ....\sigma_{\theta i}^2 = N$$

$$E_\theta = \left(N\bar{t}_i\right)E = N\frac{(N\theta)^{N-1}}{(N-1)} e^{-N\theta} \;\; ....\sigma_\theta^2 = \frac{1}{N}$$

Graphically these equations are shown in fig. the properties of the RTD curves are sketched in fig.




**RTD curves for the tanks in series model.**

**9. Finding the RTD experiment**

The concentration readings in Table E11.1 represent a continuous response to a pulse input into a closed vessel which is to be used as a chemical reactor. Calculate the mean residence time of fluid in the vessel t, and tabulate and plot the exit age distribution E.

**Table**

| Time t, min | Tracer Output Concentration, $C_{pulse}$ gm/liter fluid |
|---|---|
| 0 | 0 |
| 5 | 3 |
| 10 | 5 |
| 15 | 5 |
| 20 | 4 |
| 25 | 2 |
| 30 | 1 |
| 35 | 0 |

**Solution:**

The mean residence time, from Eq. 4, is

$$\bar{t} = \frac{\sum t_i C_i \Delta t_i}{\sum C_i \Delta t_i} \overset{\Delta t = \text{constant}}{=\!=\!=} \frac{\sum t_i C_i}{\sum C_i}$$

$$= \frac{5\times3+10\times5+15\times5+20\times4+25\times2+30\times1}{3+5+5+4+2+1} = 15 \text{ min}$$

The area under the concentration-time curve.

$$\text{Area} = \sum C\Delta t = (3+5+5+4+2+1)5 = 100 \text{ gm.min/liter}$$

gives the total amount of tracer introduced. To find E, the area under this curve must be unity; hence, the concentration readings must each be divided by the total area, giving

$$E = \frac{C}{\text{area}}$$

Thus we have

| $E = \frac{C}{\text{area}}$ t,min | 0 | 5 | 10 | 15 | 20 | 25 | 30 |
|---|---|---|---|---|---|---|---|
| $\text{min}^{-1}$ | 0 | 0.03 | 0.05 | 0.05 | 0.04 | 0.02 | 0.01 |

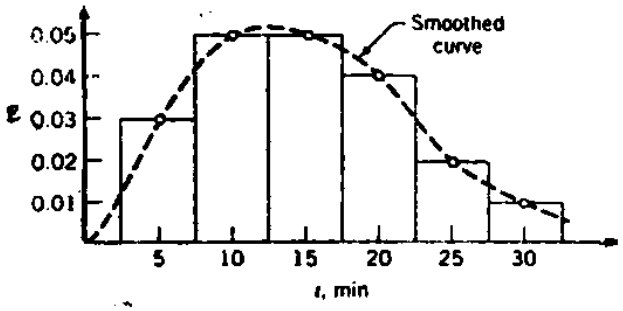



**Figure: E 11.**

Figure E11.1 is a plot of this distribution.

**10. Conversion in reactors having non-ideal flow**

The vessel of Example 11.1 is to be used as a reactor for a liquid decomposing with rate

$$-r_A = kC_A, \quad k=0.307 \text{ min}^{-1}$$

Find the fraction of reactant unconverted in the real reactor and compare this with the fraction unconverted in a plug flow reactor of the same size.

**Solution:**

For the plug flow reactor with negligible density change we have

$$\bar{t} = C_{A0}\int_0^{X_A}\frac{dX_A}{-r_A} = -\frac{1}{k}\int_{C_{A0}}^{C_A}\frac{dC_A}{C_A} = \frac{1}{k}\ln\frac{C_{A0}}{C_A}$$

and with τ from Example 11.1

$$\frac{C_A}{C_{A0}} = e^{-k\tau} = e^{-(0.307)(15)} = e^{-4.6} = \underline{\underline{0.01}}$$

Thus e fraction of reactant unconverted in a plug flow reactor equals 1.0%.

For the real reactor the fraction unconverted, given by Eq. 13 for macro fluids, is found in Table E11.4. Hence the fraction of reactant unconverted in the real reactor

$$\frac{C_A}{C_{A0}} = \underline{\underline{0.047}}$$

**Table:**

| t | E | Kt | $e^{-kt}$ | $e^{-kt}E\,\Delta t$ | |
|---|---|---|---|---|---|
| 5 | 0.03 | 1.53 | 0.2154 | (0.2154)(0.03)(5)= | 0.0323 |
| 10 | 0.05 | 3.07 | 0.0464 | | 0.0116 |
| 15 | 0.05 | 4.60 | 0.0100 | | 0.0025 |
| 20 | 0.04 | 6.14 | 0.0021 | | 0.0004 |
| 25 | 0.02 | 7.68 | 0.005 | | 0.0001 |
| 30 | 0.01 | 9.21 | 0.0001 | | 0 |
| gievn | | | | | $\frac{C_A}{C_{A0}} = \sum e^{-kt}E\Delta t = \underline{\underline{0.0469}}$ |

From the table we see that the unconverted material comes mostly from the early portion of the E curve. This suggests that channeling and short-circuiting can seriously hinder attempts to achieve high conversion in reactors.

## UNIT – II

## PART – A

**1. Write a short note on stirred tank reactor.**

In a stirred tank reactor 1) Mixing and bubble dispersion are achieved by mechanical a gitation.

1) Baffles are used in stirred reactors to reduce vortexing.
2) Impellers are used to produce different flow patterns inside the vessels.
3) Only 70-80% of the volume of stirred reactors is filled with liquid which allows head space for disengagement of droplets from the exhaust gas.
4) Temperature and hart transfer in stirred vessels can be accomplished using internal cooling coils.

**2. Draw Bubble-column bioreactor and label the parts.**

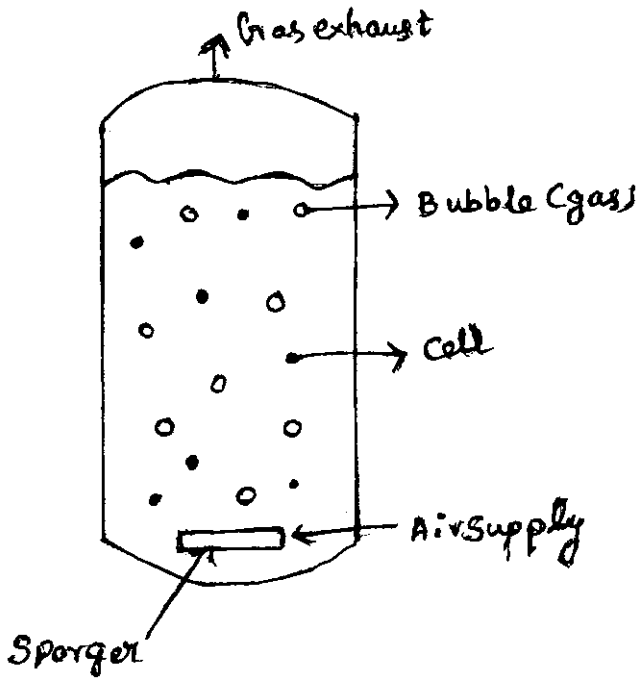



**Fig: Bubble-column bioreactor.**

**3. Write a short note on bubble column reactor.**

1) Bubble column reactor in clues vessels with no mechanical agitation.
2) It is an alternate for stirred tank reactor
3) In BCR, aeration and mixing are achieved by gas sparging.

4) Bubble columns are simple cylindrical vesels with height greater than twice the diameter.
5) It has no internal structures other than a sprayer.
6) Sparger is used for the cntry of compressed air
7) It is used for the production of baker's yeast bear and Vineger.

**4. Draw the figure showing heterogeneous flow in a bubble column and label the parts.**

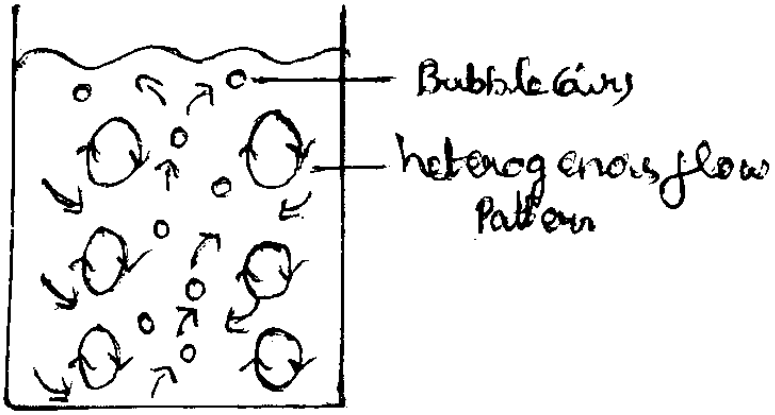
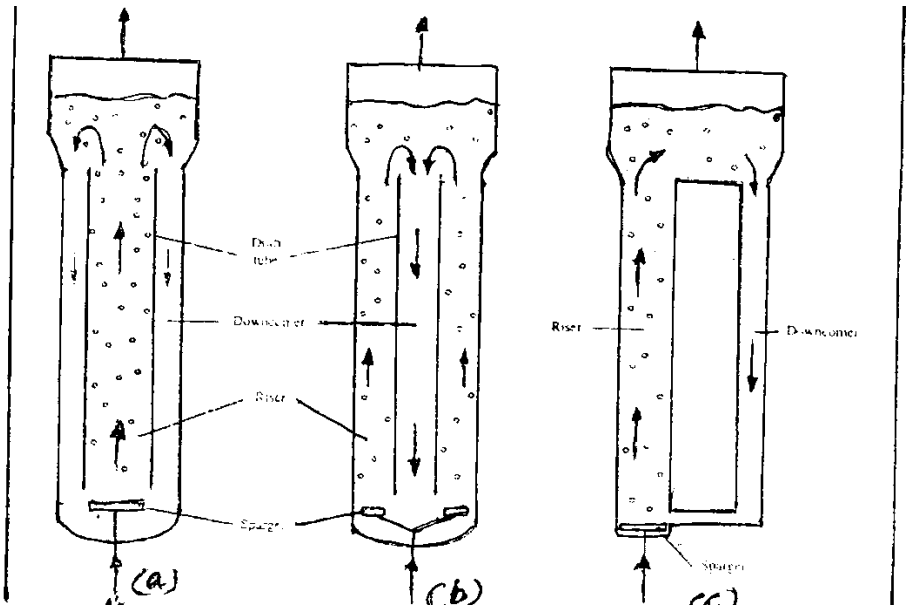



**Figure: Heterogenous flow in a bubble column reactor**

**5. Write a short note on Airlift reactor.**

1) Airlift reactors are often chosen for culture of plant and animal cells and immobilized catalysts
2) It is accomplished without mechanical a gitation.
3) Shear levels are significantly lower than in stirred vessels.
4) Here, the patterns of liquid flow are more defined owing to the physical separation of up flowing and downflowing streams.
5) Based on the direction of the rised and the down comer internal loop vessels and external loop vessels can be illustrate and explained.

**6. Draw i) Internal loop airlift reactor**
**ii) External loop air lift reactor and label the parts.**




**7. Write a short note on packed bed reactor.**

Packed-bed reactor are used with immobilized or particulate biocatalyst. The reactor consists of a tube, usually vertical, packed with catalyst particles. Medium can be fed either at the top or bottom of the column and forms a continuous liquid phase between the particles.

**8. Given any two commercial use of packed bed reactor.**

Packed-bed reactors have been used commercially with immobilized cells and enzym's for
i) Procduction of aspirate and fumarate,
ii) Conversion of penicillin to 6-amino pencillanic acid
iii) It is used for the resolution of amino acid ISO mess.

**9. Draw packed bed reactor and label the parts.**

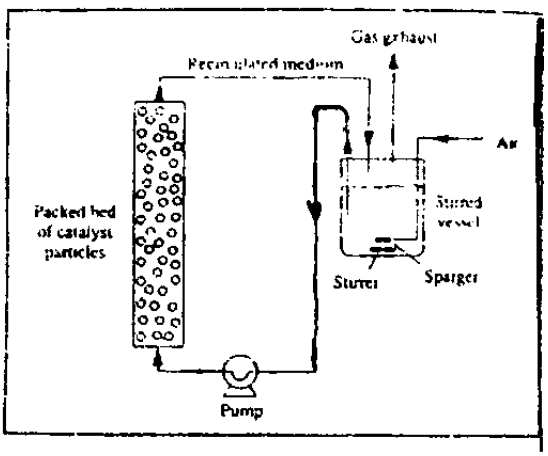



**Figure: Packed-bed reactor with medium recycle.**

**10. Write a short note on fluid sed bed reactor.**

Fluidised bed reactor are formed when packed beds are operated in up flow mode with catalyst beads of appropriate size and density, the bed expands at high liquid flow rates due to upward motion of the particles. The particles in fluidized beds are in a constant motion, channeling and clogging of the bed are avoided and air can be introduced directly in to the column.

**11. Give any two commercial use of fluidized bed reactor.**

1) Fluidised bed reactors are used in waste treatment with sand or similar material supporting mixed microbial populations.
2) They are used with flowculating organisms in brewing and for production of vinegar.

**12. Draw fluidized bed reactor and label the parts.**

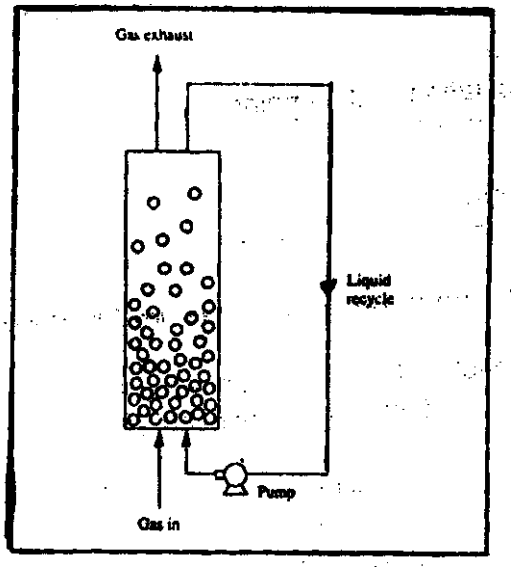



**Figure: Fluidised-bed reactor.(13.9)**

**13. Draw Trickle bed reactor and label the parts.**

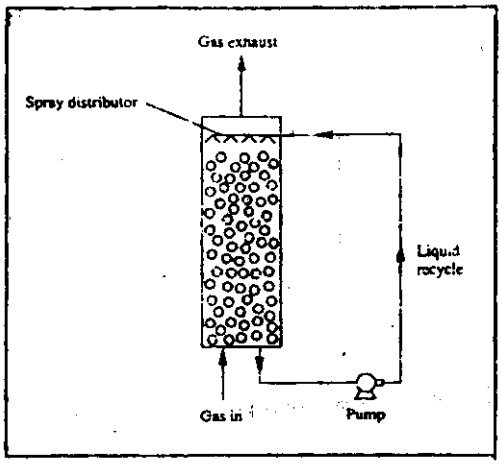



**Figure: Tricked-bed reactor**

**14. Write a shortnote on Trickle bed reactor.**

The trickle bed reactor is another variation of the packed bed reactor. Liquid is sprayed on to the top of the packing and trickles down through the bed in small rivulets. Air may be introduced at the base, because the liquid phase is not continuous throughout the column, air and other gases more with relative ease around the packing.

Use: Aerobic waste water treatment.

**15. Write any four practical considerations for Bioreactor construction.**

1) Aseptic operation
2) Fermenter inoculation and samping
3) Materials of construction
4) Sparger design
5) Evaportaion control
6) Monitoring and control of Bioreactors.

**16. How will you calculate void fraction in a packed bed reactor?**

In a packed bed reactor, the axial fluid velocity will be larger than in an open plug flow reactor and depends on the void fraction ε, defined as,

$$\varepsilon = \frac{\text{Free reactor volume}}{\text{Total reactor volume}}$$

$$\varepsilon = 1 - \frac{\text{Total particle volume}}{\text{Total reactor volume}}$$

**17. How will you calculate inter stitial fluid velocity in a packed bed reactor?**

The Interstitial fluid velocity '$V_i$' is calculated using the formula,

$$V_i = \frac{F}{\varepsilon(\text{cross sectional area})}$$

$$v_i = \frac{F}{\varepsilon V/L}$$

where '$\varepsilon$' - void fraction
'F' – volumetric flow rate
'V' – averagelinear velocity
'L' – length of the reactor

**18. How will you calculate liquid residence time in a packed bed reactor?**

The interstitial velocity ($v_i$) is employed to calculate the liquid residence time '$\tau$' .

$$\tau = \varepsilon\left(V/F\right)$$

$\varepsilon$ = 1 for enzymes
$\varepsilon$ = 0.4 for immobilized enzym's on spherical supports.

where
'$\tau$' – residence time
'F' – volumetric flow rate
'V' – Average linear velocity.

**19. Draw the model of liquid flow patterns in a bioreactor.**

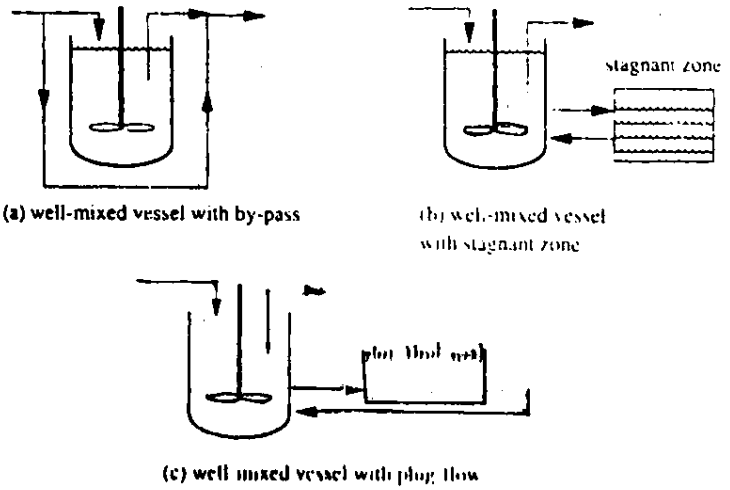



**Figure: Models of the liquid flow patterns in bioreactors. (a) a stirred tank with by-passing, (b) a stirred tank with a stagnant fluid region, (c) a stirred tank with a plug flow reactor by-pass.**

**20. Draw the two phase model to represent the bubbling fluidized bed.**

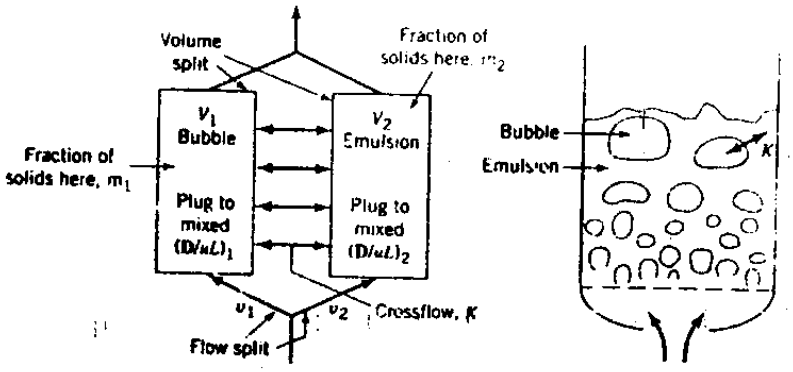



**Figure: Two-phase model to represent the bubbling fluidized bed, with its six adjustable parameters, $v_1$, $V_1$. $(D/uL)_1$, $(D/uL)_2$, $m_1$,K.**

**21. Write short note on RTD models.**

RTD models are used to calculate conversions. But since the rate of catalytic reaction of an element of gas depends on the amount of solid in its vicinity, the effective rate constant is low for bubble gas, high for emulsion g as. In order to calculate conversion from RTD assume that all element of gas, both slow and fast moving, spend the same fraction of time in each of the phases.

**22. What is the role of draft tube in an airlift reactor?**

There are two common airlift configurations namely

1) Internal – loop vessels
2) External – loop or outer loop airlift reactor

In the internal loop vessels, the riser and down comer Ares, pirated by an internal baffle or draft tube.

**<u>Bole of Draft tube</u>**

1) Air may be sparged in to either the draft tube or the annulus.
2) Baffles or draft tubes helps for better mixing and provides good aeration.

**23. What are the advantages of two chemostats in series?**

The use of two chemostats in series enables the preferred substrate to be completely consumed in the first reactor and the second substrate can then be consumed in the second reactor, minimizing the required reactor volume. Hence the smallest reactor volume for a specified degree of product conversion can be achieved.

**24. What is diauxic effect?**

When mixed substrates are employed such as two saccharides or a substrate such as lactose, which is hydrolyzed in to two saccharides where the preferential substrate is consumed first and this is called "diauxic effect"

**25. What are the main assumptions in the dispersion model?**

1) The dispersion coefficient D($m^2$/s) represents the spreading process Thus,

   large D $\rightarrow$ Rapidspreading of the tracer curve
   small D $\rightarrow$ Slowspreading
   D = o $\rightarrow$ No spreading, hence plug flow

2) (D/uL) is the dimensionless group characterizing the spread of whole vessel.
3) Varying intensitres of turbulence or intermixing the predict ions of this model should range from plug flow at one extreme to mixed flow at the other.

**26. Write any two commercial application of bubble column reactor.**

1) Production of antibiotics
2) Production of bakers yeast
3) Production of single cell protein
4) Production of vinegar.

**27. Write short note on bubble generation at an orifice.**

Sparger helps for the introduction of gas in to a liquid. There are three distinct regimes of bubble formation, based on the gas flow rate through the orifice.

1) At low gas flowrates, bubbles of constant volume are formed.
2) The bubble size depends on the orifice diameter, surface tension and buoyancy.
3) The bubble grows till the buoyancy force exceeds the surface tension force holding the bubble to the orifice.

**28. Draw the diagram illustrating the bubble generation at an orifice.**

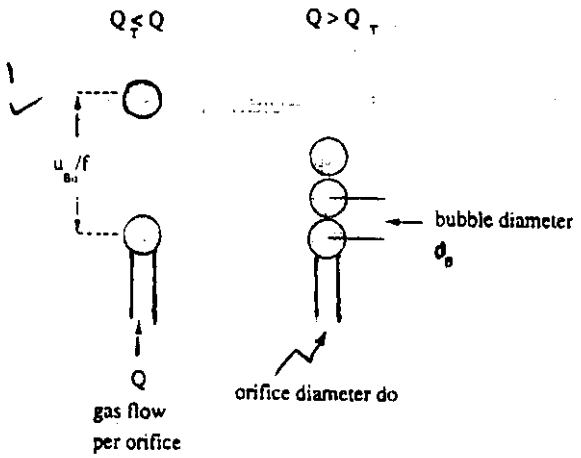



**Figure: Formation of gas bubbles at an orifice, illustrating the transition gas flow rate ($Q_T$) from the constant volume variable frequency regime to the constant frequency variable volume regime.**

**29. What is Bubble coalescence?**

Bubble breakup is caused by the dynamic pressure forces exerted on the bubble by the turbulent liquid flow. These forces are opposed by the surface tension force and the resistance of the liquid phase to deformation.

**30. What is weber number?**

Weber number is a constant at equilibrium. It is the ratio between the shearstress and the length scale of turbulent eddy to the surface tension of the bubble.

$$W_e = \frac{\tau d_B}{\sigma}$$

We = webernumber
$\tau$ = shear stress at the bubble surface
$d_B$ = length scale of turbulent eddy

$\sigma$ = Surfacetension of the bubble

**31. How will you calculate the transition orifice gas flow rates?**

The transition orifice gas flow rates can be calculate using the formula,

$$Q_T = 0.38 g^{1/2} \left( \frac{6\sigma d_0}{g(\ell_L - \ell_G)} \right)^{5/6} \text{ for } R_{eB} >> 1$$

where, $Q_T$ = orifice gas flow rate
g = acceleration due to grarity
$\sigma$ = surface tension
d = diameter of the bubble
$\ell_L$ = Density of the liquid
$\ell_G$ = Density of the gas
$R_{eB}$ = Bubble Reynolds number

## PART – B

**1. What is a bubble column reactor? Explain Bubble generation at an orifice with a neat diagram.**

**Bubble Columns**

Bubble columns are widely used for production of antibiotics, bakers yeast and single cell protein. The height to diameter ratio of may vary, but values from 3 to up to 6 to 1 are common. The gas phase rises in plug flow through the liquid, which may not be well-mixed in large tanks.

Gas is introduced into the liquid through a sparger, which may be either a concentric ring or a tree-type device. The objective is to uniformly distribute the gas over the tank cross-sectional area. The size of the gas bubbles so formed and their subsequent coalescence and breakup determine the available interfacial area in the bubble column.

**Bubble Generation at an Orifice**

The introduction of gas into a liquid through a sparger is illustrated in Figure. The important dimensions are indicated on the figure. There are three distinct regimes of bubble formation, based on the gas flow rate through the orifice. At low gas flow rates, bubble of constant volume are formed. The bubble size depends on the orifice diameter, surface tension and buoyancy. The bubble grows till the buoyancy force exceeds the surface tension force holding the bubble to the orifice. Inertial forces can be neglected.

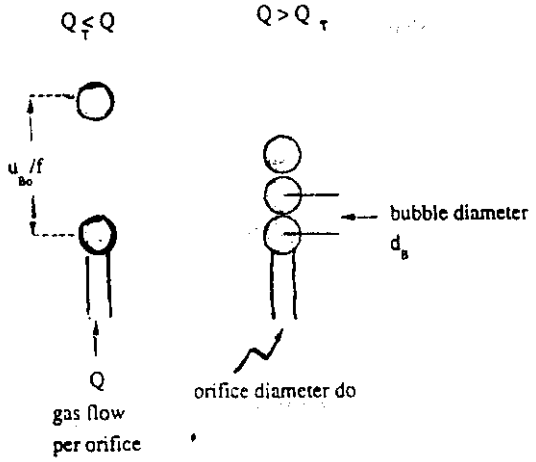



**Figure: Formation of gas bubbles at an orifice, illustrating the transition gas flow rate ($Q_T$) from the constant volume variable frequency regime to the constant frequency variable volume regime.**

A balance of buoyancy and surface tension forces yields:

$$g(\rho_L - \rho_G)\frac{\pi}{6}d_{Bo}^3 = \sigma(\pi d_0)$$

$$d_{g0} = \frac{6\sigma d_0}{g(\rho_L - \rho_G)} \qquad \rightarrow (1)$$

This equation will be valid a the orifice gas flow increases to a transition value $Q_T$. As bubbles of a constant volume rise at a constant rate. Increasing gas flow will result in generation of bubbles at an increasing frequency. This low gas flow regime can thus also be considered as a constant volume, variable frequency regime. The distance between bubbles will be inversely related to the frequency of formation. At $Q_T$, bubbles will touch, as illustrated in Figure, and the gas can no longer be transported at in a variable frequency, constant volume manner. The transition gas flow rate is given by:

$$Q_T = \frac{\pi}{6}d_{B0}^3.f_r \qquad \rightarrow (2)$$

where

$$f_T = \frac{u_{Bn}}{d_{Bn}} \qquad \rightarrow (3)$$

The rise velocity $u_{Bo}$ of the bubble with a diameter $d_{Bo}$ is give by stokes equation

$$u_{Bo} = \left(\frac{g\rho_L}{18\mu_L}\right) d_{Bo}^2 \quad \text{for } Re_B < 1 \qquad (4)$$

or by the Mendleson relationship

$$u_{Bo} = \left(\frac{2\sigma}{\rho_L d_{Bo}} + \frac{g d_{Bo}}{2}\right)^{0.5} \quad \text{for} \quad Re_B >> 1 \quad (5)$$

The bubble Reynolds number is defined with the liquid rather than the gas properties

$$Re_B = \frac{d_B u_B \rho_L}{\mu_L} \qquad \rightarrow (6)$$

The transition orifice gas flow rates are thus

$$Q_T = \frac{\pi g(\rho_L - \rho_o)}{108\mu_L}\left(\frac{6\sigma d_o}{g(\rho_L - \rho_o)}\right)^{4/3} \quad \text{for} \quad Re_B < 1 \quad \rightarrow (7a)$$

$$Q_T = 0.38 g^{1/2}\left(\frac{6\sigma d_o}{g(\rho_L - \rho_G)}\right)^{5/6} \quad \text{for} \quad Re_B >> 1 \qquad (7b)$$

Above this orifice transition gas flow rate $Q_T$, the bubble size increases but bubbles are formed at a constant frequency. The bubble size in this regime has been correlated with the liquid properties and the gas flow rate[17].

$$\frac{d_B}{d_o} = 3.23 Re_{oL}^{-0.1} Fr_o^{0.21} \qquad \rightarrow (8)$$

where $Re_{oL} = \dfrac{d_o\left(\dfrac{Q}{\pi d_o^{2/4}}\right)\rho_L}{\mu_L} = \dfrac{4\rho_L Q}{\pi d_o \mu_L}$ and $Fr_o = \dfrac{Q^2}{d_o^5 g}$

The modified orifice Reynolds number ($Re_{oL}$) describes the gas flow through the orifice with respect to the liquid properties and the Froude number ($Fr_o$) relates the inertial to gravity forces for flow through the orifice[18].

Equation indicates that the bubble size depends on the gas flow rate to the 0.32 power, so that the frequency of bubble formation, given by Equation below, is indeed constant in this regime.

$$f = \frac{Q}{\pi d_{Bo}^3 / 6} - \frac{6}{\pi}\frac{Q}{\pi\left(Q^{0.32}\right)^3} - \frac{6}{\pi} \qquad \rightarrow (9)$$

In this regime we also see from the Reynolds number dependence in Equation that the bubble size depends weakly on the liquid viscosity to the tenth power however, as viscosity may rise by several orders of magnitude in polysaccharide fermentations, this dependence is significant.

Beyond these gas flow rates, an apparent gas jet forms at the orifice and this jet grows in size with entrainment of surrounding liquid. The bubble diameter that results is weakly dependent on the gas flow rate and is given by the following expression:

$$d_{Bo} = 0.71 Re_o^{-0.05} \ (\text{in cm}) \text{ where } Re_o = \frac{4Q\rho_8}{\pi d_o \mu_g} \quad \rightarrow (10)$$

The Reynolds number $Re_o$ is base don gas flow within the orifice. The above equation applies for $Re_o$>10,000.

As bubbles rise form the orifice, they are subject to breakup or coalescence events which may alter the bubble size. The extent to which this occurs depends on the size of the vessel. In a region close to the orifice, which can be conveniently defined as having a height equal to the vessel diameter, the bubble size is given by the size generated at the orifice. Above this region, coalescence and breakup, caused by bulk liquid motion, may determine the equilibrium bubble size. For small vessels, the entire vessel may be considered to be in region I. This is illustrated in Figure.

To calculate the overall mass transfer coefficient in a bubble column, the equilibrium bubble size needs to be determined. We shall examine the factors leading to bubble breakup and coalescence as these determine the resulting equilibrium bubble size and hence the interfacial area in the vessel.

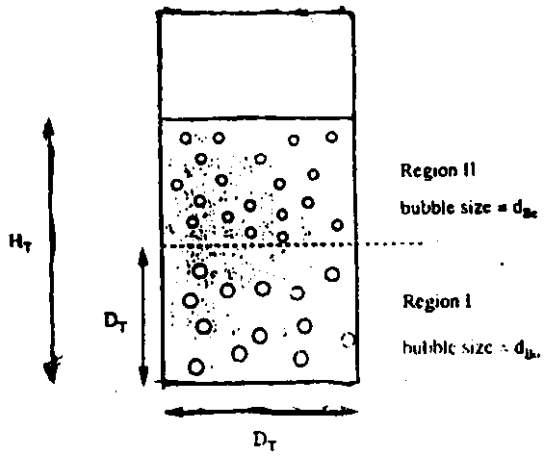



**Figure: Region I and II in a bubble column. In region I the bubble size is determined by the size generated at the orifice; in region II bulk liquid motions result in bubble coalescence and breakup, resulting in an equilibrium size $d_{Be}$.**

**2. Explain stability analysis in the transient behaviour of the bioreactors.**

**Stability Analysis**

To date we have examined the steady state solutions for various constitutive models describing the growth of the cells in the reactor. Here we shall examine whether such steady states can be attained in practice, by examining whether these steady states are stable to perturbations in the system parameters. The method used is a linearlized stability analysis, based on the method of Lyapunov[5]. The stability of the non-linear system is examined by considering the behavior of the eigenvalues of the linearized system around the steady state point. The general approach is as follows.

Consider the set of unsteady state mass balances around a stirred tank reactor. These may be written in vector notation as

$$\frac{dC}{dt} = f(C,p) \qquad \rightarrow (1)$$

where C is the vector of concentrations and p is a vector of parameters, including the kinetic parameters. Feed concentrations, etc. The state solutions may be found from

$$f(C,p) = \qquad \rightarrow (2)$$

If the vector x is introduced as representing the deviations from the steady state:

$$\underline{x}(t) = \underline{C}(t) - \underline{C} \qquad \rightarrow (3)$$

the mass balance may be rewritten in terms of deviations from the steady state

$$\frac{d\underline{x}}{dt} = \underline{f}\left(\underline{C},,+\underline{x},\underline{p}\right) \qquad \rightarrow (4)$$

We may now expand if $\left(\underline{C},,+\underline{x},\underline{p}\right)$ in a Taylor series around $\underline{C},$ Noting that $\underline{f}\left(\underline{C},,\underline{p}\right)$ is zero at steady state, we obtain

$$\frac{dx}{dt} = \underline{f}\left(\underline{C},,\underline{p}\right) + \underline{\underline{A}}\underline{x} + (\text{higher order terms}) \cong \underline{\underline{A}}\underline{x} \qquad \rightarrow (5)$$

where the elements of the matrix $\underline{\underline{A}}$ are evaluated at each steady state viz.

$$a_{i.j} = \frac{\partial f_i\left(\underline{C},,\underline{p}\right)}{\partial C_j} \qquad \rightarrow (6)$$

The solution of the above equation have the form

$$\underline{x}(t) = \sum_{i=1}^{n} \alpha_i \underline{\beta}\, e^{\lambda i} \qquad \rightarrow (7)$$

where n is the number of species for which mass balances have been written, and $\underline{\beta}$ and $\lambda_I$ are corresponding pairs of eigenvectors and eigenvalues of the matrix $\underline{\underline{A}}$. The eigenvalues are found from the n roots of

$$\det\left(\underline{\underline{A}} - \lambda\underline{\underline{\lambda}}\right) = 0 \qquad \rightarrow (8)$$

where $\underline{\underline{I}}$ is the n x identify matrix. The eigenvectors satisfy

$$\left(\underline{\underline{A}} - \lambda_i \underline{\underline{I}}\right)\underline{\beta_i} = 0 \qquad \rightarrow (9)$$

and the values of $\alpha_I$ are chosen to satisfy the initial conditions $\underline{x}(0)$

$$\sum_{i=1}^{n} \alpha_i \underline{\beta} = \underline{x}(0) \qquad \rightarrow (10)$$

The eigenvalues can be seen to have, in general, both real and imaginary parts of the form $\lambda_i = a_i \pm b_i i$. If all the eigenvalues have negative real parts and no imaginary parts ($b_i$=0), the steady state at which the eigenvalues are calculated is locally stable. This is seen from equation (7), where the vector $\underline{x}(t)$ decays to zero at sufficiently long times if the $\lambda_I$ values are all negative.

Conversely, if even one of the eigenvalues is positive, then the steady state is unstable, as the deviation from the steady state value (represented by $\underline{x}(t)$) will grow in time. If $\lambda_I$ has only imaginary parts and zero real parts, the stability is determined by the higher order terms that we have neglected in this analysis.

The behavior of the system around a steady state is given below. It should be noted that the system of equations has been linearized and that a locally unsteady state solution may not be globally unstable. Our expansion is only valid in the neighborhood of the steady state.

| Eigenvalues | | | |
|---|---|---|---|
| **Real Part** | **Imaginary Part** | **Behavior** | **Type** |
| Negative | =0 | Exponential decline to zero | Stable node |
| Positive | =0 | Exponential growth | Unstable node |
| Negative | <> 0 | Damped oscillations | Stable focus |
| Positive | <>0 | Undamped oscillations | Unstable focus |
| Zero | <>0 | Sustained oscillatins | Vortex point |

The behavior of one of the components of $\underline{x}$ for each of these five types of steady state is shown in figure.

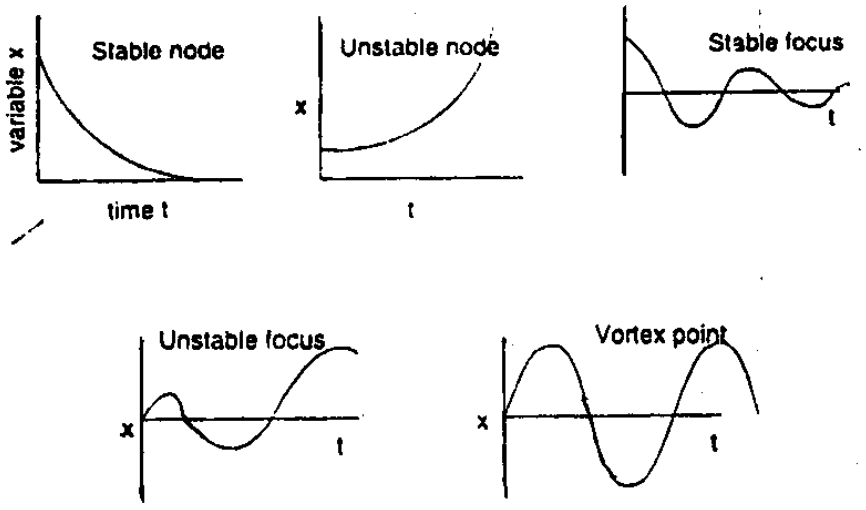



**Figure: The time course of deviations from the steady state values, illustrating the possible types of behavior.**

We need not compute all the eigenvales from Equation (8) to determine if the real parts are positive or negative. If the nth order equation for $\lambda$ is written in the following form

$$\lambda^n + B_1\lambda^{n-1} + \ldots + B_{n-1} + \lambda + B_n = 0 \qquad \rightarrow (11)$$

the Hurwitz criterion states that all the roots of the above equation for $\lambda$ will have negative real parts, fi and only if the following conditions are met:

$$B_i > 0$$

$$\det\begin{pmatrix} B_1 & B_3 \\ 1 & B_2 \end{pmatrix} > 0$$

$$\det\begin{pmatrix} B_1 & B_3 & B_5 \\ 1 & B_2 & B_4 \\ 0 & B_1 & B_3 \end{pmatrix} > 0 \qquad \rightarrow (12)$$

This test is considerably easier to apply than calculation of the eigenvalues for systems with three or more components. We shall examine the simplest case of the chemo stat to illustrate the technique.

**3. Explain stability of the chemo stat in a bioreactor.**

**Stability of the Chemo stat**

The governing mass balance for cells and substrate are given by

$$\frac{dX}{dt} = -DX + \mu(S)X \qquad \frac{dS}{dt} = D(S_o - S) - \frac{\mu(S)}{Y_{X/S}} \qquad \rightarrow (1)$$

The signs of the eigenvalues $\lambda_1$ and $\lambda_2$ can be found as follows. The matrix $\left(\underline{\underline{A}} - \underline{\underline{\lambda I}}\right)$ has elements

$$\begin{bmatrix} a_{11} & \lambda & a_{12} & \\ a_{12} & & a_{22} & -\lambda \end{bmatrix}$$

Solving $\det\left(\underline{A} - \underline{\lambda I}\right) = 0$ we obtain

$$\lambda^2 - (a_{11} + a_{22})\lambda + (a_{11}a_{22} - a_{12}a_{21}) = 0 \qquad \rightarrow (2)$$

For both eigenvalues to have negative real parts

$$(a_{11} + a_{22}) < 0 \quad \text{and} \quad (a_{11}a_{22} - a_{12}a_{21}) > 0 \qquad \rightarrow (3)$$

From the mass balances, the matrix $\underline{\underline{A}}$ has components

$$a_{11} = \frac{\partial}{\partial X}\left(\frac{dS}{dt}\right)_{ss} = \frac{\mu(S_{ss})}{Y_{X/S}} \qquad a_{22} = \frac{\partial}{\partial S}\left(\frac{\partial S}{dt}\right)_{ss} = -\left[\frac{X_{ss}}{Y_{X/S}}\left(\frac{d\mu}{dS}\right)_{ss} + D\right] \qquad \rightarrow (4)$$

which satisfies the criterion given by Equation (3). We can now introduce the various constitutive models for the specific growth rate. For the Monod model

$$\left(\frac{d\mu}{dS}\right)_{ss} = \frac{\mu_{max} K_S}{(K_S + S_{ss})} > 0 \quad \text{for all } S_{ss} \qquad \rightarrow (5)$$

The steady states are:

| Dilution rate | $S_o > \frac{DK_S}{\mu_{max} - D}$ | $S_o < \frac{DK_S}{\mu_{max} - D}$ |
|---|---|---|
| $D < \mu_{max}$ | $S_{SS} = \frac{DK_S}{\mu_{max} - D}$<br>$X_{SS} = Y_{X/S}(S_o - S_{ss})$ | $S_{ss} = S_{ss}$<br>$X_{ss} = 0$ |
| $D > \mu_{max}$ | $S_{ss} = S_o$<br>$X_{ss} = 0$ | $S_{ss} = S_0$<br>$X_{ss} = 0$ |

Generally, $S_o > \frac{DK_S}{\mu_{max} - D}$ and the matrix $\underline{\underline{A}}$ has elements

for $D < \mu_{max}$ for $D > \mu_{max}$.

$$\begin{bmatrix} 0 & X_{SS}\left(\frac{d\mu}{dS}\right) \\ -\frac{D}{Y_{X/S}} & -\left(\frac{X_{ss}}{Y_{X/S}}\left(\frac{d\mu}{dS}\right)_{ss} + D\right) \end{bmatrix} \qquad \begin{bmatrix} -D + \frac{\mu_{max} S_0}{(K_S + S_0)} & 0 \\ -\frac{\mu_{max} S_0}{(K_s + S_0)} & -D \end{bmatrix}$$

We see that in both cases the criteria for negative real parts of the eigenvalues are satisfied. We also note that both the eignevalues only contain real parts; there are no complex parts. When complex parts exist and the real parts are negative, the solution for $\underline{x}(t)$ Equation (7) can be rewritten in terms of pre-exponential sine and cosine terms, indicating the presence of damped oscillations around the steady state. In the cases where $S_0 < DK_S/(\mu_{max} - D)$ the real parts of the eigenvaleus are positive and both steady states are unstable.

The eigenvaleus yield further information about the characteristic times of the chemostat. For the non-washout steady state, we have

$$\lambda_1 = -D \qquad \lambda_2 = -\frac{(\mu_{max} - D)\left[S_0(\mu_{max} - D) - DK_s\right]}{\mu_{max} K_s} \qquad \rightarrow (6)$$

The characteristic times are given by

$$t_i = |\lambda_i|^{-1} \qquad i=1,...n \qquad \rightarrow (7)$$

Thus the response time of the chemostat to perturbation will depend on both $t_1(D^{-1})$ and $t_2$, which is function of D and the set of microbial ($K_S$ and $\mu_{max}$) and environmental ($S_o$) parameters.

**4. Explain operating diagrams in the transient behaviour of bioreactors.**

**Operating Diagrams**

In operating the chemostat, the only parameters that can be manipulated by the investigator are the inlet substrate concentration and the dilution rate. It is useful therefore to construct diagrams in ($S_o$, D) space which show the boundaries between the different steady states. For a given ($S_0$,D) we can see which steady state the system will reach. These diagrams are called operating diagrams. The boundaries between regions corresponding to each steady state can be found by using the stability criteria we have developed. When the real part of one of the eigenvalues is positive, the system is unstable. If, by changing $S_0$ or D, the real parts of the eigenvalues can be made negative, the system will be stable. Thus the stable/unstable boundary must occur at ($S_0$,D) values which make the real parts of the eigenvaleus zero. For a limited number of models of growth kinetics, we can determine these boundaries analytically. In more complex cases we must resort to numerical methods.

We shall consider substrate inhibition kinetics to illustrate the method. Consider the Jacobian matrix $\underline{\underline{A}}$ (Equation 4.96) resulting from the linearization. The eigenvalues are found from

$$\lambda^2 - (a_{11} + a_{22})\lambda + (a_{11} + a_{22} - a_{12}a_{21}) = 0 \qquad \rightarrow (1)$$

The eigenvalues will have no real parts if ($a_{11}$ + $a_{22}$) = 0 and ($a_{11}a_{22}$-$a_{12}a_{21}$) < 0. Inserting the appropriate values of $a_{jj}$ (4.113) for substrate inhibition, we have

$$\frac{X_{ss}}{Y_{X/S}}\left(\frac{d\mu}{dS}\right)_{ss} + D = 0 \quad \text{and} \quad \frac{DX_{ss}}{Y_{X/S}}\left(\frac{d\mu}{dS}\right)_{ss} < 0 \qquad \rightarrow (2)$$

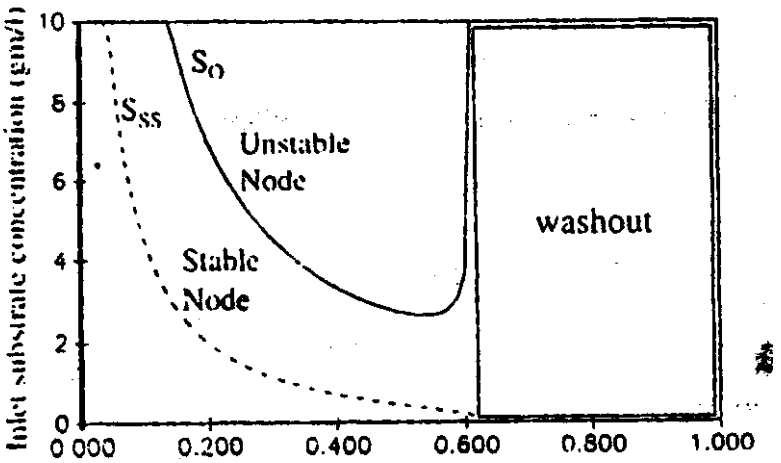



**Figure: The operating region for substrate inhibited growth. Parameter values are $\mu_{max}$ 1.0 $hr^{-1}$, $K_s$ = 0.05 gm/l, $K_i$ = 0.5 gm/l. At dilution rates greater than 0.6125 $hr^{-1}$ the culture washes out, as D > μ. The unstable node region is indicated. The dashed lien represents the unstable steady state values of the substrate concentration ($S_{ss}$), showing it is less than the inlet substrate concentration ($S_o$).**

The relationship between $S_o$ and D can be found by substituting for $S_{ss}$ as a function of D and the microbial kinetic parameters $(\mu_{max}, K_s \text{ and } K_1)$ from Equation (4.117). Values of $S_o$ which are greater than the steady state values ($S_{ss}$) can only be found when the unsteady state solution is substituted into Equation (4.119). In this case, $S_{ss}^2 > K_s K_1$. This defines the extent of the unstable steady state region, indicated by the solid line on Figure. The operating diagram is shown in Figure.

**5. Explain chemostals in series with a neat diagram.**

**Chemiostats in Series**

In batch cultures, the individual phases of growth result from the changing physiology of the cell; each phase is dependent on the one preceding it. On the other hand, in continuous culture, the physiological state is no longer determined by the one preceding it; rather it is controlled by the dilution rate. Thus the culture has no "history." In situations where the culture must pass through some physiological state in order to produce the desired product, a combination of reactor types will provide the optimal reactor configuration, defined as the smallest reactor volume for a specified degree of product conversion. In situations where the optimal rate of product formation may occur at a different temperature or pH from that of growth, two or more stirred tank reactors operated in series permit growth and product formation conditions to be optimized in each reactor. When mixed substrates are employed (such as two saccharides or a substrate such as lactose, which is hydrolyzed into two saccarides), the

preferential substrate is consumed first (diauxic effect). The use of two CSTRs in series enables the preferred substrate to be completely consumed in the first reactor and the second substrate can then be consumed in the second reactor, minimizing the required reactor volume. We shall examine the steady state behavior of chemostats in series with multiple feed streams.

Let us examine a two stage system, with a second feed stream into the second stage. The configuration is shown in Figure.

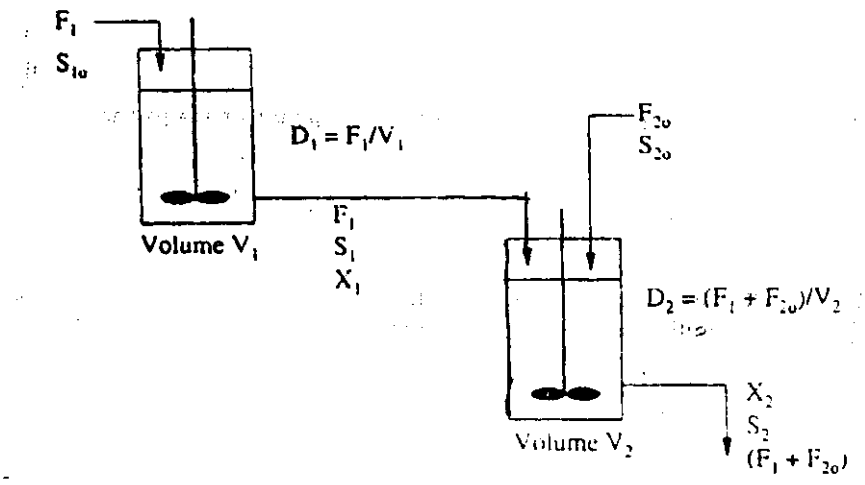



**Figure: Two chemostats in series, with a separate feed stream entering the second reactor. The steady state ccell and substrate concentrations are indicated.**

We can write steady-state mass balances for cells and substrate for each stage, noting that the specific growth rates in each stage will be different due to the different substrate concentrations and environmental conditions (such as pH) in each vessel. For simplicity we will assume that the values of $\mu_{max}$ and Ks are the same in each stage.

For the first stage:

$$F_1 X_1 = \mu_1 X_1 V_1 \qquad \rightarrow (1a)$$

$$F_1 (S_{10} - S_1) = \frac{1}{Y_{X/S}} \mu_1 X_1 V_1 \qquad \rightarrow (1b)$$

For the second stage:

$$(F_1 + F_{20}) X_2 - F_1 X_1 = \mu_2 X_2 V_2 \qquad \rightarrow (2a)$$

$$F_1 S_1 + F_{20} S_{20} - (F_1 + F_{20}) S_2 = \frac{1}{Y_{X/S}} \mu_2 X_2 V_2 \qquad \rightarrow (2b)$$

Denoting $D_1 = F_1/V_1$ and $D_2 = (F_1+F_{20})/V_2$, equation (1a) can be rearranged to show that

$$\mu_2 = D_2 - D_1 \frac{X_1}{X_2}.\frac{V_1}{V_2} \qquad \rightarrow (3)$$

i.e., the growth rate in the second stage is always less than the overall dilution rate ($D_2$) in the second stage. Combining Eqs. (2a) and (2b) and solving for $X_2$ gives

$$X_2 = Y_{X/S}\left(\frac{F_1}{V_2 D_2}S_{10} + \frac{F_{20}}{V_2 D_2}S_{20} - S_2\right) \qquad \rightarrow (4)$$

If we employ the Monod equation to describe the specific growth rate in the second stage, we see

$$\mu_2 = \frac{\mu_{max} S_2}{K_S + S_2} \qquad \rightarrow (5)$$

then the steady state substrate concentration $S_2$ is found by inserting the expression for $X_2$ into (2b) and solving. Only the positive root has significance.

$$(\mu_{max} - D_2)S_2^2 - \left\{\frac{\mu_{max}F_1S_{10}}{V_2D_2} + \frac{\mu_{max}F_{20}S_{20}}{V_2D_2} - \frac{F_1S_1}{V_2} - \frac{F_{20}S_{20}}{V_2} + K_sD_2\right\}S_2$$
$$+\left(\frac{K_SF_1S_1}{V_2} + \frac{F_{20}S_{20}K_S}{V_2}\right) = 0 \qquad \rightarrow (6)$$

Figure. Shows the effect of varying the dilution rate $D_2$ in the second vessel on the behavior of the steady state values of $X_{2ss}$ and $S_{2ss}$ ($F_1$, the volumetric feed rate to the first vessel, is constant).

If there is no separate flow into the second stage, the steady state solutions can be found from

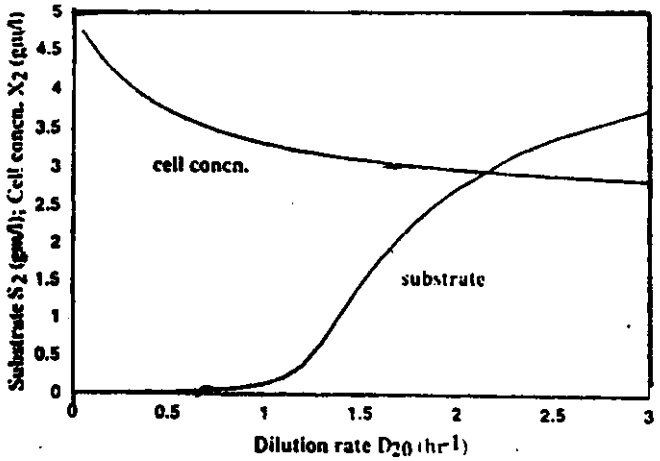



**Figure: Steady state values of substrate and cell concentrations in the second stage of two chemostats in series. The dilution rate $D_{20}$ in the second stage is varied while the feed rate into**

**the first stage is maintained constant at a dilution rate of $D_1$=0.5 $hr^{-1}$. The other parameter values are $\mu_{max}$ = 1.0 $hr^{-1}$. $K_s$ = 0.05 gm/l, $S_{10}$ = 10.0 gm/l, $S_{20}$ = 5.0 gm/l. $Y_{x/s}$ = 0.5 gm/gm. The steady-state values are obtainied from equations (4) and (6).**

$$(\mu_{max} - D)S_{2SS}^2 - \{\mu_{max}S_o + D_2(K_s - S_{1.ss})\}S_{2.ss} + K_S D_2 S_{1.ss} = 0 \qquad (7a)$$

$$X_{2.ss} = Y_{X/S}\{S_0 - S_{2.ss}\} \qquad \rightarrow (7b)$$

$$\mu_2 = \frac{D\{X_{2.ss} - X_{1.ss}\}}{X_{2.ss}} \qquad \rightarrow (7c)$$

Generally $X_{1.ss}$ and $X_{2.ss}$ are not significantly different and the specific growth rate in the second stage will be approximately zero.

**6. What is a chemostat? Explain with a neat diagram.**

**The Chemostat: the ideal CSTR**

The use of a continuous stirred tank reactor to extend the duration of culture of microbes was developed in the 1950s by Novick and Szilard[1] and Monod[2]. The realization that a CSTR could be used to maintain microbial growth at a steady state value, which could be varied from any growth rate up to the maximum $\mu_{max}$, was an important advance, as it broke the traditional thinking at the time that stable microbial growth was only possible at the maximum rate, corresponding to the minimum doubling time found in batch cultures. Subsequently, the use of a well-mixed continuous microbial reactor to study microbial physiology led to important advanced in understanding the cell cycle, metabolic regulation and microbial product formation.

The configuration of a typical well-mixed continuous reactor is shown in Figure. Agitation may be provided by an impeller or by the motion imparted to the liquid phase by rising gas bubbles. In aerobic systems, supply of oxygen to the organism generally occurs via air sparging. In the ideal case, the liquid phase is completely mixed, i.e., the liquid phase composition is uniform throughout the vessel. Similarly, temperature is maintained constant and uniform by circulation of cooling water through coils in the vessel or in a jacket surrounding the vessel. Typically the pH of the culture medium is controlled by the addition of acid or base.

We may write material balance equation for each of the important variables in the CSTR. We shall first consider the case where only one substrate (S) limits the growth rate of the organism, and that the volumetric rate of growth is given by $\mu X$. The balance equations are:

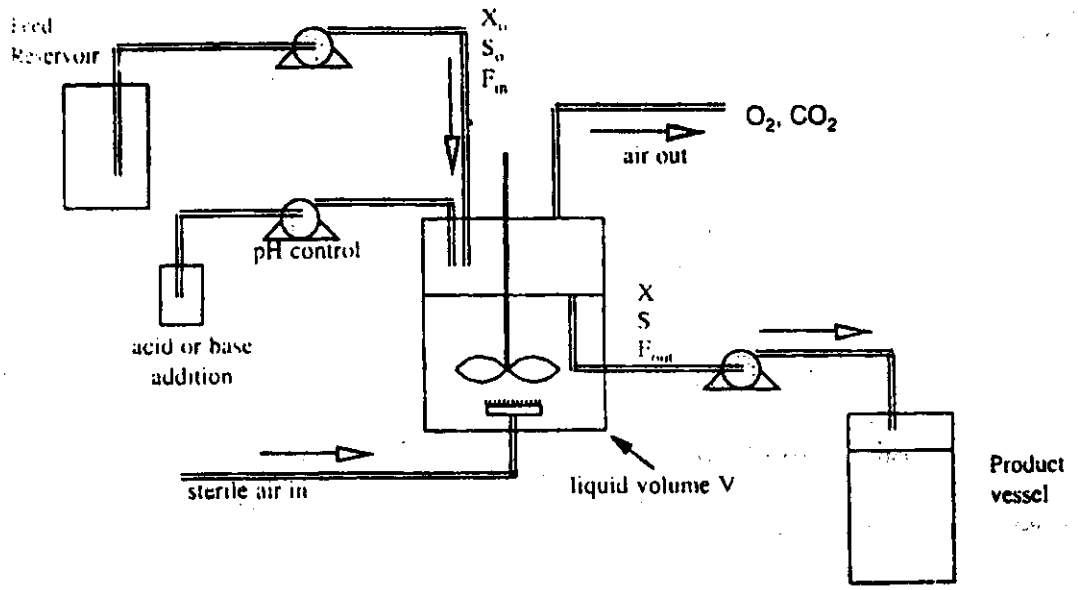



**Figure: A schematic of a continuous stirred tank bioreactor. Typically the pH and temperature are controlled, as are the flow rates of nutrients into the vessel. The notation used to model CSTR system is indicated; X is the dry cell weight, S is the substrate concentration and F is the flow rate of nutrients into the vessel.**

$$\frac{dXV}{dt} = F_{in}X_o - F_{out}X + \mu XV \qquad \rightarrow (1a)$$

$$\frac{dSV}{dt} = F_{in}S_o - F_{out}S - \frac{1}{Y_{X/S}}\mu XV \qquad \rightarrow (1b)$$

$$\frac{dV}{dt} = F_{in} - F_{out} \qquad \rightarrow (1c)$$

When the volumetric feed rates, $F_{in}$, $F_{out}$, into and from the vessel are maintained constant and equal (F), the equations simplify to (note that dX/dt no longer equals μX, as it does during batch growth)

$$\frac{dX}{dt} = \frac{F}{V}(X_o - X) + \mu X \qquad \rightarrow (2a)$$

$$\frac{dS}{dt} = \frac{F}{V}(S_o - S) - \frac{1}{Y_{X/S}}\mu X \qquad \rightarrow (2b)$$

The ratio F/V is generally referred to as the dilution rate, denoted as D, with units of reciprocal time. It is the inverse of the average residence time τ. It equals the number of reactor volumes that pass through the reactor per unit time. At steady state, the time derivatives are set to zero and the equation for cell concentration has the solution

$$DX_o = (D-\mu)\,X \qquad \rightarrow (3)$$

When the feed stream is sterile (generally the case), $X_0$ is zero and two solutions to the above equation are possible:

$$X_{ss} = 0 \qquad \text{or} \qquad \mu = D \qquad \rightarrow (4)$$

In the unusual case that the specific growth rate of the culture ($\mu(S)$) is independent of substrate concentration and is constant, the steady-state concentration of cells that results when the dilution rate is set equal to $\mu$ is indeterminate. Solution of the second mass bath $X_{ss}$ and $S_{ss}$ must satisfy

$$S_{ss} = S_o - \frac{1}{Y_{X/S}} X_{ss} \qquad \rightarrow (5)$$

Thus a range of values of cell and substrate concentrations is possible. Experimentally, this can be occasionally seen at very low inlet substrate concentrations. Time-varying cell mass and substrate concentrations are observed.

Generally however, the specific growth rate is a function of substrate concentration. When the Monod relationship between $\mu$ and S is employed, the mass balance equations are no longer indeterminate and we find

$$D = u = \frac{\mu_{max} S}{K_s + S} \qquad \rightarrow (6)$$

which can be solved for S:

$$S_{ss} = \frac{DK_s}{\mu_{max} - D} \text{ provide } \quad X_{ss} \neq 0 \qquad \rightarrow (7)$$

and noting $D = \mu$, we obtain

$$X_s = Y_{X/S}\left(S_o - \frac{DK_s}{\mu_{max} - D}\right) \qquad \rightarrow (8)$$

The second steady state solution occurs when $X_{ss} = 0$. The corresponding value of the substrate concentration is $S_{ss}=S_o$. This steady state is referred to as washout, as cells are no longer present in the reactor. The dilution rate at which washout occurs can be found by examining equation (6). When $S_{ss}$ equals the feed concentration $S_o$, the corresponding dilution rate is

$$D_{max} = \frac{\mu_{max} S_o}{K_{S+S_o}} \qquad \rightarrow (9)$$

The maximum dilution rate is thus slightly smaller than the maximum specific growth rate. If the dilution rate is greater than this value, the system moves to the second steady state solution $X_{ss} = 0$. This can be seen from the behavior of $S_s$ as $D \rightarrow \mu_{max}$, $S_{ss}$ becomes indeterminate.

If $S_o < S_{ss}$, then the specific growth rate $\mu$ is constant, the equations are indeterminate, and a variety of steady states could be observed.

The cell concentration is approximately $Y_{x/s}S_o$ for dilution rates up to values approaching $\mu_{max}$. The behavior of the steady state solutions $X_{ss}$ and $S_{ss}$ as a function of the dilution rate is shown in Figure. The operation of a CSTR under conditions where only one substrate is growth-limiting gives rise to an almost constant value of the substrate concentration over a wide range of dilution rates. Other substrates which are consumed at rates proportional to the specific growth rate of the cells will also have steady state concentrations that are independent of D and are constant. For this reason, this type of bioreactor operation is referred to as chemostant operation (i.e., the chemical environment is static).

As the dilution rate approaches $\mu_{max}$, the cell concentration decreases very rapidly. Operating the chemostat at dilution rates close to $\mu_{max}$ is experimentally difficult due to this sensitivity. Small variations in inlet substrate concentration, feed flow rate or small variations in the growth rate of the cells.

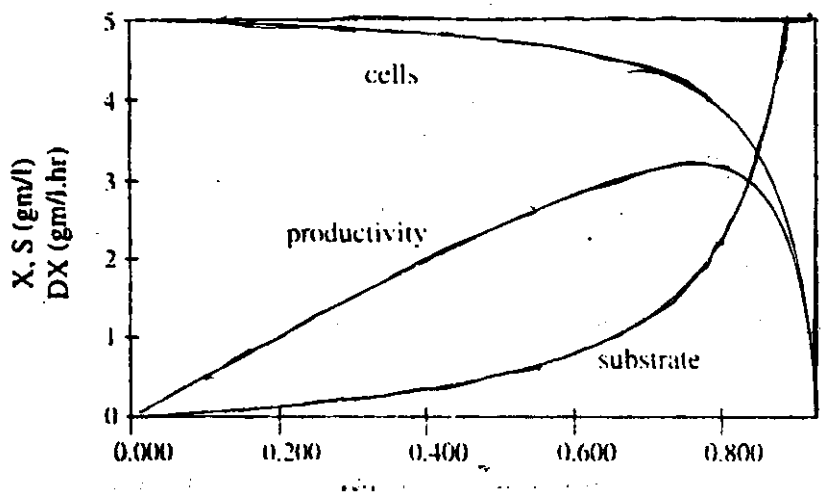



**Figure: The dependence of the steady state cell and substrate concentrations on the dilution rate D. the values of the constants in the Mono model are $\mu_{max}$= 1.0 $hr^{-1}$, $K_s$ = 0.5 gm/l, $Y_{x/s}$ = 0.5 gm cells/gm substrate with $S_o$ = 10 gm/l. Also shown is the cell productivity. $DX_{ss}$, in gm/liter-hr.**

$$\frac{dDX_{ss}}{dD} = 0 \quad \text{thus} \quad D_{max} = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + S_o}}\right) \qquad \rightarrow (10)$$

The corresponding cell concentration is

$$X_{ss,max} = Y_{x/s}\left(S_o + K_S - \sqrt{K_s\left(S_o + K_s\right)}\right) \qquad \rightarrow (11)$$

In contrast to the behavior of steady state bacterial and yeast cultures, continuous suspension cultures of animal cells show viable and total cell concentrations that decrease with increasing dilution rates, although the percentage of the cells that are viable may increase with increasing dilution rate. Hybridoma cells, which are fusion products of myeloma and spleen cells used for the production of monoclonal antibodies, become slightly larger at higher dilution rates, which may explain some of these trends. The behavior of these cells in continuous suspension cultures.

The decrease in cell viability at low dilution rates illustrates a high maintenance requirement. At low viabilities, the specific growth rate is no longer equal to the dilution rate, and we distinguish between viable and non-viable cells. The mass balance equations thus become

$$\frac{dn_v}{dt} = \mu n_v - k_d n_v - D n_v \qquad \rightarrow (12)$$

$$\frac{dn_\tau}{dt} = \mu n_v - D n_T \qquad \rightarrow (13)$$

where $n_v$ and $n_T$ are the number concentrations of viable and total cells. The viability ($n_v/n_T$) at steady state thus depends on the death rate $k_d$ and the specific growth rate $\mu$, and thus varies in an indirect manner with D.

$$\left(\frac{n_v}{n_T}\right) = \frac{\mu - k_d}{\mu} \qquad \rightarrow (14)$$

At large dilution rates, the viability is high and the specific growth rate approaches the dilution rate. The residual glucose (substrate) concentration increases with increasing dilution rate more gradually than would be predicted by the Monod model, consistent with the gradual decrease in cell concentration with increasing dilution rate. At low dilution rates, the lactate production from glucose is reduced, as more glucose is required for maintenance and production of nucleotide sugars. The relationships between specific metabolite production or consumption rates are typically not linear, as is usually the case with bacterial systems. The more complex carbon and energy metabolism of mammalian cells, particularly the requirements for two substrates, glucose and glutamine, mean that the Monod assumption that one limiting nutrient regulates the specific growth rate no longer applies.

**7. Explain Airlift reactor with a neat diagram.**

**Airlift Reactor**

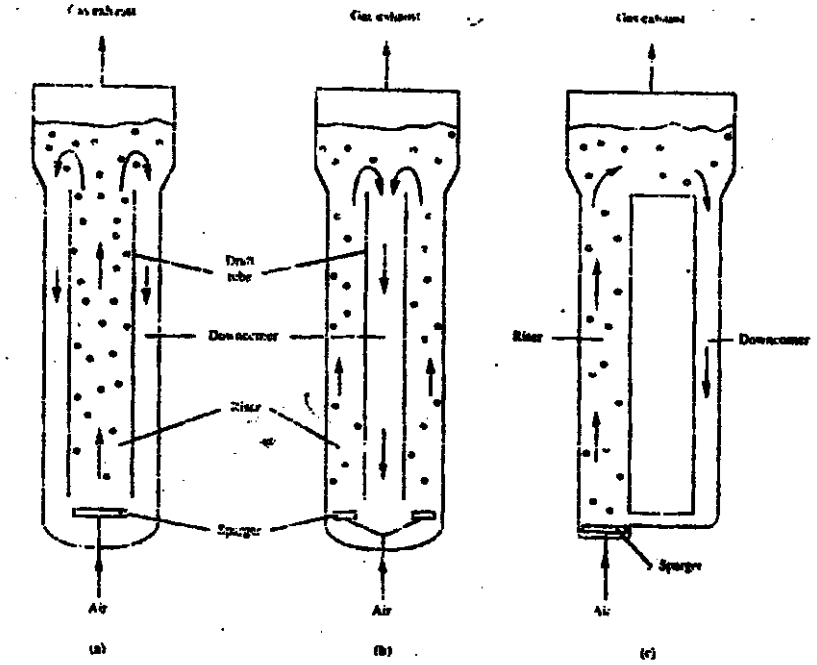
As in bubble columns, mixing in airlift reactors is accomplished without mechanical agitation. Airlift reactors are often chosen for culture of plant and animal cells and immobilized catalysts because shear levels are significantly lower than in stirred vessels.

Several types of airlift reactor are in use. Their distinguishing feature compared with the bubble column is that patterns of liquid flow are more defined owing to the physical separation of up-flowing and down-flowing streams. As shown in Figure. Gas is sparged into only part of the vessel cross section called the riser. Gas hold-up and decreased fluid density cause liquid in the riser to move upwards. Gas disengages at the top of the vessel leaving heavier bubble free liquid to recirculate through the down comer. Liquid circulates in airlift reactors as a result of the density difference between riser and down comer.

Figure illustrates the most common airlift conjurations. In the internal-loop vessels of Fgirue 9a) and (b), the riser and down comer are separated by an internal baffler or draft tube, air may be sparged into either the draft tube or the annulus. In the external-loop or outer-loop airlift of Figure(c), separate vertical tubes are connected by shott horizontal sections at the top and bottom. Because the raised and down comer are further apart in external-loop vessels gas disengagement simore effective than in internal loop devices Fewer bubbles are carried into the down comer, the density difference between fluids in the riser and down comer is greater, and circulation of liquid in the vessel is faster. Accordingly, mixing is usually better in external-loop internal-loop reactors.

Airlift reactors generally provide better mixing than bubble columns except at low liquid velocities when circulatory flow patterns similar to those shown in Fgirue develop. The airlift configuration confers a degree of stability to liquid flow compared with bubble columns; therefore, higher gas flow rates can be used without incurring operating problems such as slug flow or spray formation. Several empirical correlation have been developed for liquid velocity, circulation time and mixing time in airlift reactors; however there is considerable discrepancy between the results (6). Equations derived form hydrodynamic models are also available (6,7), these are usually relatively complex and because liquid velocity and gas hold-up are not independent, require iterative numerical solution.

**Figure. Airlift reactor configurations.**




Gas hold-up and gas-liquid mass-transfer rates in internal-loop airlifts are similar to those in bubble columns [6]. However, in external-loop devices, near-complete gas disengagement increases the liquid velocity and decreases the air hold-up (8,9) so that mass-transfer rates at identical gas velocities are lower than in bubble columns [6]. Therefore, by comparison with Eq.(13.3) for bubble columns, for external-loop airlifts.

$$k_L a < 0.32 u_G^{0.7}. \qquad \rightarrow (15)$$

Several other empirical mass-transfer correlations have been developed for Newtonian and non-Newtenian fluids in airlift reactors(6)

Performance of airlift devices is influenced significantly by the details of vessel construction [6, 10, 11]. For example, in internal-loop airlifts, changing the distance between the lower edge of the draft tube and the base of the reactor alters the pressure drop in this region and affects liquid velocity and gas hold-up. The depth of draft submersion from the top of the liquid also influences mixing and mass-transfer characteristics.

Airlift rectors have been applied in production of single-cell protein from methanol and gas oil; the are also used for plant and animal cell culture and in municipal and industrial waste treatment. Large airlift reactors with capacities of thousands of cubic meters have been constructed. Tall internal-loop airlifts built underground are known as deep shaft reactors; very

high hydrostatic pressure at the bottom of these vessels considerably improves gas-liquid mass-transfer. The height o airlift reactors is typically about 10 times the diameter; for deep-shaft systems the height-to-diameter ratio may be increased up to 100.

**8. c**

**Packed Bed**

Packed-bed reactors are used with immobilized or particulate biocatalysts. The reactor consists of a tube, usually vertical, packed with catalyst particles. Medium can be fed either at the top or bottom of the column and forms a continuous liquid phase between the particles. Damage due to particle attrition is minimal in packed beds compared with stirred reactors.

Packed-bed reactors have been used commercially with immobilized and enzymes for production of aspartame and fumarte, conversion of penicillin to 6-aminopenicillanic acid, and resolution of amino acid isomers.

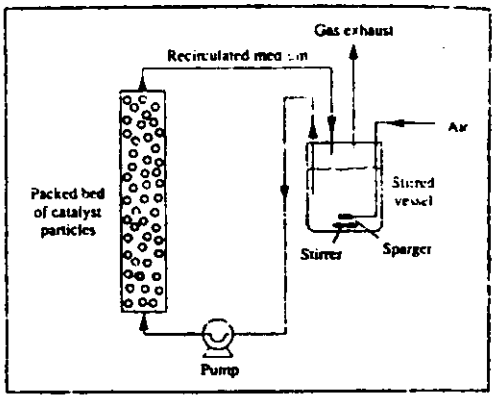
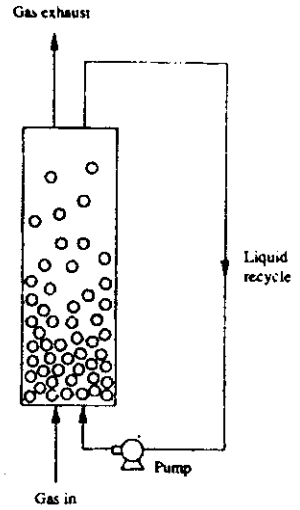



**Figure: Packed-bed reactor with medium recycle.**

Mass transfer between the liquid medium and solid catalyst; is facilitated at high liquid flow rates through the bed; to achieve this, packed beds are often operated with liquid recycle as shown in Figure. The catalyst is prevented from leaving the column by screens at the liquid exit. The particles should be relatively incompressible and able t withstand their own weight in the column without deforming and occluding liquid flow. Reticulating medium must also be clean and free of debris t avoid clogging the bed. Aeration is generally accomplished in a separate vessel; if air is spared directly into the bed, bubble coalescence produces gas pockets and flow channeling or misdistribution. Packed beds are unsuitable for processes which produce large quantities of carbon dioxide or other gases which can become trapped in the packing.

**Fluidised Bed**

When packed beds are operated in up flow mode with catalyst beads of appropriate size and density, the bed expands at high liquid flow rates due to upward motion of the particles. This is the basis for operation of fluidized-bed reactors as illustrated in Figure. Because particles in fluidized beds are in constant motion, channeling and clogging of the bed are avoided and air can be introduced directly into the column. Fluidized-bed reactors are used in waste treatment with sand or similar material supporting mixed microbial populations. They are also used with flocculating organisms in brewing and for production of vinegar.



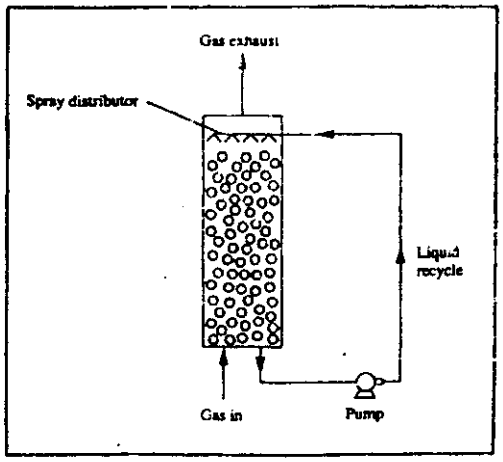




**Figure: Fluidised-bed reactor**

**Figure: Trickle-bed reactor**

**Trickle Bed**

The trickle-bed reactor is another variation of the packed bed. As illustrated in Figure, liquid is sprayed onto the top of the packing and trickles down through the bed in small rivulets. Air may be introduced at the base; because the liquid phase is not continuous throughout the column, air and other gases move with relative case around the packing. Trickle-bed reactors are used widely for aerobic wastewater treatment.

**9. With help of neat sketches explain the parts of various impeeler-less bioreactors with application areas for each.**

The various impeller less reactor includes,

1) Bubble column reactor
2) Packed bed reactor
3) Fluidised bed reactor

4) Trickle bed reactor

| S.No. | Name of the Reactor | Application |
|---|---|---|
| 1. | Bubble column reactor | 1. Production of bakerisy east ber r and vinegar<br>2. Treatment of waste water |
| 2. | Airlift reactor | 1. Production of single cell protein from methonal and gas oil.<br>2. Treatment of municipal and Industrial waste. |
| 3. | Packed bed reactor. | 1. Production of aspartate and fumarate<br>2. Conversion of penicillin to 6-aminopencillanic acid. |
| 4. | Fluidized bed reactor | 1. They are used with flocculating organisms in brewing<br>2. Production of vinegar. |
| 5. | Trickle bed reactor | Aerobic waste water treatment. |

## UNIT – III

## PART – A

**1. Write short notes on oxygen transfer.**

The growth of an aerobic organism in submerged culture requires oxygen dissolved in the liquid. Since oxygen in sparingly soluble in aqueous solution, it must be supplied continuously. The transfer of oxygen from the gas to the micro organism is called oxygen transfer.

**2. Write the equation for the oxygen transfer.**

The rate of oxygen transfer at low concentrations is proportional to the difference in oxygen concentrations. For the transfer to the interface from the bulk liquid this can be written as,

$$N_a = K_L (C^0 - C_L)$$

$N_a$ = Oxygen flux, kmol / $m^2$/s
$K_L$ = liquid side mass transfer coefficient, m/s.
$C^0$ = Oxygen concentration in the liquid at the interface, Kmol / $m^3$
$C_L$ = Oxygen concentration in the bulk liquid, kmol/$m^3$

**3. What are the methods for the measurement of $K_La$?**

Methods for measuring $K_La$ in fermentation processes can be divided in to two general types,

1. Steady state method
2. Dynamic method

**4. Short note on steady state – oxygen balance method.**

Steady state – oxygen balance method is used for measuring "$K_La$". In this method the quantities $N_a$, C* and $C_L$ are measured and then substituted in the equation,

$$N_a = K_La (C^*-C_L) \text{ to find } K_La$$

$N_a$ – The rate of oxygen transfer

$K_La$ - Overall mass transfer coefficient

C* - Liquid concentration in equilibrium (kmol/$m^3$)

$C_L$ – Oxygen concentration in bulk fluid

**5. Short note on Dynamic methods.**

1. The two methods used in dynamic methods are
   (i) Static gassing out
   (ii) Dynamic gassing out
2. Static gassing out is applicable to a non – respiring system
3. Dynamic gassing out is applicable to a respiring system

The air supplied to a respiring culture is stopped and the resulting fall in dissolved oxygen concentration is measured.

**6. Write the equation to evaluate $K_L$ a in stirred tanks.**

The general form of the correlations for evaluating $K_L a$ is,

$$K_L a = x\left(\frac{P_g}{V_L}\right)^y \left(V_g\right)^z$$

where,

$P_g$ = agitator power under gassing conditions, W
$V_L$ = Liquid volume without gassing , $m^3$
$V_g$ = Superficial gasve locity, m/s
$K_L a$ = Volumetric mass transfer coefficient, $S^{-1}$
X = constant.

**7. Write the equation to evaluate $K_L a$ in airlift bioreactors.**

A typical correlation for a wide range of configurations is,

$$K_L a = 5.5\times10^{-4}\left(1+\frac{Ad}{Ar}\right)^{-1.2}\left(\frac{P_g}{V_L}\right)^{0.8}$$

Ad= down comer cross sectional area, $m^2$
Ar = riser cross – sectional area, $m^2$
$P_g$ = agitator power under gassing conditions, w
$V_L$ = Liquid volume without gassing, $m^3$

**8. What is gas hold up?**

The volume fraction of gas – phase in the gas – liquid dispersion is known as the gas – hold up or the gas void fraction,

**9. Write the formula to calculate gas hold up.**

The overall gas hold up (ε) is given by,

$$\varepsilon = \frac{V_g}{V_g + VL}$$

where $V_g$ and $V_L$ are the gas and liquid volumes in the reactor.

**10. What are the objectives of mixing?**

The object of mixing in a gas / liquid / solid system are to:

a) Create small bubbles and so increase the interfacial area
b) Disperse the bubbles throughout the liquid

**11. Short note on Reynolds number.**

The Reynolds number is to characterize the flow. As mixing is extremely complex, the variables involved are lumped together in to dimensionless groups to obtain correlations that describes the system Reynold's number "NRe" is a dimensionless number.

**12. Write the formula to calculate Reynolds number.**

$$Re = \frac{Di^2 N\rho}{\mu}$$

where

$D_i$ = impeller diameter, m
N = impeller speed, $H_z$
ρ = fluid density , $kg/m^3$
μ = fluid viscosity , kg/m/s

**13. Write any one use of Reynolds number.**

Reynolds number is to characterize the flow. Fully turbulent flow exists above a Reynolds number of 104, while fully laminar flow exists below 100 , and in between the transition region.

**14. What is froude number?**

Froude number is a dimensionless group. It is used to characterize mixing in a vessel which takes in to account gravitational accounts. Froude number is abbreviated as "Fr".

**15. Write the formula to calculate froude number.**

$$Fr = \frac{N^2 Di}{g}$$

where,

g = gravitational acceleration, $m/s^2$

N = Impeller speed, Hz

$D_i$ = Impeller diameter, m

**16. What is power number?**

Power number is a dimensionless group. It relates to the energy required by the agitator. The power refers to the shaft power, and so allowances must be made for electrical and friction losses when calculating motor power input.

**17. Write the formula to calculate power number.**

$$P_0 = \frac{P}{N^3 D_i^{\,5} P}$$

P = agitator power, w

N = impelled speed, Hz

Di = Impeller diameter, m

$\delta$ = fluid density, kg/m/s

**18. Write the formula to calculate the head number.**

$$H = \frac{gH^1}{N^2 Di^2}$$

where

g = gravitational acceleration , $m/s^2$

$H^1$ = the head which is dissipated on us culation through the ressel

N = impeller speed , Hz

Di = Impeller diameter, m

**19. Write the formula to calculate flow number.**

$$Fl_g = \frac{Q_g}{ND_i^{\,3}}$$

$Q_g$ = gas flow in the reactor, $m^3\ s^{-1}$

N = impeller speed, Hz

Di = impeller diameter,m

**20. What are the types of agitator?**

Three common types of agitator have been used in bio reactors,

1. Turbine (Rushton or inclined)
2. Propeller
3. "INTERMIG"

## PART – B

**1. Explain oxygen mass transfer in bioreactors.**

**Mass Transfer:**

**Oxygen transfer:**

The growth of an aerobic organism in submerged culture requires oxygen dissolved in the liquid. Since oxygen is sparingly soluble in aqueous solution, it must be supplied continuously. The transfer of oxygen from the gas to the microorganism takes place in several steps. Firstly, the oxygen must travel through the gas to the gas – liquid interface, then through the interface, through the bulk liquid, and finally into the organism. This entire process is driven by the difference between the oxygen concentration in the gas and in the organism.

It has been found that the rate of oxygen transfer at low concentrations is proportional to the difference in oxygen concentrations. For the transfer to the interface from the bulk liquid this can be written,

$$N_a = k_L \left( C^0 - C_L \right)$$

$N_a$ = Oxygen flux, kmol/$m^2$/s
$K_L$ = liquid side mass transfer coefficient, m/s
$C^0$ = oxygen concentration in the liquid at the interface, kmol/$m^3$
$C_L$ = oxygen concentration in the bulk liquid, kmol / $m^3$

A gas side mass transfer coefficient can be similarly defined in terms of the gas side partial pressure.

$$N_a = K_g (p - P_0)$$

Where $K_g$ = gas side mass transfer coefficient kmol/$m^2$/s/bar
$p, P_0$ = oxygen partial pressure in the gas (bulk and interface respectively), bar

In general it is impossible to measure the interface concentration, so an overall mass transfer coefficient based on the difference in bulk concentrations is used:

$$N_a = K_L (C^* - C_L)$$

Where $K_L$ = overall mass transfer co efficient based on the difference in equivalent bulk liquid concentrations, m.s.
$C^*$ = equivalent liquid concentration in equilibrium with the bulk gas partial pressure, kmol/$m^3$.

If Henry's law is obeyed (for small values of C)

$$p = H C^*$$

where H = Henry's law constant

so

$$\frac{1}{k_L} = \frac{1}{H.K_g} + \frac{1}{K_L}$$

For slightly solube gases the rate of diffusion is controlling in the liquid phase, consequently H is large ($4.2 \times 10^4$ bar mol $^{-1}$ fraction for the solution of oxygen in water), so $k_L \approx K_L$.

Since it is usually impossible to determine local concentrations, every where in a bioreactor, average values of the concentrations and mass transfer coefficients must be used. To known the total oxygen transfer rate in a vessel, the total surface area available for oxygen transfer has to be determined. This is a difficult quantity to evaluate, so an overall mass transfer coefficient incorporating the surface area of the bubbles is used, namely $K_La$.

The transfer of oxygen into the bioreactor or the oxygen uptake rate is,

$$N_a = K_La (C^* - C)$$

a = surface area of bubbles / unit volume, $m^2$ / $m^3$

The $K_La$ value is dependent on the physicochemical properties of the bioreactor media and on the physical properties and operating conditions of the vessel. The magnitude of $K_La$ can be controlled by the agitation conditions and the air flow rate. Oxygen is a substrate which limits

growth; however, above a certain concentration, growth will become independent of oxygen concentration. Knowledge of $K_La$ behaviour allows the operation of bioreactors at conditions where oxygen is not a limiting factor for growth.

In many cases such as fungi, bacteria, and yeasts this will mean that the agitation is very intense, which in turn will also mean that the shear stresses in the vessel will be large. Plant and animal cells are particularly susceptible to shear, and this has led to different designs of bioreactor other than the conventional stirred tank vessel.

**2. Explain in detail the two methods for the determination of $K_La$.**

**Measurement of $K_La$:**

Methods for measuring $K_La$ in fermentation process can be divided into two general types: steady state methods and dynamic methods.

**Steady state – Oxygen balance method:**

In this method the quantities Na, C*, and measured and then substituted into the equation of this volume to find $K_La$.

$$Na = K_La (C^* - C_L)$$

The rate of oxygen transfer, Na, into the system is obtained by measuring the difference between the amount of oxygen in the streams to and from the bioreactor, and the respective flow rates. As the difference in oxygen concentration between the entrance and exit of the reactor is small, the exit concentration has to measured very accurately. A mass spectrometer is usually used.

The dissolved oxygen concentration can be measured at one or several points in the vessel (depends on vessel size), using a dissolved oxygen probe. The equilibrium concentration is evaluated from Henry's law.

In large reactors the partial pressure of oxygen in the gas will fall as it passes through the vessel. Therefore a logarithmic mean oxygen concentration of the inlet and outlet gas streams should be used as given below.

$$p_{lm} = \frac{p_{in} - p_{out}}{In\left(p_{in} / p_{out}\right)}$$

and so the equilibrium concentration

$$C^* = \frac{p_{lm}}{H}$$

This is the most accurate method of measuring $K_La$, and it can be used on an actual fermentation system; however, it does depend on accurate $O_2$ analyzers which are expensive, and also on accurate measurement of temperature and pressure.

**Dynamic methods:**

(a) Static gassing out – This is applicable to a non – respiring system. The oxygen content of the liquid in the bioreactor is reduced to zero by gassing out with nitrogen. The system is then aerated and the value of the dissolved oxygen concentration, $C_L$, is measured as a function of time. The equation representing this operation is:

$$\frac{dC_L}{dt} = K_L a\left(C^* - C_L\right)$$

which integrates to (if $C_L = 0$ at time, t)

$$\text{In}\left(I - \frac{C_L}{C^*}\right) = -K_L at$$

A graph of $\text{In}\left(I - \frac{C_L}{C^*}\right)$ against t will give a straight line of slope – $K_La$.

The advantage of this method is that it is simple; however, it can, as stated earlier, be used only on a non – respiring system, and it also requires an oxygen probe with a fast response. The method assumes that the oxygen concentration in the gas phase will be the same in both the inlet and outlet streams, which will not be the case, particularly in the initial stages of the re – oxygenation of the liquid. A modified dynamic response technique has been developed by Chapman et al. (1982) to overcome this problem.

(b) Dynamic gassing out – In this method air supplied to a respiring culture is stopped and the resulting fall in dissolved oxygen concentration is measured. The air supply in switched on before the critical dissolved oxygen concentration is reached, and the increasing dissolved oxygen is recorded as a function of time.

The mass balance of dissolved oxygen in the bioreactor is given by

$$\frac{dC_L}{dt} = K_L a\left(C^* - C_L\right) - rX$$

where r = specific rate of oxygen uptake by the cell (mol $O_2$/g cell/s)

X = cell concentration (g cell/I)

Which on rearranging gives,

$$C_L = -\frac{1}{K_L a}\left(rX + \frac{dC_L}{dt}\right) + C^*$$

A graph of $C_L$ against $\left(rX + \frac{dC_L}{dt}\right)$ will give a straight line of slop - $-\frac{1}{K_L a}$

This method determines $K_La$ for an actual fermentation; however, it assumes that the culture is rapidly degassed when the air is stopped, which is not always the case, particularly for a filamentous fungi fermentation broth. The accuracy of the method is also dependent on the dynamic response of the oxygen probe.

**3. Explain the correlations for evaluating $K_La$ in stirred tank reactors.**

**Correlation for $K_La$ – stirred tanks:**

Increasing power input reduces bubble size, and so increases interfacial area. Correlations, therefore, for evaluating $K_La$ as a function of power input/unit volume and also superficial gas velocity, which is the volumetric gas flow rate divided by the cross – sectional area, exist.

The general form of the correlations for evaluating $K_La$ is

$$K_L a = x\left(\frac{P_g}{V_L}\right)^y \left(v_g\right)^z$$

where $P_g$ = agitator power under gassing conditions, W

$V_L$ = liquid volume without gassing, $m^3$

$v_g$ = superficial gas velocity,m/s

$K_La$ = volumetric mass transfer coefficient, $s^{-1}$

X = constant

Many studies of gas – liquid mass transfer in low – viscosity fluids in agitated vessels have been reviewed by Van't Riet (1983). Results of experiments in many different vessels with different agitator configurations all fit within 20 -40% of the correlations

(a) For coalescing – air – water dispersion

$$K_L a = 2.6\times10^{-2}\left(\frac{P_g}{V_L}\right)^{0.4}\left(\nu_g\right)^{0.5}$$

$$V_L \le 2.6 m^3;\ 500 < \frac{P_g}{V_L} < 10\ 000.$$

(b) For non – coalescing air – electrolyte dispersion

$$K_L a = 2\times10^{-3}\left(\frac{P_g}{V_L}\right)^{0.7}\left(\nu_g\right)^{0.5}$$

$$V_L \le 4 m^3;\ 500 < \frac{P_g}{V_L} < 10\ 000.$$

In general, coalescing systems are one where the water is relatively pure, and non – coalescing systems are ones where a small amount of electrolyte is in the system. These correlations do not take into account the non – Newtonian behaviour of bio logical fluids, nor the effect of antifoam and the presence of solids. A correlation that has been shown to apply to a non – Newtonian filamentous fermentation is of the form:

$$K_L a = x\left(\frac{P_g}{V_L}\right)^{0.33}\left(\nu_g\right)^{0.56}$$

Comparing this with the equation for Newtonian fluids shows that the oxygen transfer coefficient for non – Newtonian fluids is less sensitive to increases in power input, so more power is required to reach the same $K_L a$ value than in a Newtonian fluid.

The addition of antifoam has a significant effect on the $K_L a$ value. The effect of the antifoam is to reduce the interfacial free energy at the phase interface between the water and air, so reducing surface tension and decreasing the bubble diameter, leading to higher values of the interfacial area per unit volume, a. However, the tendency for a to increase is countered by the effect of the surfactant film on the mass transfer coefficient $K_L$. This decrease may be due to either or both of the following mechanisms; the liquid movement near the interface is reduced because of the decreased mobility of the interface, and the increased resistance of the molecular film results in a different vapour / liquid equilibrium at the interface.

In general, with modern silicon – based antifoams, the decrease in $K_L$ seems to be larger than the increase in a, which means the overall $K_La$ value is reduced at all concentrations. However, with other surfactants (sodium dodecy1 sulphate and sodium laury1 sulphate) it has been shown that the $K_La$ value increased with increasing concentration.

**4. Explain the correlations for evaluating $K_La$ in airlift bioreactors.**

**Correlations for $K_La$ – airlift bioreactors:**

There are a wide range of correlations dependent on the configurations of the bioreactor, that is, whether there is an internal draught tube or an external loop, whether the draught tube or the annulus is sparged.

A typical correlation for a wide range of configurations is

$$K_L a = 5.5\times10^{-4}\left(1+\frac{A_d}{A_r}\right)^{-1.2}\left(\frac{P_g}{V_L}\right)^{0.8} \qquad \text{---------- (1)}$$

or in terms of the superficial gas velocity in the riser $D_{gr}$ m/s

$$K_L a = 0.76\left[1+\frac{A_d}{A_r}\right]^{-2} v_{gr}^{0.8}, \qquad \text{---------- (2)}$$

where $A_d$ = down comer cross – sectional area, $m^2$

$A_r$ = riser cross – sectional area, $m^2$

$v_{gr}$ = superficial gas velocity in the riser, $m.s^{-1}$.

The correlation has been tested in various bioreactors at differing air flow rates, and the results are shown in figure. It can be sent hat agreement was within 20% of the measured values; however, in a real system with antifoam and dissolved solids, the results could be different. The aspect ratio of the fermenters used was 5 to 1 (10 litres), 12 to 1 (30 litres), 6.5 to 1 (100 litres) and 8.5 to 1 (1000 litres).

**5. Briefly explain gas hold up.**

**Gas hold – up:**

The volume fraction of gas – phase in the gas – liquid dispersion is known as the gas hold – up or the gas void traction. The overall gas hold – up (ε) is given by

$$\varepsilon = \frac{V_g}{V_g + V_L}$$

where $V_g$ and $V_L$ are the gas the liquid volumes in the reactor.

In airlift bioreactors the individual riser and down comer gas holdups, $\varepsilon_r$ and $\varepsilon_d$ can be evaluated and these are related to the overall holdup by

$$\varepsilon = \frac{A_r\varepsilon_r + A_d\varepsilon_d}{A_r + A_d}$$

This equation is valid for both internal – loop airlifts and external loops where the dispersion height in the riser and down comer and nearly equal.

Gas hold – up determines the residence time of the gas in the liquid, and in combination with the bubble size, it influences the gas – liquid interfacial area available for mass transfer. The relationship between the interfacial area/ volume reactor and hold – up is given by

$$a = \frac{6\varepsilon}{d}$$

where d = bubble diameter.

So, the interfacial area may be increased by increasing the gas hold – up or decreasing the bubble size.

Because of the difficulties in direct measurement of interfacial areas, the parameter is little used for bioreactor design purposes. Instead, the easily measured overall volumetric mass transfer coefficient $K_La$ is usually used.

**6. Briefly explain Agitation in a stirred tank reactor.**

**Agitation in a stirred tank:**

There are more data available on turbine agitators than on any of the other agitators mentioned, so the discussion below concentrates on turbines. If a gas – liquid two phase system is considered, the effect of the flow regimes on increasing agitation can be seen in figure with a low agitator speed, the flow pattern is dominated by the gas – liquid flow up the middle, that is, the impeller is said to be flooded.

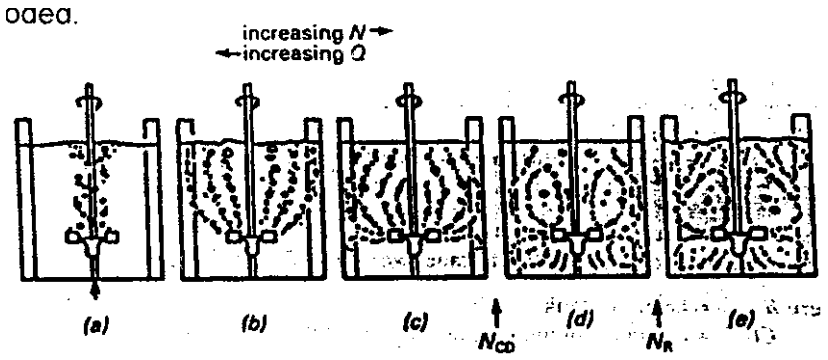



**Figure: Flow regimes in a stirred – tank bioreactor with increasing impeller speeds (N), and increasing flow rate produced by the impeller (Q). $N_{CD}$ is the impeller speed below which the gas does not penetrate below the impeller, and $N_g$ is the impeller speed at which gross recirculation of gas back into the agitator occurs (from Nienow et al. 1985).**

Increasing the speed creates a flow pattern dominated by the agitator in which the flow is horizontally dispersed from the agitator, and then above a certain speed, the flow is distributed to all parts of the vessel. Increasing the gassing rate $Q_g$ at constant N leads to the opposite sequence of events.

The flooding $N_F$ and the point at which complete dispersion occurs $N_{CD}$ can be calculated from the following correlations:

$$N_F = \left( \frac{Q_g T}{3D_i^4} \right)$$

where T = vessel diameter, m.

For pipe spargers

$$N_{CD} = \frac{\left(4Q_g^{0.5}\ T^{0.25}\right)}{D_i^2}$$

For ring spargers

$$N_{CD} = \frac{\left(3Q_g^{0.5} T^{0.25}\right)}{D_i^2}$$

The speed $N_{CD}$ is important as it gives the speed below which the gas does not penetrate beneath the agitator and the whole tank is not used. A further critical speed can also calculated, $N_R$, which is the speed which gross recirculation of gas back into the agitator occurs.

$N_R$ is given by

$$N_R \frac{\left(1.5Q_g^{0.2}T\right)}{D_i^2}$$

These correlation are in SI units and are valid for coalescing systems in tanks up to 1.8 m diameter, with a 6 – bladed disk turbine, where h = T, and Cl = T/4

Where h = height of tank

Cl = clearance of impeller above the bottom of the tank.

**7. Explain the effect of gas and solids on power consumption.**

**Effect of gas and solids on power consumption:**

Knowledge of the power, $P_g$ absorbed by the system is required for the determination of mass transfer rates, gas hold – up and interfacial area on the large scale from small – scale tests. The un gassed power consumption can be calculated from the power number, see figure when gas is sparged into the system at a given speed, the power decreases, as shown in figure because of the formation of gas cavities behind the blades.

The gassed power consumption can be evaluated from the following equation obtained by Michel & miller (1962)

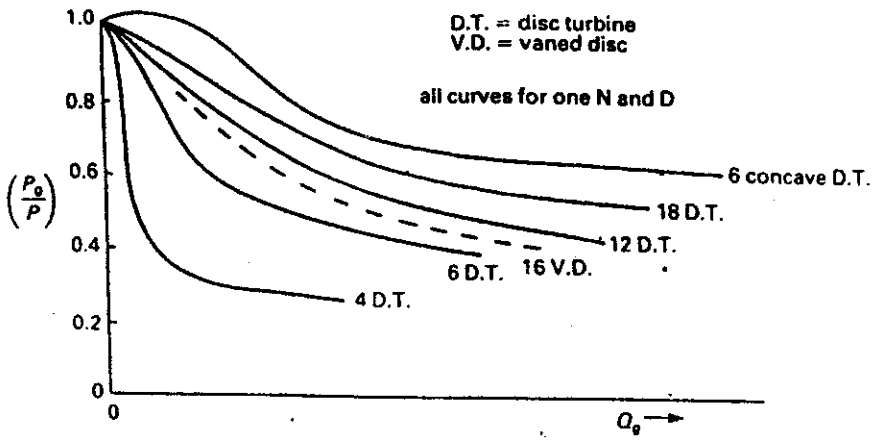



**Figure: Agitator power for various impellers with increasing gas flow ($Q_g$). $P_g$ agitator power under gassing, P, agitator power without gassing; D.T., disk turbine with 4 – 18 vanes; V.D., vaned disk (from Middlecton, J.C. (1985)**

$$P_g = 0.706\left(\frac{P^2ND_i^3}{Q_g^{0.56}}\right)^{0.45}$$

If a plot of $P_g$ against $Fl_g$ is made as shown in figure at constant $Q_g$ and increasing N, then a minimum is found at $N_{CD}$. The explanation of the shape of the curve (Middleton 1985) is that in the region at the lowest speed, the gas passes mainly through the agitator without dispersion and the liquid flows around the outer part of the blades undisturbed by the gas. Therefore, the gassed power is not much different to the single phase liquid system. As N increases, gas is captured by the vortices behind the agitator blades, and dispersed and so $P_g$ decreases as the captured gas 'streamlines' the blades forming large cavities. Further increases of N diminishes the cavities which allows the air to be dispersed and results in large values of $P_g$. The power continues its rise with increasing speed until $N_R$ is reached at which gross circulation of gas back into the agitator arises.

The previous discussion refers to a system where solid is not present. In bio reactors containing cultures, the system becomes three – phase and so more complex. It is important to prevent the solid settling on the bottom of the vessel and to keep an even distribution. It has been found that the clearance of the agitator above the base of the vessel is important, and that an optimum is reached when this distance is of the tank diameter.

There are considerable advantages in using angled blade agitators for three phase systems, in that less power is required and greater stability may be achieved. Also, the maximum shear will be reduced. However, the interaction with the sparger are more complex than with disk turbines, and more work is necessary to determine optimum geometries.

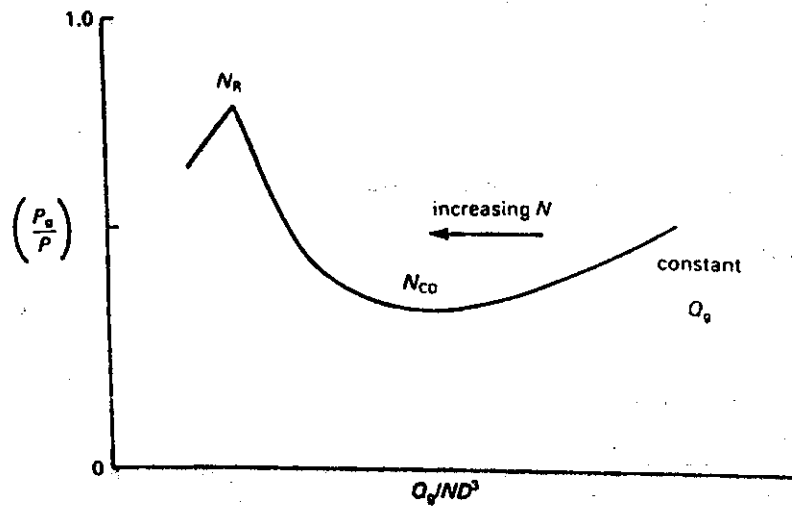



**Figure: Agitator power curve at a constant gassing rate ($Q_g$). $P_g$ agitator power under gassing; P, agitator power without gassing; $Q_g$ / $ND_i^3$ = modified flow number $Fl_g$ where $Q_g$ gas flow rate; N, impeller speed; $D_i$ impeller; $N_{CD}$ the impeller speed below which the gas does not penetrate below the impeller; $N_g$ the impeller speed at which gross recirculation of gas back into the impeller occurs (from Middleton, J.C. (1985).**

Inclined blade agitators are extremely sensitive to aeration rates at $D_i$ / T ratios of 0.33 or less, which leads to poor suspension. At values of $D_i$ / T of between 0.4 and 0.5, these instabilities disappear and particle suspension efficiency is satisfactory.

**8. Explain the rheological characteristics of fluids.**

Fluids normally encountered in bio reactors can be defined as Newtonian or non – Newtonian. The rheological characteristics of these fluids can be described by the following general equation:

$$\tau = \tau_0 + k(\gamma)^n \qquad \text{---------- (1)}$$

where

$\tau$ = shear stress, $N/m^2$
$\tau_0$ = yield stress, $N/m^2$
$\gamma$ = shear rate, $s^{-1}$
k = consistency index
n = flow behaviour index

The shear rate $\gamma$ is the velocity gradient and can be written for the case of laminar flow in the x direction as dv / dy where v is the velocity and y is the distance perpendicular to the x direction.

**Newtonian fluids:**

These exhibit to yield stress, so n=1, $\tau_o = 0$ , and k becomes $\mu$ the dynamic viscosity, so,

$$\tau = \mu\gamma. \qquad \text{---------- (2)}$$

Newtonian fluids have a viscosity which is independent of shear rate. A number of bacterial and yeast fermentation fluids exhibit Newtonian behaviour.

**Non – Newtonian fluids:**

**(a) Pseudoplastics**

These fluids follow a power law model

$$\tau = k\,(\gamma)^n$$

where k and n change during the course of the fermentation and will decrease with increasing shear rate.

The apparent viscosity can be defined by the equation.

$$\mu_{app} = \frac{\tau}{\gamma} = k\gamma^{n-1}$$

many filamentous organisms exhibit this characteristic.

**(b) Dilatants**

These fluids also obey the power law model, but in this case n > 1.

**(c) Bingham plastics and Casson fluids**

These fluids are characterized by the relatively high yield stress required to make the fluids flow. Very few organisms grown in bioreactors exhibit this behaviour.

The rheological characteristics of all these different fluids are shown on the shear-stress diagram,

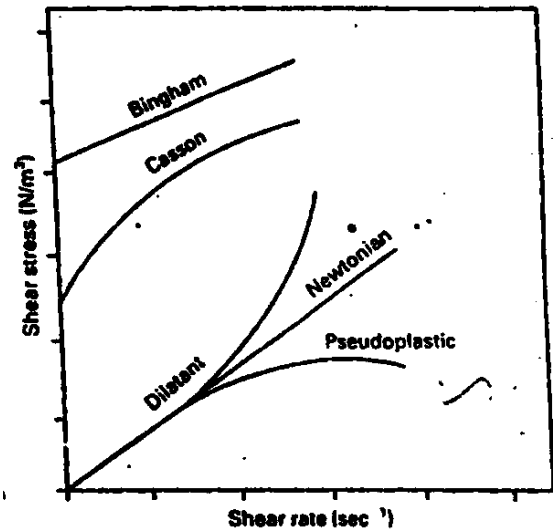



**Figure: Rheological characteristics of fluids**

**9. Briefly explain shear in airlift bioreactors.**

**Shear in airlift bioreactors:**

In an airlift bioreactor, the stresses generated are less than in a stirred – tank bio reactor because the intensity of turbulence is much lower. Time – average or laminar shear values will give the maximum shear values attained in the vessel.

The maximum shear rate will occur at the vessel wall, and the Blasius equation can be used to evaluate the shear stress where,

$$\frac{\tau}{\rho v_{LR}^2} = 0.0396\ Re^{-0.25}$$

where $2500 < Re < 100\ 000$ and $V_{LR}$ = liquid velocity in the reser.

The shear rate can be calculated from the standard relationship $\tau = \mu\gamma$ , for Newtonian fluids and the power law relationship $\tau = K\gamma^n$ for non – Newtonian fluids.

The liquid circulation velocity is a function of the power input to the bioreactor, which is also related to the overall mass transfer coefficient, $K_La$. Therefore, it is possible to evaluate the maximum achievable oxygen transfer rate if the maximum permissible shear stress is known. Figure (Wood & Thompson 1986) shows results for three size of airlift bioreactor. As can be seen, with increasing size of the bio reactor for the same oxygen transfer rate, the level of shear is decreased.

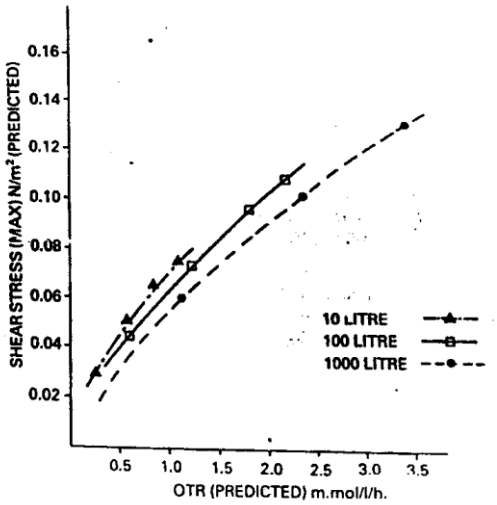



**Figure: The relationship between predicted oxygen transfer rate (OTR) and predicted shear stress ($N/m^2$) in 3 sizes of airlift bioreactor (from Wood, L.A. & Thompson, P.W. 1986).**

The average shear rate in a column is a function of the turbulence generated by the rising gas bubbles and can be related the gas velocity. There are a wide range of correlations quoted in the literature to evaluate average shear rates, the majority, however, are of the form.

$$\gamma = kv_{gr}$$

where k varies between 1500 and 5000.

## UNIT – IV

## PART – A

**1. Short note on modeling.**

Simple methods can be used to describe the results of practical fermentation studies of cell growth to very great effect, since it becomes possible to assign numerical values to such kinetic parameters as the specific growth rate (μ), the monod saturation constant ($K_s$), the biomass yield coefficient (Y×ls), and maintenance coefficient (m), for any given fermentation models helps to increase the product formation.

**2. What is Digital simulation?**

Digital simulation provides a very easy method of simulating most types of fermentation phenomena, since the computed solutions follows simply and directly from the basic model, and the actual numerical details of the solution procedure are handled automatically by the computer.

**3. What are the advantages of digital simulation?**

Digital simulation is based on the use of non – steady – state or time dependent model relationships, an additional advantage of the digital simulation method is that the models solved dynamically, and the transient response characteristics of the system are obtained as a natural consequence of the solution procedure.

**4. What are the model variables in stirred batch fermenter?**

1. 'V' – volume
2. 'X' – biomass
3. 'S' – substrate
4. 'P' – product

**5. What are the reasons for incorrect results in computer simulation?**

1. Inaccuracies in the model
2. Inaccuracies in the parameter values
3. Numerical errors in the solution

**6. Short note on Numerical solutions.**

In digital simulation, the computer solves the equations by carrying out a sequence of arithmetical and logical operation on model variables. To solve differential equations, many of these operations are associated with numerical methods of integration.

**7. Short note on digital simulation programming.**

Digital simulation programming grew in the 1950s from the desire to match the relative simplicity of analogue computing by digital means. This was achieved by incorporation a block structure in to the digital simulation program.

**8. Write any two digital simulation programming languages.**

1. BEDSOCS
2. I SIM

} developed at British universities, are good examples of interactive CSSL type languages.

**9. What is "STELLA"?**

STELLA (1985), uses a non – mathematical, graphical set-up of the model in a block structure and runs on Apple Machintosh computer. It is a typical digital simulation programming language.

**10. Short note on ISIM.**

ISIM (Interactive Simulation Language)
ISIM conforms to the basic structure of a CSSL language in having three regions,

1. The INITIAL
2. The DYNAMIC
3. The TERMINAL regions

} CONTROL REGION

- ✓ Control region is optional

**11. What are the function of various regions in ISIM?**

1. The INITIAL region is used for setting values of constants, initial values, etc.
2. The DYNAMIC region contains the model equation.
3. The terminal region may be used for end of run calculations.

**12. Write the model equation for mass balances.**

The mass balance equation can be written as,

Rate of accumulation = Rate of production

**13. Write the model equation for cells.**

$$\text{For cells, } V.\frac{dx}{dt} = r_x V$$

(or)

$$\frac{dx}{dt} = r_x$$

**14. Write the model equation for substrate.**

$$\text{For substrate, } V.\frac{ds}{dt} = r_s V$$

(or)

$$\frac{ds}{dt} = r_s$$

**15. Write the model equation for product.**

$$\text{For product, } V.\frac{dP}{dt} = r_P V$$

(or)

$$\frac{dx}{dt} = r_x$$

**16. Write the model equation for kinetics.**

Kinetics: $r_x = \mu_x$

$$\text{Where, } \mu = \mu_m \frac{S}{(K_S + S)}$$

$$r_S = \frac{\mu}{y \times ls}$$

$$\boxed{r_P = (K_1 + K_2 \mu) X}$$

where, $K_1$ is the non growth associated coefficient

$K_2$ – the coefficient – associated with growth

**17. What are line numbers?**

Line numbers are shown only for reference, and do not belong to the actual program.

**18. What is the use of line number?**

1. Line numbers can be used as reference
2. Line numbers help to refer the other functions in ISIM Program.
3. Any program line starts with a line number.

**19. What is SIM?**

1. SIM is used in ISIM program
2. SIM is a mandatory statement when control parameters are specified.

**20. What is INITIAL?**

'INITIAL' is used in ISIM Program
INITIAL specifies the start of the initial program

## PART – B

**1. Briefly explain simulation of Bioprocesses.**

A further aspect of the use of models in then to solve the model, in conjunction with appropriate numerical data values, and to compare the results that are predicted by the model to those obtained form the actual fermentation. This procedure is known as simulation and is very important, since it enables:

(1) A check that the from of model used to describe experimental results is ' correct', in the sense that eh model when combined with experimentally derived parameter values does at least give a reasonable representation of observed behaviour.
(2) A check on the accuracy of the model parameter values, this may be particularly important in batch fermentations for example, where because of the time varying nature of the process, it is sometimes rather difficult to determine kinetic parameters very accurately. This may be important when attempting to establish the exact dependency of specific growth rate on substrate concentration, where there are difficulties in obtaining rate data at high substrate concentrations, during the initial part of the batch, and these difficulties are compounded by the reciprocal nature the Line weaver- Burk plot. Thus good agreement between the experimental results and the simulated output provides extra confidence both with regard to the applicability of the model and to the accuracy of the data, obtained in the fermentation.
(3) A better understanding of the ways in which fermentations behave. This is probably the greatest benefit of carrying out a simulation exercise, since it is through understanding that progress is made.

One of the major benefits of any modeling exercise is the degree of thought that must accompany it. Thus the very act of sitting down and trying to formulate the formation in mathematical form is one which brings substantial benefits in its own right, since one is then forced to consider the complex cause and effect sequences in detail, together with all the complex interrelationships that there may be.

The comparison of the computer simulated results with these of the fermentation is often very meaningful, s this can lead to discussion on the ways that model may perhaps be inappropriate and thus lead on to an improved understanding of the process. Alternatively, it may suggest ways in which the fermentation conditions may have changed and hence be no longer appropriate to the model.

Fermentation is a very complex process, and it is often very difficult to get a complete picture of what is actually going on in a particular fermentation. The process of deriving the

model is going to be of help in this, but the model itself, by allowing repeated simulations to be carried out under a wide range of operating conditions, can lead to much better understanding by suggesting various reasons as to why certain apparently unexplainable phenomena may occur.

(1) An extension to process conditions. Once a realistic model has been obtained, it should then be capable of being extended to cover further investigations of, say, alternative feeding strategies, alternative control strategies, and process optimization studies.

(2) An experimental tool. Often proposed experimental changes can be stimulated in advance of the actual experiment, and this can give very useful information on the sensitivity of the system to the proposed changes and may suggest whether the particular experiment is worth carrying out or indeed whether or not the experiment should be redesigned. As a further experimental too, new forms of computer software are now available that enable direct prediction of model parameter values by direct comparison of experimental data and the corresponding model prediction, obtained by computer simulation.

(3) Operator training. Many important aspects of fermentation operations can be easily simulated by the use of simple models. These include such concepts as linear growth, double substrate limitations, changeover policies for batch to fed bath cooperation, fed-batch feeding strategies, oxygen aeration dynamics, dissolved oxygen probe dynamics, cell retention systems, microbial interactions, and biofilm diffusion limitations and control. Many such models can now be found in the general literature, and most of these can be adapted very easily to fit any desired situation.

Having developed the model, the problem them, however, consists of how to solve it, and this usually demands some form of numerical solution. For general application, the most appropriate methods is probably digital simulation (Dunn et al. 1988) as this allows rapid solutions to be obtained, without the necessity of having to be fully conversant with the numerical solution procedure. The solution of even very complex fermentation models, using digital simulation is very simple.

**2. Explain the Digital simulation of Bio processes.**

**DIGITAL SIMUATION**

Digital simulation via its simplicity and versatility provides a very easy method of simulating most types of fermentation phenomenon, since the computed solution follows simply and directly from the basic model, and the actual numerical details of the solution procedure are handled automatically by the computer. This means that the bio chemical engineer or fermentation technologist is thus completely free to concentrate on the actual fermentation problem and does not need bother about the method of solution.

Many digital simulation programming languages are available commercially, but all share the basic aim of providing a direct and simple means of solving the sets of simultaneous differential and algebraic equations that comprise the model. It is the tasks of the modeler to for formulate the basic mass balance equations, the rate of transfer equations, kinetic equations, and the controller equations that comprise the model. But the, having done this, the aim of the simulation language is to eliminate any difficulty in obtaining the resultant computer solution, so that the modeler can progress quickly and easily from formulating the model to obtaining the computer solution.

Since the method of solution is based on the use of non – steady – state or time – dependent model relationships, an additional advantage of the digital simulation method is that the model is solved dynamically, and the transient response characteristics of the system are obtained as a natural consequence of the solution procedure. This makes the method ideal for solving unsteady – state batch or semi – batch fermentation problems and for the application of control studied. Steady – state conditions, such as continuous chemostate operation, can also obtained, but this must be done by formulating the problem in dynamic terms. In this, unsteady - state model relationships are solved, with the solution starting from some initial conditions and following the system transient response up to eventual steady state. With this solution procedure, the simulation actually mirrors the start-up, cause and effect sequences of an actual fermenter.

**3. Explain the steps in the modelling procedure.**

(i) At the initial stage, the general problem is formulated, different physical modes are examined, and the objectives of the simulation specified.

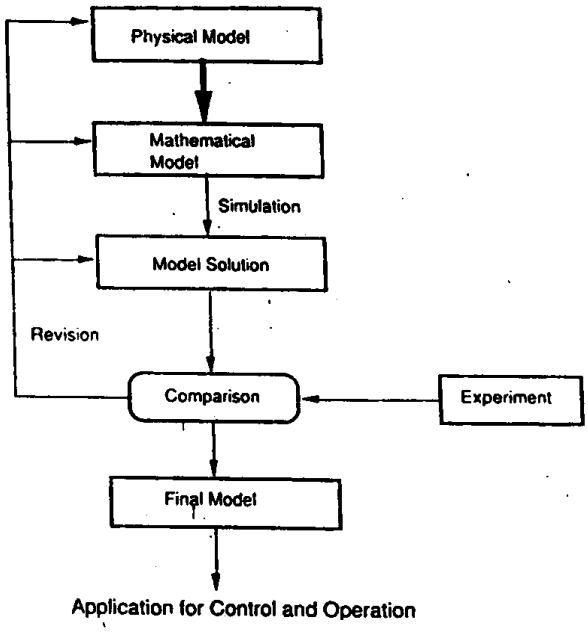



**Fig. The steps in the modelling procedure.**

(ii) The available theory must be formulated in terms of mathematical relationships. Usually these will be composed as sets of simultaneous first – order differential and algebraic equations. Often these may be represented as a block information flow diagram which aids in visualizing the interdependencies of the model questions. Fig. is an example of this, as shown for the case of the batch fermentation example, considered later.

(iii) The computer model is composed essentially as a set of variables which correspond to those of the problem, but which are constrained in such a way that they can vary only in accordance with the model equations.

(iv) Digital simulation languages provide an easy and direct method of solving the model equations, and output is easily provided in graphical or tabular form.

(v) It is essential to check the validity of the computer prediction against qualitative reasoning and eventually with experiment, and steps (i) to (iv) should be checked and, if necessary, revised at frequent intervals during the course of the setting up the model simulation, as fig. suggests.

**4. What are the reasons for the incorrect results in computer simulation?**

In correct results can be obtained from the computer simulation, owing to a variety of different causes. Amongst these are:

(1) In accuracies in the model. In some cases the fermentation system may not be fully understood and the model is therefore incorrectly formulated.

(2) Inaccuracies in the parameter values. Care and judgement often have to be used to prevent the model becoming overly complex, with many unmeasurable or difficult to determine parameters.

(3) Numerical errors in the solution. These may be caused by limitations in the integration step length and in the numerical precision of the computer, and should be checked by the use of an alternative numerical integration routine.

**5. Briefly explain Digital simulation programming languages.**

**DIGITAL SIMULATION PROGRAMMING LANGUAGES**

Digital simulation programming grew in the 1950s from the desire to match the relative simplicity of analogue computing by digital means. This was achieve by incorporating a block structure into the digital simulation program. Thus the main program was built up of sub – program blocks, in exactly the same way as for the equivalent electrical wiring connections used in analogue computation, improvements in the 1960s led to more developed languages such as MIMIC and CSMP. Although these remained block – oriented, the formation of a block network

solution became no longer essential, and the programming could be effected easily by the writing down simple FORTRAN like statements. This was then followed by the development of modern, primarily expression based, language such as DSL and CSSL.

Owing to the proliferation in the number of simulation languages a specification or CSSL was commissioned. Practically all digital simulation programming languages follow this common structure, and as a result the programming in any language is very similar to that of any other.

BEDSOCS and ISIM, both developed at British universities, are good examples of interactive CSSL type languages, with ISIM (program manual ) being generally available for PC use. Another standard simulation program called ACSL (1986), more suited to larger industrial problems, also follows CSSL standards. ESL (program manual) is a recent development of ISIM people, and it competes with ACSL for large, complex problems. One recent development, STELLA (1985), uses a non – mathematical, graphical, set-up of the model in a block structure and runs on the Apple Macintosh computer.

**6. Briefly explain ISIM.**

**ISIM INTERACTIVE SIMULATIN LANGUAGE**

In demonstrating the application of modelling and simulation to fermentation, use will be made of the ISIM digital simulation programming language.

ISIM conforms to the basic structure of a CSSL language in having three regions; the INITIAL. DYNAMIC, and TERMINAL regions, together with the optimal CONTROL region. The INITIAL regions is used for setting values of constants, initial values, etc. The DYNAMIC region contains the model equations, and the TERMINAL region may be used for end of run calculations. During execution, data values are output in tabular form by the command OUTPUT, and a graphical output of one variable by the command PLOT. Post mortem plots of all variables, stored by the command PREPARE, can be shown by using the command GRAPH. Particularly valuable is the fact that the programs can be interrupted during execution by pressing any key, values of constants or parameters changed, and the simulation continued.

**7. Example 2: Chemostat operation.**

**Chemostat operation**

The previous program may be easily modified to allow the chemostat operation with sterile feed by modifying the mass balance relationships. The corresponding equations as shown in Chapter 2 are then:

For cells $\frac{dX}{dt} = -DX + r_x$

For substrate $\frac{dS}{dt} = D(S_F - S) + r_s$

For product $\frac{dP}{dt} = -DP + r_P$

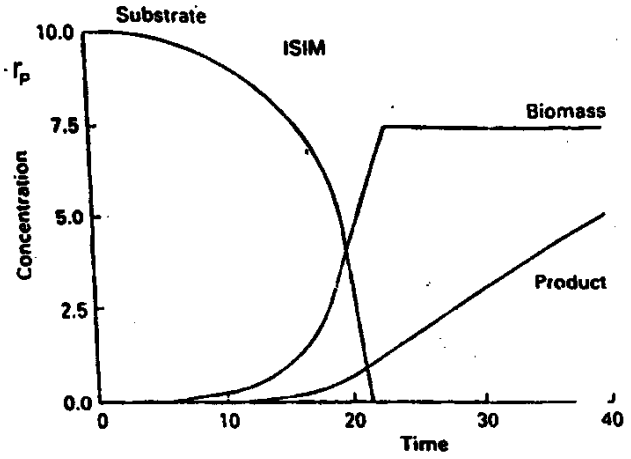



**Fig. Batch bioreactor simulation graphical results.**

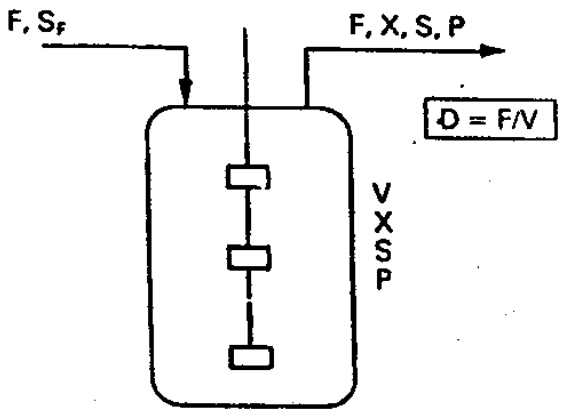



**Fig. continuous bioreactor with model variables**

Where D is the dilution rate and $S_F$ the concentration of the limiting substrate in the feed. New CONSTANT values for D and $S_F$ can be inserted into the program and lines changed by simple EDIT instructions. The value for TFIN also needs to be increased.

The modified ISIM program is shown in fig. It allows the effect of varying D on X,S, and P to be studied. Starting from initial concentrations $X_0$, $s_0$, and $P_0$ and up to the eventual final steady state. The graphical output shown in fig. mirrors

| |
|---|
| 1. : CHEMOSTAT START UP AND OPERATION |
| 2. : FILE, "CHEMO" |
| 3. CONSTANT UM = 0.3, KS = 0.1, K1=0.03K2=0.08,Y=0.8 |
| 4. CONSTANT SF=10 |
| 5. CONSTANT CINT'=1,TFIN=60 |
| 6. CONSTANT D=0 |
| 7. SIM |
| 8. INITIAL |
| 9. X=0.01 |
| 10. S=10 |
| 11. P=0 |
| 12. DYNAMIC |
| 13. X'=-D*+RX : BIOMASS BALANCE EQUATION |
| 14. S'=D* ( SF-S)+RS :SUBSTRATE BALANCE EQUATION |
| 15. P'=-D*P+RP : PRODUCT BALANCE EQUATION |
| 16. RX=U*X :KINETIC EQUATIONS |
| 17. U=UM*S/(KS+S) |
| 18. RS=-RX/Y |
| 19. RP =(k1+k2*U)*x |
| 20.PLOT T,x.0,10 |
| 21. OUTPUT T,S,X.P |
| 22. PREPARE T,S,X,P |
| $ VAL D =0.20000. |

**Fig. Continuous bioresctor ISIM simulation program**

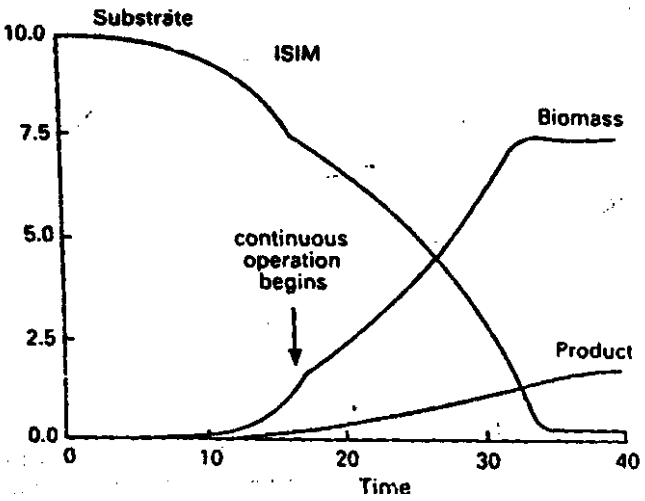



**Fig Continuous bioreactor simulation graphical results**

The start up of the bioreactor under initially batch growth conditions; the reactive interrupt facility is sued to infinite the change over the batch to continuous separation. Thus, starting with D=0 simulate batch growth. Pressing any key i.e., interrupt program execution, followed by HOLD and VALID = (new value) followed by the GO command simulates the change to contiguous operation at any appropriate time at which the cell concentration is considered to be sufficiently high. In this case the new value of D is taken as 0.2 for the simulation results shown in fig. and compares with the value of $\mu_{max}$=0.3. The break in the concentration tune dependency as feeding start is quite apparent, and the new transient then continues up to the eventual steady – state chemo stat operation condition.

**8. Explain briefly about Fed – batch.**

In this case the model equations are modified to allow for the continuous feeding of sterile substrate, the absence of outflow from the bioreactor, and the increase in volume ( total accumulation of mass) in the bioreactor. Seliematically as shown in fig. For fed batch operation, the equations become.

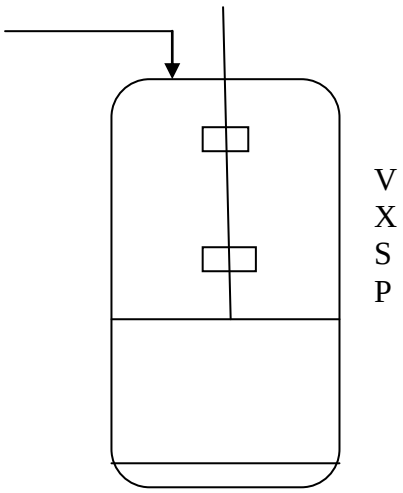



**Fig. Fed-batch bioreactor with model variables**

Total balance $\frac{dV}{dt} = F$

For cells $\frac{d(V,X)}{dt} = r_x V$

For substrate $\frac{d(VS)}{dt} = ES_F + r_s V$

For Product $\frac{d(VP)}{dt} = r_P V$

where F is the volumetric feed rate, $S_F$ is the feed concentration, and V is the volume of the bio reactor contents at time t. As in Example (2), operation begins under initial batch conditions and at some subsequent time (approximately 20h), feeding of substrate starts. The ISIM program and typical graphical output are shown in fig. and Again the break in the batch growth transient, as semi – batch – feeding starts,

```
1. :FED BATCH FERMENTATION WITH BATCH START UP
2. :FILE, "FEDBAT"
3. CONSTANT UM = 0.3,KS=0.1,K1=0.03k2=0.08,y=0.8
4. CONSTANT SF=10
5. CONSTANT F=0
6. CONSTANT CINT=1,TFIN=160
7. SIM
8. INITIAL
9. x=0.01;S=10;P=0;V=1
10. VX=V*X
11. VS=V*S
12. VP=V*+P
13. DYNAMIC
14. V'=F                    :TOTAL MASS BALANCE
15. VX'=RX*V
16. VS'=F*SF+RS*V
17. VP'=RP*v
18. X=VX/V
19. S=VS/V
20. P=VP/V
21. RX=U*X                  :KINETIC EQUATIONS
22. U=UM*S/(KS+S)
23. RS=-RX/Y
24. RP=(K1+K2*U)*X
25. D=F/V                   :NOMINAL DIUTION RATE
26. PLOT T,X,0,TFIN,0,10
27. OUTPUT T,S,X,P
28. PREPARE T,S,X,P,D,U
$VALF          0.50000E=01
```

**FIG. Fed – batch bioreactor ISIM simulation program**

Is very apparent, with the transient continuing to an apparent 'quasi' steady state operating condition. Under these conditions the biomass concentration becomes constant, while the substrate concentration is below the $K_s$ value and decreases very slowly. The values of D (=F/V) also decrease since V increases owing to the incoming feed, and it scan be seen from the simulation that X eventually becomes constant and D eventually becomes equal to μ when S falls below $K_s$.

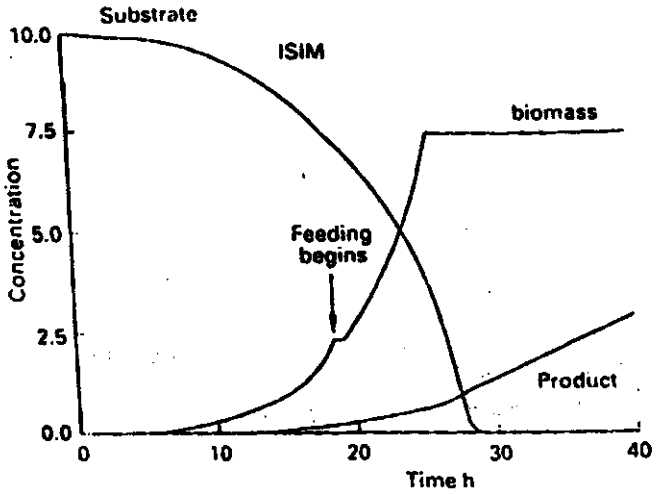



**Fig. Fed batch bioreactor simulation graphical results.**

This is similar to the chemostate situation except that here both are decreasing. The total biomass is determined by the yield coefficient times the total amount of substrate that ahs been consumed equal to the amount in the bioreactor initially plus the amount added during the feeding period. As the mass balances state, the concentrations at any time are equal to the total masses divided by the volume. During the quasi – steady – stat the total biomass will increase linearly with time if, as in the case, the feeding flow rate is constant. This is a linear growth' situation in which the growth rate is limited by the feeding rate. The product production rate depends linearly on biomass concentration, and thus even when μ becomes very low, will continue to increase linearly.

**9. Explain growth patterns and Kinetics in a Batch culture.**

**TRANSIENT GROWTH KINETICS**

During certain intervals in the batch cultivation or during the start up or disturbances to continuous flow reactors, cell population grows in a transient state.

**Growth Patterns and Kinetics in a Batch Culture**

When a liquid nutrient medium in inoculated with a seed culture, the organisms selectively take up the dissolved nutrient from the medium and convert them into biomass.

A typical batch growth curve that includes the following phases is depicted in figure.

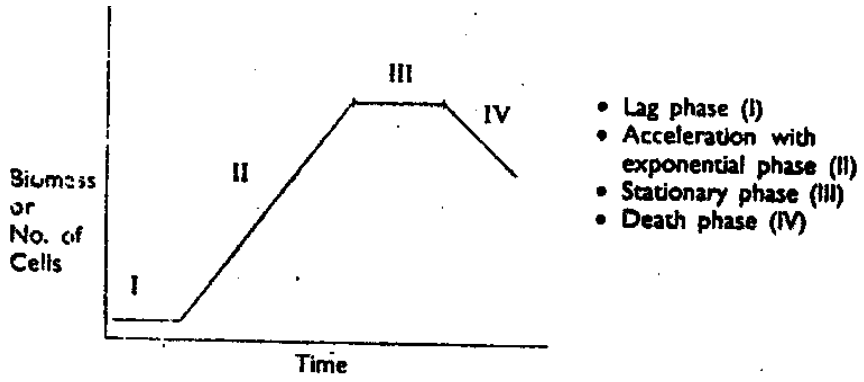



**Figure Biomass vs time.**

**Lag phases:** in the lag phase there is no increase in microbial density with time. The length of the phase depends upon changes in the nutrient composition, age and the size of the inoculum. For instance, if the size of the inoculum is small, there will be outward diffusion of the nutrients into the bulk medium. There could be a sudden shift from the old to the new environment known as adaptability.

**Acceleration with exponential phase:** This phase is also called the logarithmic phase. In this phase, biomass increases exponentially with time.

**Mathematical expression:** We know that the growth rate is proportional to existing population. Here existing population means the microbial density X, which has been defined as mass of cells per unit culture volume.

We can write it as

$$\frac{dX}{dt} \alpha X$$

or

$$\frac{dX}{dt} \mu X$$

The above equation is called Malthus Law.

Rearranging, we get

$$\frac{dX}{X} \mu dt$$

Integrating Eq. using the limits X =$X_0$ at t=0 and X=X at t=t, and simplifying, we get the resultant as

$$X = X_0 e^{\mu(t-t_{lag})}$$

Note: t=0=$t_{lag}$

Equation shows that the growth is exponential.

**Doubling time:** At t=$t_d$, the time for doubling biomass, X= $2X_0$. Using the expression of the form in $(X/X_0)=\mu t$ and replacing the values of X and t by $2X_0$ and $t_d$ respectively, we get

$$t_d = \frac{\ln 2}{\mu}$$

It is an expression for the doubling time of the biomass. In a CSTR, it is used to characterize the population during the growth.

**Stationary phase:** The stationary phase starts at the end of the exponential phase. In this phase, the net growth rate is zero as there is no cell division where we can say the growth rate is balanced by the death rate. Though the net growth rate is zero, the cells are still metabolically active and produce secondary metabolites. During the stationary phase the process called endogenous metabolism takes place, i.e., cell catabolizes the cellular reserves for new building blocks and for energy producing monomers.

The cell must expend some energy to maintain an energized membrane and also fro the transport of nutrients for essential metabolic activities. The metabolic activities refer to the motility and repair of the damaged cells. This energy expenditure is called Maintenance energy.

Conversion of cell mass to maintenance energy or the loss of cell mass due to the lysis during the stationary phase is given by

$$\frac{dX}{dt} \alpha X$$

or $$\frac{dX}{dt} = -K_d X$$

or $$\frac{dX}{X} = -K_d dt$$

Further integrating, using the limits as X= $X_{s0}$ at t =0 and X=X at t=t, we get

$$X = X_{s0} e^{-K_d t}$$

where $K_d$=first –order rate constant for the endogenous metabolism
$X_{s0}$= cell mass concentration at the start of the stationary phase

Death or decline phase: it follows the stationary phase. There is no clear demarcation between the stationary phase and the death phase, since some cells even die in the stationary phase because of exhaustion of essential nutrients or the accumulation of the toxic products.

The rate of death can be given as

$$\frac{dN}{dt} \alpha N$$

where N is the cell concentration

Also

$$\frac{dN}{dt} - K'_d N$$

or

$$\frac{dN}{N} K'_d dt$$

Integrating the above expression using the limits as $N=N_s$ at t=0 and N=N at t=t,

$$N = N_s e^{-K'_d t}$$

where $K'_d$ = first order death rate constant

**Multiple lag phases**: Multiple lag phases may sometimes be observed when the medium contains multiple carbon sources. Multiple carbon sources can be in the form of glucose, xylose, sucrose, etc.

During the growth of the cell or a microbe feeding on one particular carbon source hat nears the exhaustion, the cell must divert its energy from growth to retool for the new source of carbon supply, i.e. second carbon source. This phenomenon known as diauxic growth which is caused by a shift in the metabolic patterns in the midst of growth. Figure depicts multiple lag phases during the growth.

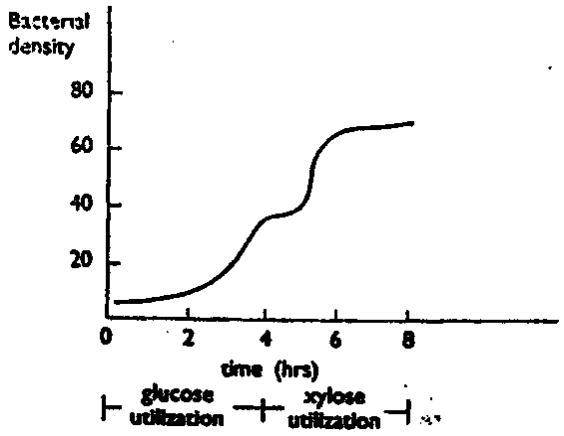



**Fig. Multiple lag phase.**

In a medium containing initially equal amounts of glucose and xylose, diauxic growth of E-coli is observed in a batch culture.

The design to minimize the culture and process times normally includes minimization of lag times associated with each new batch culture. A good design is to minimize the length of the lag phase and maximize the rate and length of the exponential phase.

To comply with, the following generalizations are drawn:

1. The inoculating culture should be as active as possible and the inoculation should be carried out in the exponential growth phase.
2. The culture medium used to grow the inoculum should be s close as possible to the final full – scale fermentation composition.
3. The use of a reasonably large inoculum of the order of 5-10 percent of the new medium volume, is recommended to avoid the wastage by diffusion of the required intermediates or activators.

At the end of the lag phase, the population of microorganisms is well adjusted to its new environment. The cells can then multiply rapidly, and cell mass, or the number of living cells, doubles regularly with time.

**10. Explain pearl and Reed Model.**

**Pearl and Reed Model (Unstructed)**

Pearl and Reed in 1920, contributed to a theory which included an inhibiting factor to population growth. It is unstructured model and depicts microbial density X versus time t, with the profile as shown in figure.

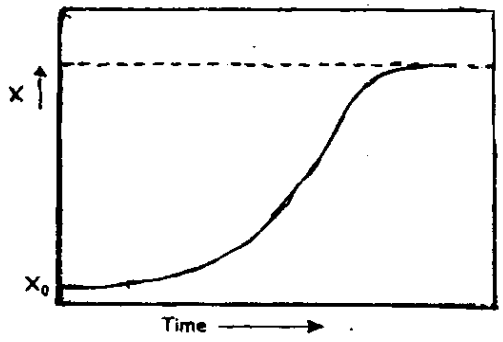



**Fig. Microbial density vs time.**

In this case, the relation $\frac{dX}{dt} = \mu X$ called the Malthus Law is modified as:

$$\frac{dX}{dt} = KX - K\gamma X^2 \text{(assuming that inhibition is proportional to } X^2\text{)}$$

$$\frac{dX}{dt} = KX(1-\gamma X); X(0) = X_0$$

The above equation is called the Ricalli equation

Here is an inclusion of $\gamma$ on account of inhibition at high biomass concentration. Inhibition is proportional to the square of biomass concentration.

On integration; we get

$$X = \frac{X_0 e^{kt}}{1-\gamma X_0\left(1-e^{kt}\right)}$$

This is called Logistic equation and the profile shown in figure is called the Logistic curve.

## UNIT – V

## PART – A

**1. What is Immobilization?**

Immobilization is the restriction of cell mobility with in a defined space. Immobilization of cells as biocatalysts is almost as common as enzyme immobilization.

**2. What are the advantages of immobilization?**

1. Immobilization provides high cell concentrations
2. Immobilization provides cell recess and eliminates the costly processes of cell recovery and cell recycle

**3. What are the limitations of Immobilization?**

1. The major limitation of immobilization is that the product of interest should be excreted by the cells
2. Immobilization often leads to systems for which diffusional limitation are important.

**4. What is active immobilization of cells?**

Active immobilization is entrapment or binding of cells by physical or chemical forces. The two major methods of active immobilization are entrapment and binding.

**5. What is physical entrapment?**

Physical entrapment within porous matrices is the most widely used method of cell immobilization various matrices can be used for the immobilization of cells.

**6. What are the matrices commonly used in physical entrapment?**

1. Porous polymers (agar, alginate, K – Carrageenan, polyacry lamide, chitosan, gelatin, collagen)
2. Porous metal screens.
3. Polyurethane.

**7. What is gelation of polymers?**

Gelation and agar beads may be prepared by mixing the liquid form of these polymers with cell suspensions and using a template to form beads. Reduction of temperature in the templates causes solidification of the polymers with the cell entrapped.

**8. What is precipation of polymers?**

Cell are dispersed in a polymer solution, and by changing the $p^H$ or the solvent, the polymer can be precipitated. The starting solution of the polymers has to be prepared with an organic solvent mixture.

**9. What is Jon exchange gelation?**

Jon exchange gelation takes place when a water – soluble poly electrolyte is mixed with a salt solution. Solidification occurs when the poly electrolyte reacts with salt solution to form a solid gel.

**10. What is poly condensation?**

Epoxy resins are prepared by poly condensation and can be used for cell immobilization poly condensation produces covalent networks with high chemical and mechanical stability.

**11. What is polymerization?**

Polymeric networks can be prepared by cross linking copolymers of a vinyl group containing monomers. Poly acrylamide beads are the most widely used polymer beads, prepared by co polymerization of acrylamide and bisacrylamide.

**12. What is encapsulation?**

Encapsulation is another method of cell entrapment Micro capsules are hollow, spherical particles bound by semi permeable membranes.

Cells are entrapped within the hollow capsule volume.

**13. What are the polymers used in micro encapsulation?**

Various polymers can be used as capsule membranes among these are nylon, collodion, polystyrene, acrylate, poly lysine – alginate hydrogel, cellulose acetate – ethyl cellulose, and polyester membranes.

**14. What is adsorption?**

Adsorption of cells on inert support surface has been widely used for cell immobilization. The major advantage of immobilization by adsorption is direct between nutrient and support materials.

**15. What are the factors affecting the strength of the support materials?**

1. Adsorption capacity
2. Strength of binding

**16. What is passive immobilization?**

Biological films are the multilayer growth of cells on solid support surfaces. The support material can be inert or biologically active. Bio film formation is common in natural and industrial fermentation systems.

**17. Wire any two applications of passive immobilization.**

1. Biological waste – water treatment
2. Mold fermentations

**18. Write any two examples of cell immobilization by entrapment using different support materials.**

| **Cells** | **Support matrix** | **Conversion** |
|---|---|---|
| 1. S. cerevisiae | Polyacrylamide | Glucose to ethanol |
| 2. E. aerogenes | K – Carrageenan | Glucose to 2,3 butanediol |

**19. Write any tow examples of cell immobilization by surface attachment.**

| **Cells** | **Supports surface** | **Conversion** |
|---|---|---|
| 1. Lactobacillu sp | Gelation (adsorption ) | Glucose to lactic acid |
| 2. clostridium ace to butylicum | Jon exchange resins | Glucose to acetone, butanol |

**20. What is Damkobler number?**

The presence and significance of diffusional limitations depend on the relative rates of bioconversion and diffusion which can be described by the damkohler number (Da)

$$\text{Da}=\frac{\text{Maximum rate of bio cenversion}}{\text{Maximum rate of diffusion}}=\frac{\gamma\max}{(\text{De}/\delta)\text{So}}$$

## PART – B

**1. Briefly explain Immobilization of cells.**

Immobilization of cells as biocatalysts is almost as common as enzyme immobilization. Immobilization is the restriction of cell mobility within a defined space. Immobilized cell cultures have the following potential advantages over suspension cultures.

1) Immobilization provides high cell concentrations.
2) Immobilization provides cell reuse and eliminates the costly process of cell recovery and cell recycles.
3) Immobilization eliminates cell washout problems at high dilution rates.
4) The combination of high cell concentrations and high flow rates (no washout restrictions) allows high volumetric productivities.
5) Immobilization may also provide favorable micro environmental conditions (i.e., cell – cell contact, nutrient – product gradients, pH gradients) for cells, resulting better performance of the biocatalysts (e.g., higher product yields and rates).
6) In some cases, immobilization improves genetic stability.
7) For some cells, protection against shear damage is important.

The major limitation on immobilization is that the product of interest should be excreted by the cells. A further complication is that immobilization often leads to systems for which diffusional limitations are important. In such cases the control of micro environmental conditions is difficult, owing to the resulting heterogeneity in the system. With living cells, growth and gas evolution present significant problems in some system and can lead to significant mechanical disruption of the immobilizing matrix.

Many of the ideas in enzyme immobilization have a direct counter pert in whole cells. However, the maintenance of a living cell in such system is more complex than maintaining enzymatic activity. The primary advantage of immobilized cells over immobilized enzymes is that immobilized cells can perform multi step, cofactor – requiring, biosynthetic reactions that are not practical using purified enzyme preparations.

**2. Write short notes on'**
**(i) Active immobilization**
**(ii) Encapsulation**

**Active immobilization:**

Active immobilization is entrapment or binding of cells by physical or chemical forces. The two major methods of active immobilization are entrapment and binding.

Physical entrapment within porous matrices is the most widely used method of cell immobilization. Various matrices can be used for the immobilization of cells. Among these are porous polymers (agar, alginate, k-carrageenan, polyacrylamide, chitosan, gelatin, collagen), porous metal screens, polyurethane, silica gel, polystyrene, and cellulose triacetate.

Polymer beads should be porous enough to allow the transport of substrates and products in and out of the bead.

**Encapsulation:**

Encapsulation is another method of cell entrapment. Microcapsules are hollow, spherical particles bound by semi permeable membranes. Cells are entrapped within the hollow capsule volume. The transport of nutrients and products is and out of the capsule takes place through the capsule membrane. Micro capsules have certain advantages over gel beads. More cells can be packed per unit volume of support material into capsules, and intraparticle diffusion limitations are less severe in capsules due to the presence of liquid cell suspension in the intracapsule space. Various polymers can be used as capsule membranes. Among these are nylon, collodion, polystyrene, acrylate, polylysine – alginate hydrogel, cellulose acetate – ethyl cellulose, and polyester membranes. Different membrane (composition and MW cut off) may need to be used for different applications in order to retain some high – MW products inside capsules and provide passage to low – MW nutrients and products.

**3. Write short notes on,**
**(i) Adsorption**
**(ii) Coralent binding**

Immobilization of cells on the surface of support materials can be achieved by physical adsorption or covalent binding.

Adsorption of cell on inert support surfaces has been widely used for cell immobilization. The major advantage of immobilization by adsorption is direct contact between nutrient and support materials. High cell loadings can be obtained using micro porous support materials. However, porous support materials may cause intraparticles pore diffusion limitations at high cell densities, as is also the case with polymer-entrapped cell systems. Also, the control of micro environmental conditions is a problem with porous support materials. A ratio of pore to cell diameter of 4 to 5 is recommended for the immobilization of cells on to the inner surface of porous support particles. At small pore sizes, accessibility of the nutrient into inner surfaces of pores may be the limiting factor, whereas at large pore sizes the specific surfaces of pores may be the limiting factor, whereas at large pore sizes the specific surface area may be limiting factor. Therefore, there may be an optimal pore size, resulting in the maximum rate of bio conversion.

Adsorption capacity and strength of binding are the two major factors that affect the selection of a suitable support material. Adsorption capacity varies between 2 mg / g (porous silica) and 250 mg/g (wood chips). Porous glass carriers provide adsorption capacities ($10^8$ to $10^9$ cells/g) that are less than or comparable to those of gel – entrapped cell concentrations ($10^9$ to $10^{11}$ cells /ml). The binding forces between the cell and support surfaces may vary, depending on the surface properties of the support material and the type of cells. Electrostatic forces are dominant when positively charged support surfaces (ion exchange resins, gelatin) are used. Cells also adhere on negatively charged surfaces by covalent binding or H bonding. The adsorption of cells on neutral polymer support surfaces may be mediated by chemical bonding, such as covalent bonding, H bonds, or van der Waals forces. Some specific chelating agents may be used to develop stronger cell – surface interactions. Among the support materials used for cell adsorption and porous glass, porous silica, alumina, ceramics, gelatin, chitosan, activated carbon, wood chips, polypropylene ion – exchange resins (DEAE – Sephades, CMC-) and Sepharose.

Adsorption is a simple, inexpensive method of cell immobilization. However, limited cell loadings and rather weak binding forces reduce the attractiveness of this method. Hydrodynamic shear around adsorbed cells should be very mild to avoid the removal of cells from support surfaces.

Covalent binding is the most widely used method for enzyme immobilization. However, it is not as widely used for cell immobilization. Functional groups on cell and support material surfaces are not usually suitable for covalent binding. Binding surface need to be specially treated with coupling agents (e.g., glutaraldehyde or carbodiimide) or reactive groups for covalent binding. These reactive groups may be toxic to cells. A number of inorganic carries (metal oxides such as titanium and zirconium oxide) have been developed that provide satisfactory functional groups for covalent binding.

Covalent binding forces are stronger that adsorption forces, resulting in more stable binding. However, with growing cells, large numbers of cell progeny must be lost. Support materials with desired functional groups are rather limited. Among the support materials used for covalent binding are CMC plus carbodiimide; carriers with aldehyde, amine, epoxy, or halocarbonyl groups; Zr(IV) oxide; Ti(IV) oxide; and cellulose plus cyanuric chloride. Support materials with – OH groups are treated with CNBr, materials with – $NH_2$ are treated with glutaraldehyde, and supports with COOH groups are treated with carbodiimide for covalent binding with protein groups on cell surfaces.

**4. Briefly explain passive immobilization.**

**Passive Immobilization: Biological Films:**

Biological films are the multilayer growth of cells on solid support surfaces. The support material can be inert or biologically active. Bio film formation is common in natural and industrial

fermentation systems, such as biological waste – water treatment and mold fermentations. The interaction among cells and the binding forces between the cell and support material may be very complication.

In mixed – culture microbial films, the presence of some polymer – producing organisms facilitates bio film formation and enhances the stability of the bio films. Micro environmental conditions inside a thick bio film vary with position and affect the physiology of the cells.

In a stagnant biological films, nutrients diffuse into the bio film and products diffuse out into liquid nutrient medium. Nutrient and product profiles within the bio film are important factors affecting cellular physiology and metabolism. A schematic of bio film is depicted in figure. Bio film cultures have almost the same advantages as those of the immobilized cell systems over suspension cultures, as listed in the previous section.

The thickness of a bio film is an important factor affecting the performance of the biotic phase. Thin bio films will have low rates of conversion due to low bio mass concentration, and thick bio films may experience diffusion ally limited growth, which may or may not be beneficial depending on the cellular system and objectives. Nutrient – depleted regions may also develop within the bio film for thick bio films. In may cases, an optimal bio film thickness resulting in the maximum rate of bio conversion exists and can be determined. In some cases, growth under diffusiton limitations may result in higher yields of products as a result of changes in cell physiology and cell – cell interactions.

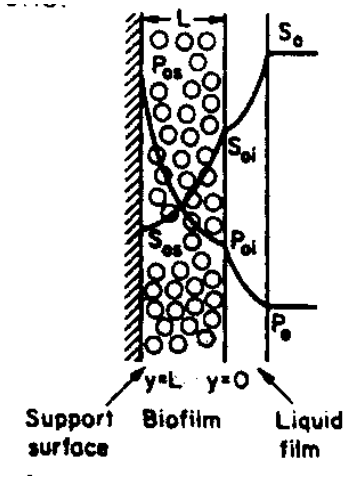



**Figure: Schematic representation of a bio film**

Improvement in reaction stoichiometry (e.g., high yield) may overcome the reduction in reaction rate, and it may be more beneficial to operate the system under diffusion limitations. Usually, the most sparingly soluble nutrient, such as dissolved oxygen, is the rate – limiting nutrient within the bio film.

**5. What is Damkohler number? Explain diffusional limitations in Immobilized cell systems.**

Immobilization of cells may cause extra diffusional limitations as compared to suspension cultures. The presence and significance of diffusional limitations depend on the relative rates of bio conversion and diffusion, which can be described by the Damkohler number

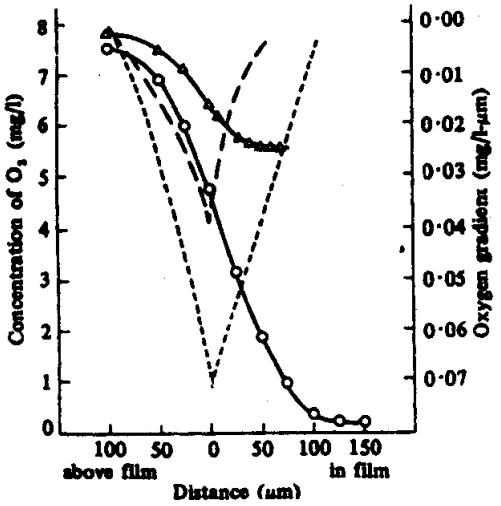



**Figure: Dissolved – oxygen profiles and oxygen gradients in a microbial slime bathed in flowing medium: - Δ - Δ oxygen profile for 20 ppm nutrient broth, 27.5$^0$C; -- oxygen gradient for this profile; - O – O – oxygen profile for 500 ppm nutrient broth, 26$^0$ C; ----- oxygen gradient for this profile. (With permission, from H.R. Bungay and others, Biotechnol. Bioeng. 11:765, 1969, John Wiley & Sons, Inc., New York).**

$$D_a = \frac{\text{maximum rate of bioconversion}}{\text{maximum rate of diffusion}} = \frac{r_{max}}{(D_e/\delta)S_o}$$

Where $r_{max}$ is the maximum rate of bio conversion (mg S/I h), $D_e$ is the effective diffusivity of the rate – limiting substrate, δ is the thickness of diffusion path (or liquid film), and $S_0$ is the bulk substrate concentration in liquid phase. When the film – theory model applies, $D_e$ / δ is the mass transfer coefficient (i.e., $k_L = D_e / \delta$).

If Da > > 1, the rate of bio conversion is diffusion limited; for Da << 1, the rate is limited by the rate of bio conversion; and for Da ≈ 1, the diffusion and bio reaction rates are comparable. It is desirable to keep Da < 1 to eliminate diffusion limitations when the productivity of a cell population does not improve upon immobilization due to cell – cell contact and nutrient gradients.

Diffusional limitations may be external (that is, between fluid and support surface in adsorption and covalent binding), intraparticle (i.e., inside particles in entrapment, encapsulation,

or immobilization in porous particles), or both. If the external mass transfer is limiting, an increase in liquid – phase turbulence should result in an increase in the reaction rate. In case of intraparticles mass – transfer limitations, a reduction in particle size or an increase in the porous void fraction of the support material should result in an increase in the rate of the bio reactions.

A mathematical model of the interaction of diffusion and reaction for surface immobilized or entrapped bio catalysts. These models apply directly to immobilized cells when the kinetics of bioconversion are described by a Michaelis – Menten type of kinetic expression. Thus, the reader may wish to consult chapter 3 again.

Another interesting case is to consider bio films where we allow cell growth. Models for immobilized enzymes have no terms for bio catalyst replication, so this case presents a new problem.

The thickness of a bio film or the size of microbial floc increases with time during the growth phase. A microbial floc is an aggregation of many cells, and in some processes these aggregates can be more than 1 mm in diameter. However, since the rate of increase in bio film thickness is much slower than the rate of substrate uptake, the system can be assumed to be at quasi – steady state for relatively short periods. The simplest case is to assume that the system is at quasi – steady state and all the cells inside the bio film are in the same physiological state. In this situation we write a steady – state substrate balance within the bio film by using average kinetic constants for the biotic phase (living cells)

A differential material balance for the rate – limiting substrate within the bio film yields at steady state.

$$D_e \frac{d^2S}{dy^2} = \frac{1}{Y_{x/s}} \frac{\mu_m S}{K_s + S} X \qquad \text{---------- (1)}$$

where $D_e$ is the effective diffusivity ($cm^2/S$) and $Y_{x/s}$ is the growth yield coefficient (g cells /g substrate)

The boundary conditions are

$$S = S_{0i} \qquad \text{at } y = 0$$

$$\frac{dS}{dy} = 0 \qquad \text{at } y = L$$

where L is the thickness of bio film.

If it is also assumed that the liquid nutrient phase is vigorously agitated and the liquid film resistance is negligible, then $S_0 \approx S_{0i}$. By defining a maximum rate of substrate utilization as $r_m = \mu_m X / Y_{x/s}$ (g subs / $cm^3$ h), we rewrite equation 1.

$$D_e \frac{d^2S}{dy^2} = \frac{r_m S}{K_s + S} \qquad \text{---------- (2)}$$

In dimensionless form, equation 2 can be written as

$$\frac{d^2\bar{S}}{d\bar{y}^2} = \frac{\phi^2 \bar{S}}{1 + \beta \bar{S}} \qquad \text{---------- (3)}$$

where

$$\bar{S} = \frac{S}{S_0}, \qquad \bar{y} = \frac{y}{L}, \qquad \beta = \frac{S_0}{Kls}$$

and

$$\phi = L\sqrt{\frac{\mu_m X}{Y_{x/s} D_e K_s}} = L\sqrt{\frac{r_m}{D_e s}} \qquad \text{---------- (4)}$$

equation 3 can be solved numerically. An analytical solution can be derived for the limiting cases of zero or first order reaction rates.

The maximum rate of substrate flux in the absence of diffusion limitations is given by the following equation

$$N_s A_s = -A_S D_E \left.\frac{dS}{dy}\right|_{y=0} = \frac{r_m S_o}{K_s + S_0}\left(LA_s\right) \qquad \text{---------- (5)}$$

where $A_s$ is a surface area of bio film available for substrate flux, $N_s$ is the substrate flux, and L is the thickness of the bio film.

In the presence of diffusion limitation, the rate of substrate consumption of flux is expressed in term of the effectiveness factor.

$$N_s = -D_e \left.\frac{dS}{dy}\right|_{y=0} = \eta\left(\frac{r_m S_0}{K_s + S_0}\right)L \qquad \text{---------- (6)}$$

where $\eta$ is the effectiveness factor, defined as the ration of the rate of substrate consumption in the presence of diffusion limitation to the rate of substrate consumption in the absence of diffusion

limitation. In the absence of diffusion limitation, $\eta \cong 1$, and in the presence of diffusion limitations, $\eta < 1$, The effectiveness factor is a function of $\phi$ and $\beta$. Figure is a plot of $\eta$ versus $\beta$ for various values of $\phi$. The $\phi$ value should be low

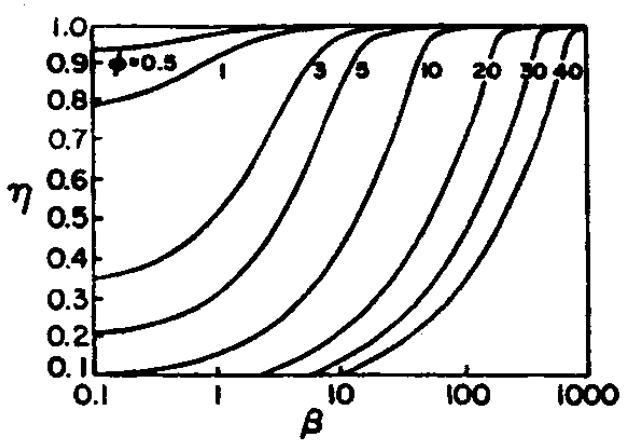



**Figure: Effectiveness factor for a flat bio film as a function of β the dimensionless initial substrate concentration, and ϕ , the Thiele modulus. (with permission, redrawn from B. Atkinson, Biochemical Reactors Pion Ltd, London 1974, p. 81)**

($\phi < 1$) to eliminate diffusion limitations. As the bio film grows (slowly), the value of $\phi$ will gradually increase. If shear forces cause a portion of the film to detach, then $\phi$ will decrease abruptly.

The effectiveness factor ($\eta$) can be calculated as

$$\eta = 1 - \frac{\tanh\phi}{\phi}\left(\frac{\omega}{\tanh\omega} - 1\right), \qquad \text{for } \omega \leq 1 \qquad \text{----------}(7)$$

$$\eta = \frac{1}{\omega} - \frac{\tanh\phi}{\phi}\left(\frac{1}{\tanh\phi} - 1\right), \qquad \text{for } \omega \geq 1 \qquad \text{----------}(8)$$

where $\omega$ is the modified Thiele modulus and is given by

$$\omega = \frac{\phi\left(S_o / K_s\right)}{\sqrt{2}\left(1 + \frac{S_0}{K_s}\right)}\left[\frac{S_0}{K_s} - \text{In}\left(1 + \frac{S_0}{K_s}\right)\right]^{-1/2} \qquad \text{---------- (9)}$$

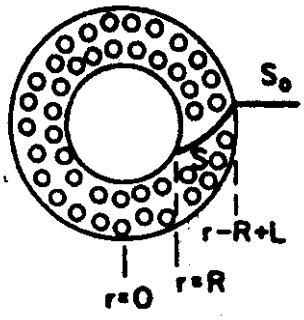
**6. Briefly explain analysis of micro bial film on inert spherical support particle and a spherical microbial floc.**

Some cells such as molds (A. niger) form pellets in a fermentation broth, and substrates need to diffuse inside pellets to be available for microbial consumption. Cell may form bio films on spherical support particles, as depicted in figure. Similar equations need to be solved in spherical geometry in this case to determine the substrate profile within the floc and the substrate consumption rate. The dimensionless substrate transport equation within the microbial floc is

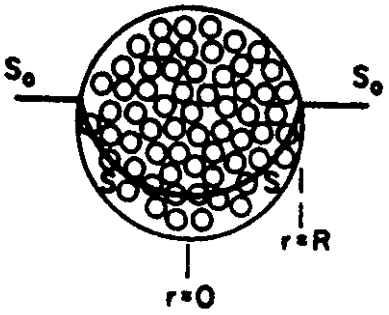
$$\frac{d^2\bar{S}}{d\bar{r}^2} + \frac{2}{\bar{r}}\frac{d\bar{S}}{d\bar{r}} = \frac{\phi^2\bar{S}}{1+\bar{S}/\beta'} \qquad \text{---------- (1)}$$

where

$$\bar{S} = \frac{S}{S_0}, \quad \bar{r} = \frac{r}{R}, \quad \beta' \frac{S_0}{K_s}$$




**(a) Microbial film on inert spherical support particle**




**(b) Spherical microbial floc**

and

$$\phi = \sqrt{\frac{\mu_m X}{Y_{x/s} D_e K_s}} = R\sqrt{\frac{r_m}{D_e K_s}} \qquad \text{---------- (2)}$$

The boundary conditions are

$$\bar{S} = 1, \qquad \text{at } \bar{r} = 1$$

$$\frac{d\bar{S}}{d\bar{r}} = 0, \qquad \text{at } \bar{r} = 0$$

For non spherical particles, a characteristic length is defined as

$$L = \frac{V_p}{A_p} \qquad \text{---------- (3)}$$

where $V_P$ and $A_P$ are the volume and surface area of microbial pellet.

The rate of substrate consumption by a single microbial floc is

$$N_S A_P = -A_P D_e \left.\frac{dS}{dr}\right|_{r=R} = \eta \frac{r_m S_0}{K_s + S_0} V_p \qquad \text{---------- (4)}$$

The effectiveness factor (η) is a function of ϕ and β. Variation of η with ϕ and β is similar to that of figure. However, η values for spherical geometry are slightly lower than those of rectangular geometry for intermediate values of ϕ ($1 < \phi < 10$). An analytical solution to (1) is possible for first – and zero – order reaction kinetics.

The reaction rate can be approximated to first order at low substrate concentrations.

$$r_s = \frac{\mu_m S}{Y_{x/s} K_s} X = \frac{r_m}{K_s} S \qquad \text{---------- (5)}$$

where $r_m = \left(\mu_m / Y_{x/s}\right) X$. The effectiveness factor in this case is given by

$$\eta = \frac{1}{\phi}\left[\frac{1}{\tanh\ 3\phi} - \frac{1}{3\phi}\right] \qquad \text{---------- (6)}$$

where

$$\phi = \frac{V_p}{A_p}\sqrt{\frac{r_m / K_s}{D_e}}$$

The rate of bio reaction can be approximated to zero order at values of $S >> K_s$. Because $K_s$ is often very small, the zero – order limit usefully describes many systems of practical interest.

$$r_s = \frac{\mu_m X}{Y_{x/s}} = r_m \qquad \text{---------- (7)}$$

the solution to equation 1 in this case is

$$S = S_0 - \frac{r_m}{6D_e}\left(R^2 - r^2\right) \qquad \text{---------- (8)}$$

Substrate concentration may be zero at a certain radial distance from the center of the floc according to equation 8. This distance is called the critical radius ($r_{cr}$) and is determined by setting S=0 at $r_{cr}$ in equation 8.

$$\left(\frac{r_{cr}}{R}\right)^2 = 1 - \frac{6D_e S_0}{r_m R^2} \qquad \text{---------- (9)}$$

When $r_{cr} > 0$ – that is $R > (6D_e S_0/r_m)^{1/2}$ – then the concentration of the limiting substrate is zero for $0 < r < r_{cr}$. In this case, the limiting substrate is consumed only in the outer shell of the floc, and the effectiveness factor is given by

$$\eta = \frac{r_m \frac{4}{3}\pi\left(R^3 - r_{cr}^3\right)}{\frac{4}{3}\pi R^3 . r_m} = 1 - \left(\frac{r_{cr}}{R}\right)^3 \qquad \text{---------- (10)}$$

or

$$\eta = 1 - \left(1 - \frac{6D_e S_0}{r_m R^2}\right)^{3/2}$$

**7. Draw and explain the design of immobilized enzyme reactors**
**(i) Packed bed reactors**
**(ii) Fluidized bed reactors**

Various reactor configurations can be used for immobilized cell systems. Since the support matrices used for cell immobilization are often mechanically fragile, bioreactors with low hydrodynamic shear, such as packed – column, fluidized – bed, or airlift reactors, are preferred. Mechanically agitated fermenters can be used for some immobilized – cell systems if the support matrix is strong and durable. Any of these reactors can usually be operated in a perfusion mode by passing nutrient solution through a column of immobilized cells. Schematic diagrams of immobilized – cell packed – column and fluidized – bed reactors are depicted in figure. These reactors can be operated in bath or continuous mode.

Consider the reactors shown in figure when the fluid recirculation rate is high, the system approaches CRSTR behavior. One commercial fluidized – bed, immobilized – animal – cell bioreactor system requires high recirculation to maintain uniform conditions in the reactor. The models we have discussed so far can be applied to such systems. The other extreme involves some waste – treatment systems where the rate of fluid recirculation is low or even zero. In the latter case, the system cannot to modeled as a CFSTR but must be treated as a PFR. To analyze such a system, consider a material balance on the rate limiting substrate over a differential element.

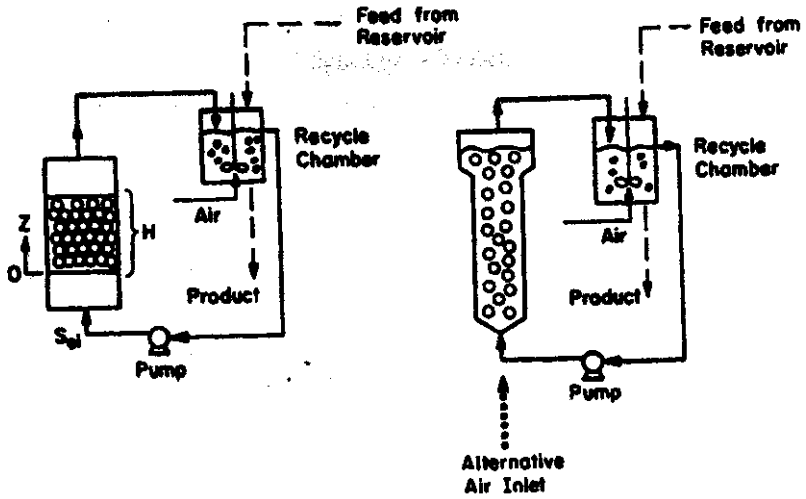



**Figure:** Schematics of packed – bed and fluidized – bed bio film or immobilized cell bioreactors are shown. In batch operation, only the streams with solid lines exist. In continuous operation, the streams shown by dashed lines are added. For the fluidized bed, fluidization can be accomplished by liquid recirculation only or a mixture of liquid and gas flows.

$$-F\, dS_0 = N_s a A\, dz$$

where $S_0$ is the bulk liquid – phase substrate concentration (mg S/cm$^3$) and is a function of height, F is the liquid nutrient flow rate (cm$^3$/h), $N_s$ is flux of substrate into the bio film (mg S/cm$^2$ h), a is the bio film or support particle surface area per unit reactor volume (cm$^2$ / cm$^3$), A is the cross – sectional area of the bed (cm$^2$) and dz is the differential height of an element of the column (cm) Substituting equation yields the following equation:

$$-F\frac{dS_0}{dz} = \eta \frac{r_m S_0}{K_s + S_0} LaA$$

Integration of equation yields

$$K_s In\frac{S_{0i}}{S_0} + \left(S_{0i} - S_0\right) = \frac{\eta r_m LaA}{F} H$$

where $S_{0i}$ is the inlet bulk substrate concentration, L is the bio film thickness or the characteristic length of the support particle ($L = V_P / A_P$) and H is the total height of the packed bed.

For low substrate concentrations in the feed, the rate of the substrate consumption is first order and equation has the following form:

$$\text{In}\frac{S_0}{S_{0i}} = -\frac{\eta r_m LaA}{FK_s}H$$

Substrate concentration drops exponentially with the height of the column in this case, and a plot of In $S_0$ versus H results in a straight line. Equation can be used as the design equation for immobilized – bio film column reactors to determine the height of the column for a desired level of substrate conversion.

**8. Glucose is converted to enthanol by immobilized S. cerevisiae cell entrapped in Ca-alginate beads in a packed column. The specific rate of ethanol production is qp = 0.2 g ethanol / g cell – h, and the average dry –weight cell concentration in the bed is $\overline{X} = 25$ g/l bed. Assumed that growth is negligible (i.e., almost all glucose is converted to ethanol) and the bead size is sufficiently small that $\eta \cong 1$. The feed flow rate is F = 400 1/h, and glucose concentration in the feed is $S_{0i}$ = 100 g glucose / 1. The diameter of the column is 1 m, and the product yield coefficient is $Y_{p/s} \approx 0.49$ g ethanol/g glucose.**
**a. Write a material balance on the glucose concentration over a differential height of the column and integrate it to determine S = Sz) at steady state.**
**b. Determine the column height for 98% glucose conversion at the exit of the column.**
**c. Determine the ethanol concentration in the effluent.**

**Solution:**

a. A material balance on the glucose concentration over a differential height of the column yields.

$$-F\,dS_0 = \frac{q_p\overline{X}}{Y_{p/s}}dV = \frac{q_p\overline{X}}{Y_{p/s}}A\,dz$$

Integration yields

$$-F\int_{S_{oi}}^{S_n} dS_o = \frac{q_p\overline{X}}{Y_{p/s}}A\int_0^H dz$$

$$S_{oi} - S_0 = \frac{q_p\overline{X}}{Y_{p/s}}\frac{A}{F}H$$

This equation differs from the form of equation because $S_{oi}$ is high and the reaction rate is effectively zero order.

b. $S_0$ = 0.02 (100) = 2 g glucose / 1. Substituting the given values into the equation yields

$$(100-2)=\frac{(0.2)(25)}{0.49}\frac{(\pi/4)(10)^2}{400}H$$

H =49 dm=4.9m

c. $P=Y_{p/s}(S_{oi}-S_o)=049(98)=48g/1.$

**9. Explain Immobilization Enzymes Reactions.**

**Immobilization Enzymes Reactions:**

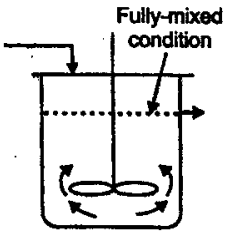
Inspite of the low enzyme activity, immobilized enzymes systems are useful for various process application. Mainly the advantages considered include, their amenability to use in continuous system, ease in removal of immobilized pellets for reuse or recycling and separation of enzyme free product in the final step.

The immobilization enzyme systems are mostly used in continuous operations. The commonly used continuous type of bioreactors are (a) Plug flow reactors (b) Continuous stirred tank reactor (c) Packed bed reactor and (d) fluidized bed reactors.

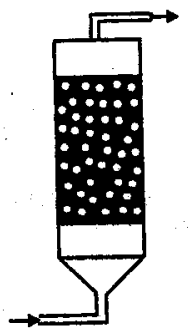
In continuous stirred tank reactors fully mixed zones are available for proper interaction between the enzymes and substrate. The only disadvantage would be disruption of enzyme pellets due to shear force.

The bioreactors with low hydrodynamic shear will be useful. These bioreactors include, fluidized bed, air lift or packed column.

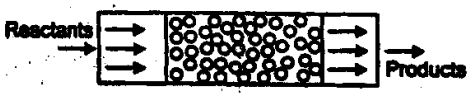
Fluidized bed bioreactors exhibit the good features of continuous stirred tank and packed bed bioreactor.

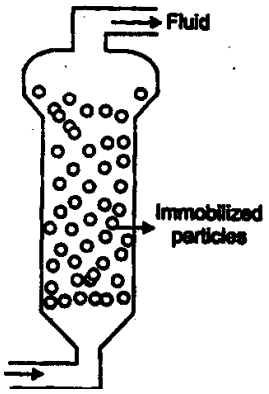



**(a) Continuous stirred – tank fermenter**



**(b) Packed – bed reactor**




**(c) Plug – flow reactor** **(d) Fluidized – bed reactor**

**Figure: various types immobilized reactors**

**10. Write short notes on,**
**(i) Passive diffusion**
**(ii) Transport diffusion**
**(iii) Active transport**

**Transport Molecules Across Cellular Membranes:**

To retain metabolic activity and to grow, the cell has to take nutrient from its extra cellular environment. The regulation of metabolic activity depends on which nutrients enter the cell and at what rate. The energy independent or energy dependent mechanism will be useful for molecules to enter the cell. Passive diffusion and facilitated diffusion are two examples of energy independent uptake. Active transport and group translocation are the examples of energy dependent uptake mechanism.

**Passive diffusion:**

In passive diffusion molecules move from high to low concentration

$$J_A = K_P (C_{AE} - C_{AI}) \quad \text{---------- (1)}$$

Where $J_A$ = flux of species 'A' across membrane

$C_{AE}$ = Extracelluar concentration of species A ($mol/cm^3$)

$C_{AI}$ = Intracellular concentration

The cytoplasimic membrane consists of a lipid core. The permeability $K_p$ is very low for charged or large molecules with very small flow of material across the membrane. Passive diffusion facilities the cellular uptake of water and oxygen. Lipids or highly hydrophobic compounds have diffusion in cellular membranes due to passive diffusion.

**Facilitated (Transport) diffusion:**

In facilitated diffusion, a carrier molecule (protein) combines specifically and reversibly with molecules. The membrane may b e embedded with a carrier protein. Probably the carrier protein after binding the target molecule, undergoes conformational changes resulting in the release of the molecule on the intracellular side of the membrane. The carrier protein binds to the target molecules on the intercellular side of the membrane and releases the molecule from the cell. So that net flux of a molecules depends on the gradient of concentration. The solubility target molecule is increased due to carrier protein. Since the binding of molecule to carrier protein can be saturated, the dependence of flux rate of the target molecule on the concentration will be different than as shown in equation (1).

The equation for the uptake of facilities transport is

$$J_A = J_{A\,max}\left\{\left[\frac{C_{AE}}{K_{MT}+C_{AE}}\right]-\left[\frac{C_{AI}}{K_{MT}+C_{AZ}}\right]\right\} \qquad \text{---------- (2)}$$

where

$K_{MT}$ = The binding affinity of substrate of substrate (mol/cm$^3$)
$J_{A\,max}$ = Maximum flux rate of A (mol/cm$^2$ – s)

The net flux will be into the cell when $C_{AE} > C_{AI}$

When

$C_{AE} > C_{AI,}$ there will be a net flux A from the cell

In eukaryotic cells, sugars and other low molecular weight organic compounds are diffused due to facilitated transport, but is not very common in prokaryotic cells. Nevertheless, the uptake of glycerol in E – coli is due to facilitated transport.

**Active Transport:**

In active transport the proteins are embedded in cellular membrane like facilitate transport. The active transport across against a concentration gradients. The intercellular concentration of molecule is much more greater than the extra cellular concentration. The movement of molecule

up concentration gradient is not favorable thermodynamically and will not happed spontaneously, so energy must be supplied. Various energy sources that can be applied in active transport are,

(a) Electrostatic or pH gradients of the proton motive force
(b) Secondary gradients derived from the proton motive fore by over active proton systems i.e., $Na^+$ or other ions and hydrolysis of ATP. The proton motive force, results from the extrusion of hydrogen as protons. Such gradients are formed in the respiratory system of cells.

For active transport an equation analogus to Michaelis – Menten Kinetics can be written to describe uptake,

$$J_A = J_{A\,max}\left[\frac{C_{AE}}{K_{MT} + C_{AE}}\right] \qquad \text{---------- (3)}$$

Equation 3 is apt, when the cell is in energy – sufficient state.

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# BT3501-GENETIC ENGINEERING

## UNIT – I BASICS OF RECOMBINANT TECHNOLOGY

Role of genes with cells, Genetic elements that control gene expression, Restriction and modifying enzymes, safety guidelines of recombinant DNA research

## PART – A

**1. Define gene.**

A Gene is the length of a DNA strand that contains all the bases required to dictate the synthesis of particular polypeptide. Polypeptides are the basic unit of proteins. Individual genes contain promoter and operator region which are required for expression of proteins.

**2. Describe the role of genes in synthesis of proteins.**

The gene in length of DNA in cruder set of codons i.e., coding sequences that corresponds to the amino acid sequence of polypeptide to be synthesized. The other gene sequences called non – coding sequences which are involve in regulation of gene expression.

**3. Define regulation of Gene expression.**

In all the organisms like bacteria, virus, bacteria – phages and higher organisms, the development of call and organisms is a reflection of co-ordinated and/or sequential expression of specific genes, and this chronological pattern of gene expression. This models were presented by authors example operant model.

**4. Although all human cell to have the same genes, they are not identical in their expression – why?**

All the genes present in the human cell is almost identical from one cell to other cell. But the expression of individual genes of the human cell is not uniform, because different gene products [Protein (or) enzymes] are required by cell in different period of time of situation and not required at the same period and same location for cellular functions. Hence, the expression of individual genes are not identical in human cell.

**5. Identify the DNA elements responsible for transcription of eukaryotic gene.**

1. Promoter for RNAP - I

It is GC rich elements in upstream control element (UCE). The region is – 180 to – 107

2. Promoters for RNAP – II

A core promoter region, - 40 bp direct transition of RNAP II

3. TATA BOX (A – T rich sequence) located – 30 upstream of start site.

4. This TF II B recognizing element located immediately after the upstream of the TATA box.

5. Inr (Initiator) element – conserved sequence

6. DPE (Downstream promoter element) – conserved sequence located of – 30 bp downstream site.

**6. Define the structure and function of gene.**

A gene, is a stretch of DNA (RNA in some viruses) that contain the coding sequences for the amino acids in a polypeptide as well as sequence modifies recognized by various enzymes and protein factors that are required to transaxle and translate the gene.

**7. What are coding sequences (CS)?**

A typical simple gene consists of a coding sequence (CS) that is flanked on both sides by DNA that contains the regulatory motifs. The most essential motif is upstream (towards the 5′ – end of the DNA) of the CS and is that of the promoter (P).

**8. Write short notes on promoters.**

The promoters in bacterial and viral genes usually contain a consensus sequences of 7 bases, named the Pribnow box, after its discover. Eukaryotic genes have one or more RNA Pol – binding motifs that include the TATA and the CAAT motifs. The TATA is proximal to the CS and the CAAT is distal to it.

**9. What are 5′ flanking and 3′ flanking regions?**

The unstranslated region between the 5′ – end and P and between T and the 3′ end of the gene are referred to as the 5′ flanking and 3′ – flanking regions, respectively.

**10. What are mobile or transposable genes?**

The mobile gene is on a transposable element in the DNA that has characteristic repeated sequences at its two ends. A copy of the transposal element is integrated into another region of the same DNA or different DNA. Some transposable elements i.e., the transposans, carry

sequences of one or more genes.

**11. What are extra chromosomal genes?**

Some species of bacteria posses additional small circular chromosome in their cell cytoplasm in addition to regular chromosome. These small, circular extra chromosomes are called plasmids and their respective genes are extra chromosomes genes. These extra chromosomes (or) plasmid genes have certain special functions as drug – resistant genes, antibiotic genes etc,

**12. What are plasmids?**

Plasmids are small, circular extra – chromosomal DNA present in certain species of bacteria in addition to that of regular genomic chromosomal DNA. The plasmid genes are having some special functions as tax in producing genes, antibiotic producing genes, drug resistant genes, Nitrogen fixing genes etc.

**13. Write the special features of plasmids.**

Plasmids are extra chromosomal mini DNA present in certain bacterial species. The genes present in plasmids are useful for survival of bacteria is certain adverted conditions. Plasmids are having multiple cloning sites; hence it is used for cloning of foreign genes.

Plasmids are also used as vector molecules to transfer the foreign genes in organisms. Plasmids are self replicative DNA molecules.

**14. What are a multiple cloning sites in a plasmid vector? Give an example.**

Multiple cloning sites (MCS) is a short DNA sequence, 2.8 kb in the case of PUE19 canijing sites for many different restriction genes to insect in to them without existing vector sequences. Many different foreign genes form various sources are possible to clone in planned vector MCS and for cloning.

Example: PBR 322
PUE 18

**15. What are restriction enzymes? Give an example?**

Restriction enzymes or restriction endonucleases are the enzymes used in genetic engineering technique to cut or leave the DNA molecule. These enzymes recognize unique base sequence motifs in a DNA strand and leave the backbone of the of the DNA molecule at a place

within or, at 'some' distance from the recognition site. The DNA is thus cleaved into defined and discrete fragments by a particular restriction enzyme.

Example: Eco RI, Hin d III

**16. Compare the exonucleases and endonucleases.**

Exonucleases and endonucleases are two types of nuclease enzymes or restriction enzymes. The exonucleases remove one nucleatide at a time, starting with the 5′ – or 3′ – end of a DNA strand.

The endonucleases cleave the DNA backborne between two nucleotides – one type at the bond between the 3′ – end of a nucleotide and the phosphate and the other type between 5′ – end and the phosphate.

**17. Explain briefly on type I, type II & type III restriction endonucleases.**

i) Type I enzymes have fairly long recognition motifs, but lave the DNA perhaps at random points at least more than 1 kb away from the motif. Type I enzymes require ATP, $mg^{++}$ and s – adenosyl methionine besides the catalyst.

ii) Type II enzymes recognize motifs that are 4 – 7 bases long, and mostly cut at a specific place within this motif; a few are known to leave a few bases to the 3′ – end of the motif. The site of cleavage by Type II restriction enzymes is specific. These were the enzymes that launched recombinant DNA techniques. Type II enzymes need only $mg^{++}$.

**18. Write short notes on EcoR I and Hind III.**

EcoR I and Hind III are restriction endonucleases or restriction enzymes. EcoR I obtained from E.Coli, hence the name EcoR I. R for restriction enzymes and Roman letter I for type.

Hind III is obtained from Heamophilus indicus, hence the name called.

EcoR I recognize 5′ GAATTC 3′ and cut on left side of the central axis generate free 5′ ended trails.

Hind III recognize 5′ AAGCTT 3′ and cut on left side of the central axis.

**19. Where the DNA fragment having EcoR I end and blunt end can be cloned into a plasmid**

**having the MCS Bam HI – EcoR I – Hind III – Sma I – Not I?**

EcoR I recognize

5'–*GAA*↓*TTC*–3'
3'–*CTT*↑*AAG*–5'

*Bam HI – G*↓*GATCC*

*EcoR I – G*↓ *AATTC*

*Hind III – A*↓ *AGCTT*

*Sma I – CCC*↓ *aaa*

*Not I – GC*↓ *GGCC*

**20. What are blunt ends? Give Examples.**

When the DNA molecule is cleaved by a particular restriction enzymes, the free ends or tails produced are not aligned or not co-hesive themselves. These blunt ends are not sticky and joining or linking of these cut DNA molecules require special step with certain molecules called linkers.

Example: Sma I fields 5′ CCC↓-------
--------↑GGG 5′

**21. Write the significance of sticky or co-hesive ends.**

The tails of two generated frequents are complementary each other and it allowed to join with a ligase enzyme, the frequents will align themselves by the hydrogen bonding between the complementary bases. These tails are referred as 'cohesive' or 'sticky' ends. The new DNA molecules or cloned DNA molecular are produced when these type of frequents generated with particular restriction enzymes and it is much significant in genetic engineering.

Example: EcoRI & Hind III

**22. What is mean by chromosome walking?**

By cutting a length of DNA with one or more restriction enzymes by recognition of motifs spatially defined locations in the genome. This is otherwise restriction mapping or physical map. These fragments identified with loci of particular genes. Thus the restriction fragments are used also for charting the complete sequence of DNA by what is known as chromosome walking.

**23. Write short notes on ligase.**

Ligase is a another important enzymes used in genetic engineering experiments to produce cloned DNA molecules. Here, the ends of DNA strands are joined by an enzyme polynucleotide ligase generally called ligase. Ligase catalyses the formation of a phosphodiester bond between the 3′ – hydroxyl and the 5′ – phosphate terminals of the two nucleotides. Thus, the enzyme able to joined unrelated DNA, repair nicks in single strands of a DNA and join the sugar – phosphate backbones of the newly repaired and resident region of a DNA strand.

**24. How the ligation of DNA fragments performed?**

The ligation reaction can be performed in a test tube or inside a cell. In this reaction, the ends of DNA fragments must posses cohesive or sticky ends. This reaction is controlled by $p^H$, temperature, concentration and kinds of sticky or cohesive ends and etc., As the cohesive ends are held together only by hydrogen bonds, the temperature at which ligation is performed becomes very important. At temperature above 15°C, the single strand tails are liable to dissociate.

**25. What are the enzymes which modify the DNA molecular?**

i) Alkaline phosphate
ii) Polynucleotide kinage
iii) Exonuclease III
iv) DNA I
v) Mung Bean and SI nucleases
vi) DNA polymerases and SI nucleases
vii) DNA polymerases and the klenon fragment
viii) Termingl deoxynucleotidyl transferees
ix) RNA dependent DNA polymerase
x) RNAses
xi) RNAse H

**26. Write the function of alkaline phosphates.**

The enzyme alkaline phosphatase removes the phosphate moiety at the 5′ – end of a DNA strand, whether it is part of a blunt end single strand extension or a recessed end of double – stranded DNA. The $PO_4$ at an RNA terminal is also removed by this enzyme.

**27. How the microorganisms classified for its pathogenecity?**

i) Class – 1 - Not pathogenic
ii) Class – 2 - May cause human disease
iii) Class – 3 - Threat to the health of lab workers but small risk.

iv) Class – 4 - Cause severe illness in human beings
v) Class – 5 - Heavy threat to population lead economic loses.

**28. What are the different biosafety levels available in recombinant DNA research?**

| | | |
|---|---|---|
| i) Biosafety level – 1 | - | Provides safety equipment and facility design of construction where viable organism are not known to cause disease. |
| ii) Biosafety level – 2 | - | This is for risk agents are available in the environment |
| iii) Biosafety level – 3 | - | This practice available where the potential letter infection caused by organism |
| iv) Biosafety level – 4 | - | Safety available for work done with dangerous organism are used. |

**29. What are isoshizomeres?**

Isoshizomers are the type of restriction enzymes with same sequence specificity and cut site. These are novel DNA sequence specification occasionally occur in enzymes which prove to have same specificity.

Neoshizomers are enzymes that recognize the same sequence but leave at different points.

**30. What are Simple genes?**

Simple genes consist of only coding sequences in the gene region. This coding sequences flanked in both sides of by DNA. DNA or gene contains regulatory motifs. Regulatory motifs consist of upstream elements and downstream elements.

**31. What are split genes?**

Split genes contain both the coding sequences (exons) and non-coding sequences (introns) in the gene region. This involve in synthesis of fashion specific proteins in different developmental periods or in tissues.

**32. What is overlapping of genes?**

More than one gene present in the same stretch of gene region or DNA region. There the gene is collinear with the Amino acids. It appears to maximize the genetic information. Overlapping genes not expressed simultaneously.

**33. Write short notes on gene cloning.**

Gene cloning is a technique of Genetic Engineering or recombinant DNA technology (rDNA) in which foreign genes are introduced into the DNA of organisms through vectors or any other methods. The outcome is the production of identical copies of genes in the DNA of organisms which are more value than the normal one.

**34. Write any two applications of gene cloning.**

(i) To produce hybrid vigours of plants which are high value for society.

Eg. Bt Cotton – insect resistant cotton.

(ii) To produce high quality protein contents its animals.

Eg. Meat, Milk & Poultry.

# PART – B

**1. Discuss the structure and functions of genes.**

**Anatomy of a Gene**

A gene, as mentioned earlier, is a stretch of DNA (RNA in some viruses) that contains the coding sequences for the amino acids in a polypeptide (or the complementary sequences for an rRNA or tRNA product) as well as sequence otifs recognized by various enzymes and protein factors that are required to transcribe and translate the gene into its product. These latter are the control or regulatory sequences.

A typical simple gene consists or a coding sequence (CS) that is flanked on both sides by DNA that contains the regulatory motifs (Figure 1.11). The most essential motif is upstream (towards the 5′ – end of the DNA) of the CS and is that of the promoter (P); the transcribing enzyme RNA polymerase (RNA Pol) binds to the promoter before it begins transcription.

The promoters in bacterial and viral genes usually contain a consensus sequence of 7 bases, named the Pribnow box, after its discoverer. Eukaryotic genes have one or more RNA Pol-binding motifs that include the TATA and the CAAT motifs. The former is proximal to the CS and the latter distal to it.

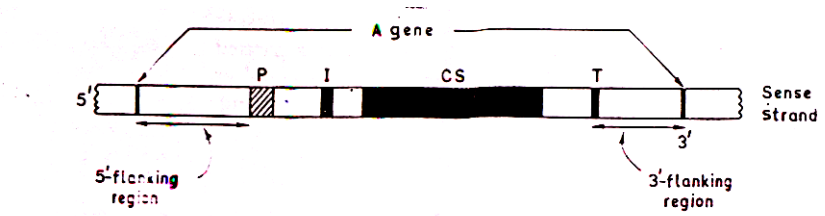



**Fig. 1.11 Anatomy of a simple gene. The coding sequence (CS) is uninterrupted, A promoter (P) and other transcription initiation/repression control sequences are present upstream (towards the 5′-end of the sense strand) of the CS. Termination (T) and other signals are present downstream (on the 3′-side) of the CS. The untranslated regions between the 5′-end and P and between T and the 3′-end of a gene are referred to as the 5′-flanking and 3′-flanking regions, respectively.**

About 10 bases from the centre of the Pribnow box (ATAPyTAPy, where by is a pyrimidine) and between the motif and the start of the CS is the initiation (I) base. The first nucleotide in an RNA is the complement of this base. The region between I and CS is called the antileader region and is transcribed into the leader region in the RNA. The leader attaches itself to the translating apparatus (small unit of the ribonucleic acid – protein complex called the ribosome) by virtue of a sequence (called the Shine – Dalgarno or SD) that is complementary to a region of the rRNA which is part of the small ribosomal unit.

The region upstream of P (5′ flanking region) usually contains one or more recognition sequences for transcription factors and other regulatory signals.

The region downstream (towards the 3′ – end of the molecule) of the CS may carry one or more recognition motifs that are involved in the termination of transcription and in most eukaryotic genes for a step in the post – translational processing of the mRNA. The region downstream of the termination sequences may also possess regulatory sequences which are not clear. A gene without the 3′ – flanking region (between the T or termination signal), and the 3′ – end of the gene, is not transcribed. Hence, this uncharacterized region is also an integral part of the gene.

The preceding discussion has been centered on what is known as the 'simple gene'. In such a gene the CS region is one block of sequences that does not contain any intervening non – coding bases. Most eukaryotic genes appear to be what have been named as split genes (Figure 1.12). In these genes, the CS consists of blocks of coding sequences (exons) alternating with blocks of sequences that do not specify amino acids (introns). These genes are versatile, as different sets of exons may be utilized to fashion specific proteins in different developmental periods and/or tissues. The exons required for a particular polypeptide are joined together and the polypeptide translated from the tailored sequence. This operation is carried out on the RNA and not on the original DNA. The gene is initially transcribed fully into a heterogeneous on hnRNA, which is then 'spliced' appropriately (Figure 1.13). The introns and exons, if any, between two exons that are to be juxtaposed are cut out and degraded. The joining is precise, so that there is not shift in the reading frame in the RNA message. Addition or loss of even one base at the exon – exon junction would result in a garbled message that would specify an abnormal sequence of amino acids.

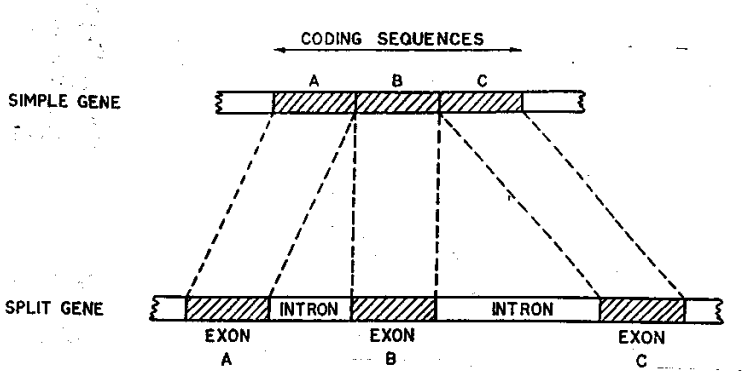



**Fig. 1.12 A split gene versus a simple gene. The former possesses modules of coding sequences (exons) separated by intervening sequences (introns). All sets or a particular set of the exons may be joined together (in the mRNA) to form a functional transcript that is translated.**

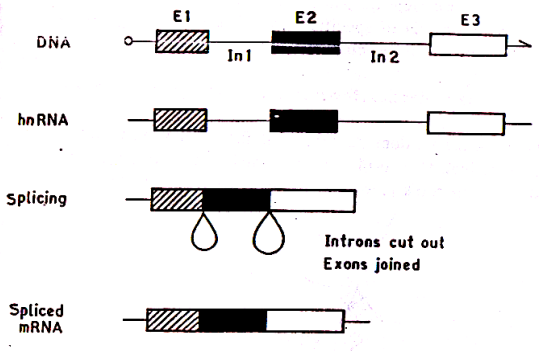



**Fig. 1.13 Splicing of the mRNA of a split gene. Different ways of splicing have been discovered. In all of them, the ends of exons and regions of the introns possess consensus sequences which are involved in the exact splicing of the exons. The introns are cleaved out and degraded.**

The coding sequence of a gene is collinear with the amino acids that they represent. This does not prevent the occupation of a common stretch of DNA by the sequences of more than one gene. Several viruses possess overlapping genes. It appears to be one way of maximizing the genetic material as a source of genetic information. Of course, the overlapping genes are not expressed simultaneously. Developmental control regulates the sequences and time of transcription of the separate genes.

A further concession had to be made in the molecular definition of gene, when the anatomy of immunoglobulin genes was exposed. An immunoglobulin (Ig, or antibody, in common parlance molecule consists of one or more units. Each unit contains two identical subunits. Each subunit is composed of two polypeptides, a longer or heavy (H) chain and a shower or light (L) chain. The H and L chains of each subunit are held together by disulphide bonds, so are the two H chains of the Ig molecule. Each subunit has one end (the aminoterminal) that binds itself to a matching antigen (a molecular moiety foreign to the animal in question). The end called the variable or V region has several alternate blocks of codons of which only appropriate ones are joined together (in response to the antigen) to form a functional gene. (Here, the unwanted DNA is cleaved off, in contrast to the situation in RNA splicing). The tailored V region is translated to form the series of amino acids that bind themselves to the antigen.

Both H and L chains have V regions ($V_H$ and $V_L$). The remainder of the Ig polypeptides are called constant (C) regions ($C_H$ and $C_L$). The V and C regions are specified by distinctly different genes, but the latter are not expressed separately. The $V_H$ and $C_H$ regions are linked through a joining (J) region on the same stretch of DNA. After formation of the functional V region, it is linked to a particular alternative subset of the J gene. The V – J joint DNA is then joined to an appropriate C region (which also possesses alternate blocks of coding sequences). The final V – J – C DNA is transcribed and translated. A similar processing occurs in the C chain gene. The Ig genes have been named as variable genes. In this case, different genes (each with variable components) are first linked together before producing one final polypeptide.

Two other types of genes (in terms of behavior and not in terms of the molecular definition) have been identified. These are the mobile or transposable genes and the processed genes. The mobile gene is on a transposable element in the DNA that has characteristic repeated sequences at its two ends (Figure 1.14). A copy of the transposable element is integrated into another region of the same DNA or a different DNA. Some transposable elements, i.e., the transposons, carry sequences of one or more genes. The latter may thus be transferred to a new site in the same genome or a different genome. Some of these elements appear to regulate certain developmental events by stationing a gene next to the one on the recipient DNA.

Now we come to the discovery of extrachromosomal genes and DNA. The essential blueprint for a species resides in the chromosome(s) of its cell. Some species, particularly bacteria, possess, in addition, DNA molecules in the cytoplasm. These are small circular molecules called plasmids. Plasmids may carry transposons containing genes for certain special functions that include drug – resistant genes, toxin – producing genes, tumour – inducing genes, nitrogen – fixing genes and others. These genes are not essential for the survival of the individuals carrying them, but do provide certain extra advantages in particular environments (Figure 1.15).

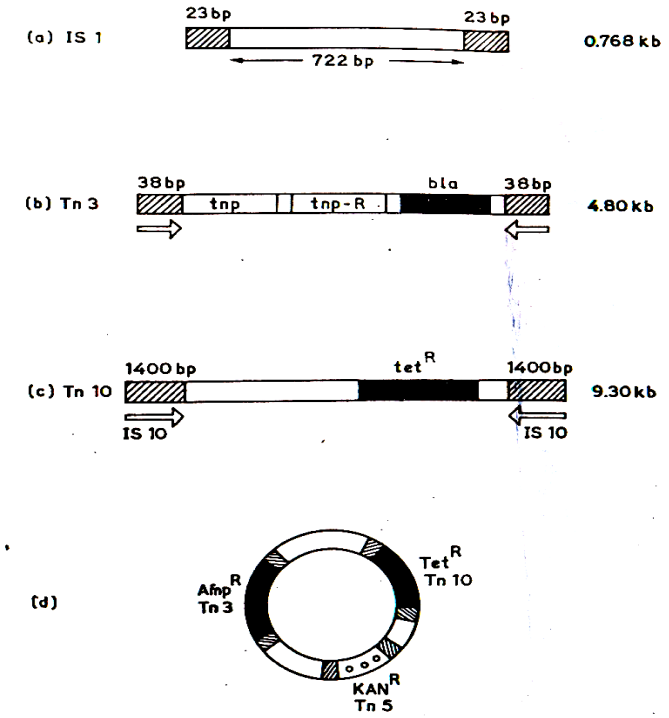



**Fig. 1.14 A transposon is a DNA unit with characteristic repeated sequences (direct, inverted, simple, compound) at the two terminals and a gene sequence. Terminal repeats are involved in the transposition of a copy of a transposon to a different site in the same or a different DNA. Many drug-resistant genes (Amp$^R$, Tet$^R$, Kan$^R$) are on transposons, which, in turn, are within bacterial plasmids or on chromosomes. Transposition may result in different base alterations in the host DNA at the juncture of the transposon and host DNA. Transposition may also join two non-homologous circular DNA into one molecule (cointegration).**

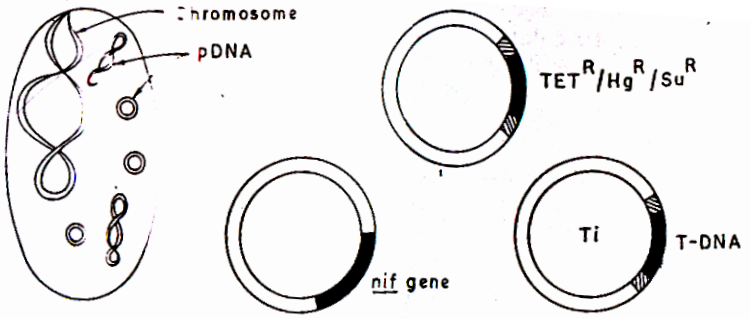



**Fig. 1.15** Plasmids are small covalently closed circular (CCC) ds DNA molecules found mainly in prokaryotic cells. Some eukaryotes have also been discovered to harbour plasmids 2 µm of yeast, Ddp of slime mold, P element of *Drosophila*, and possibly in maize. sorghum and *Vicia faba*). Plasmids usually possess one or more genes which provide special advantages to the cell carrying them. They may carry antibiotic, drug or toxic metal-resistant genes, as well as ones with specialized functions, such as the *nif*-genes of nitrogen-fixing organisms, and tumour-causing genes of the Ti plasmid of *Agrobacterium tumefaciens*. Plasmids are usually in a supercoiled state.

**2. Discuss in detail on Gene expression and regulation of gene expression.**

As the two strands of a gene are complementary, it is obvious that the coding sequences in one strand will be different from the codons that are complementary to them. This was proved conclusively in the case of two genes in a bacterial virus, the bacteriophage T4. A gene, therefore, is only on one strand of a DNA.

RNA Pol binds itself to the P region of a gene and begins the synthesis of an RNA polymer starting with a base complementary to 1 (Figure 1.16). Initiation of transcription involves a complex set of events in which one of the subunits (the sigma) of the RNA Pol is required to bind the enzyme firmly to the DNA. Soon after transcription is initiated, the sigma subunit gets detached from the core enzyme. The latter proceeds to elongate the RNA until it reaches the T signal in the 3′ – region of the gene. At this point, the enzyme, the RNA and the DNA become separated; the core enzyme reunites with the sigma and begins another round of transcription. This is the basic process of transcribing the message of a gene into an RNA molecule.

RNAs of enkaryotes are processed before they can be translated. This involves adding a methylated ‘cap’ to the first few bases in the RNA, a polyadenine tail in the trailer region (between the CS and T) and splicing, if the gene is a split one.

The mature of functional mRNA is then translated. The translation apparatus consists of the mRNA, the ribosome (with a smaller and large subunit), and tRNAs, each specific for binding to a particular amino acid.

The initiation of translation is a complex process, at the end of which the 5′ – SD region of the mRNA becomes bound to the 16S rRNA in the smaller subunit of the ribosome (in bacteria). The larger subunit joins the initiation complex to form the ribosome with the mRNA running through a groove – like space between the two subunits. There is space in the groove for only two codons.

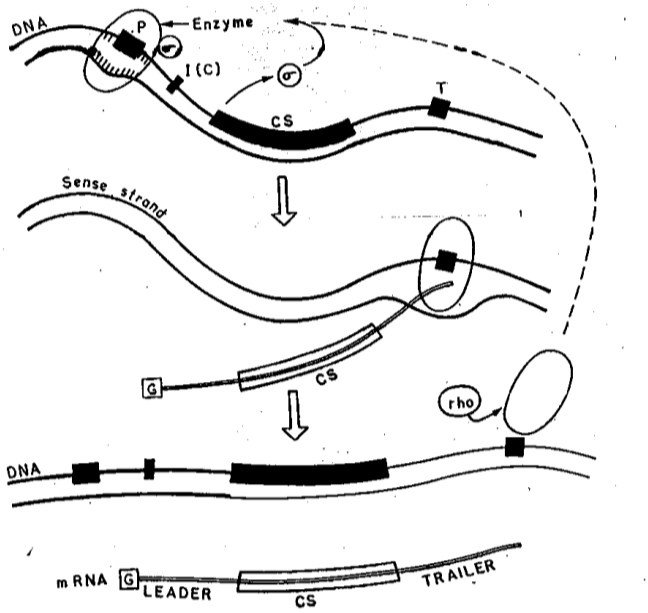



**Fig. 1.16 Transcription or copying of a DNA instruction into an RNA message.**

The first codon in a CS is always that (AUG) for methionine. When the ribosome is fixed with the mRNA, the AUG is in the second position. The amino acid methionine is brought by the methionine – specific tRNA to the ribosome. Further, tRNAs possess three loops each in their secondary structure, the middle one of which has the anticodon (complementary bases) for the codon for the amino acid they represent. So, in the preceding example, the anticodon of $tRNA_{met}$ forms hydrogen bonds with the AUG. The ribosome slides backwards by one codon length. The $tRNA_{met}$ is now in the 1st position in the groove. The tRNA with the anticodon for codon 2 now binds itself to the latter; it is bound to its specific amino acid. When amino acids 1 and 2 are side by side, a peptide bond is formed between them. This releases the first tRNA from its amino acid and the ribosome moves one codon backwards. The third codon is bound to the anticodon of the tRNA for the third amino acid. The second and third amino acids are joined. In this way, the entire coding sequence is decoded into the amino acid chain, that begins with the $–NH_2$ end of the molecule.

The last codon in the CS has no representative tRNA. Hence the polypeptide chain stops elongating when it reaches this last or stop codon. At this point, the ribosomal subunits, the mRNA the tRNAs and the new polypeptide are released from bondage. The apparatus is free to initiate a fresh polypeptide along the same mRNA.

The foregoing description presents in the briefest of outlines, the picture of how a gene is

expressed.

**Regulation of Gene Expression**

Jacob and Monod, while studying the induction and repression of certain enzymes in the bacterium E. Coli, discovered the molecular mechanism by which a gene is switched 'ON' or 'OFF'. In their particular case, they were examining the induction of genes the products of which are needed to metabolize the sugar lactose. Normally, E.coli thrives on glucose. When glucose is not available, the bacterium is able to use other sugars when induced with a minute quantity of the sugar in question. When glucose is available, the genes for utilization of the other sugars are in an 'OFF' position. In the absence of glucose and presence of the other sugar, the genes for the latter come into operation.

Jacob and Monod postulated that the protein decoded from a particular gene (inhibitor or regulator gene) competed with the RNA Pol for a place to bind itself on the DNA. It was discovered that the regulator protein can bind itself to a sequence that partially overlaps the promoter sequence. When the regulator, is bound to this region (called the operator) the RNA Pol cannot achieve transcription. The gene(s) down – stream of the P are thus shut 'OFF'. In the case of lactose utilization there are three genes which are turned 'ON' or 'OFF' by the same regulator, in one operation.

Jacob and Monod presented the Operon Theory on the basis of the detailed study of the lactose utilization genes of E.coli.

The three genes in this case are parts of the lactose or lac operon (figure 1.17). It is operated by the protein from the laclgene, which is not part of the operon. Binding of the I protein to the operator prevents transcription of the lac operon genes lac Z, lac Y and lac A. Of these, lac Z specifies the enzyme galactosidase – that mediates the separation of the glucose and galactose components in the lactose disaccharide molecule.

Extensive investigations have since revealed other factors that regulate the expression of a gene. The basic molecular principle is, however, still the same. A regulator protein either blocks the binding of the RNA Pol by itself or by complexing with another (effector) molecule. Conversely, complexing of the regulator with an effector molecule may also make it unsuitable for binding to the operator region. In such a case, the RNA Pol is free to initiate transcription.

In higher organisms the signal for a particular gene to be expressed may be in the form of chemical messenger, or hormone, antigen, mitogen (mitoses – generating substance) or others. Homones may be polypeptides or steroid based. The former bind themselves to the cell surface; the signal is transduced through the cell membrane to a second messenger which sets in motion a

series of biochemical steps that eventually trigger a particular gene to be expressed. A steroid hormone moves into the cell and forms a complex with some receptor molecule, and it appears that it is this complex that makes a beeline for the DNA of the gene to be expressed.

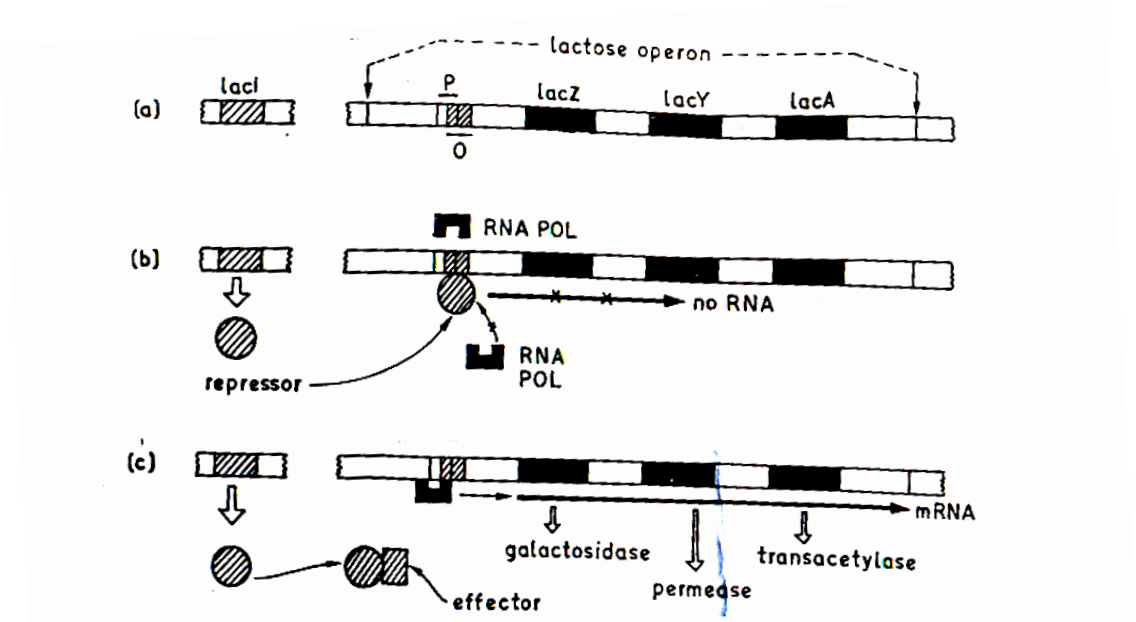



**Fig. 1.17** The *lac* (lactose) operon of *E. coli*. Three genes *lacZ*, *lacY* and *lacA* are under the control of *cis*-acting DNA elements *lacP* (promoter) and *lacO* (operator). The RNA polymerase binds itself to P, and the product of a gene *lacI* (repressor) binds itself to *O*. The *lac* operon may be induced to express the three genes by supplying a little lactose to the system. In practice an analogue IPTG is used as this, unlike lactose, is not degraded by β-galactosidase, *lacZ* encodes α- and β-subunits of the polypeptide β-galactosidase — an enzyme that mediates the conversion of the disaccharide (lactose) into the monosaccharides glucose and galactose.

**3. Write an essay on Restriction Endonucleases and it functions in recombinant DNA technology?**

**Restriction Endonucleases**

Ordinary nucleases are exonucleases or endonucleases. The former remove one nucleotide at a time, starting with the 5′ – or 3′ – end of a DNA strand. The latter cleave the DNA backbone between two nucleotides – one type at the bond between the 3′ – end of a nucleotide and the phosphate, and the other between the 5′ – end and the phosphate. In either case the polynucleotide chain is reduced to free nucleotides. A restriction endonuclease cleaves only at specific regions in a particular DNA, so that discrete and defined fragments, instead of free nucleotides, are obtained at the end of total digestion. These enzymes recognize certain short motifs of base sequences and cut the DNA within it or at some specified or unspecified distance from it.

The name ‘restriction’ endonuclease originated from an observation of a system of

restriction of the growth of the phage lambda in particular strains of the E. coli host cell. G. Bertain and J.J. Weigle (1953) had observed that when the phage lambda is grown in strain C of E. Coli, it does not fare well in strain K12. On the other hand, those grown in E. coli K 12 remain unaffected when grown in a fresh batch of K 12 cells. This was the first indication that E.coli or bacteria possess some system that preferentially restricts growth of phages as well as modifies the mechanism of restriction.

The system itself was identified by M.Meselson and R.Yuan in 1968. This time the DNA, and not the cells from strains C and K12 of E.coli were taken and treated with an extract from K12 cells. The DNA from the C strains was fragmented, but not that from the K12. The enzyme in the extract that cleaved the DNA was named 'restriction endonuclease'. This discovery was soon followed by another one: the protection from cleavage is due to methylated bases in the motifs or sites of recognition for the enzyme. All newly synthesized strands of DNA become methylated almost simultaneously with strand elongation, with the help of methylases. Phage DNA replicating in a host become methylated by the same enzymes. A phage DNA thus modified in one host strain is immune to the restriction enzyme of the same strain, but not to enzymes of others against which phage DNA have not been protected.

More than 600 different restriction enzymes have been identified, although all of them have not been characterized as yet. These enzymes have been classified into Type I, II and III. Type I enzymes have fairly long recognition motifs, but cleave the DNA perhaps at random points at least more than 1 kb away from the motif. This was the type of enzyme identified by Meselson and Yuan.-

Type II enzymes were first discovered by Hamilton O. Smith and associates, in 1968 in Hemophilus influenzae. Members of this class recognize motifs that are 4 – 7 bases long, and mostly cut at a specific place within this motif; a few are known to cleave a few bases to the 3' – end of the motif. In either case, the site of cleavage by Type II restriction enzymes is specific. These were the enzymes that launched recombinant DNA techniques.

Type III enzymes also cleave in a site – specific manner, but their other requirements for the cutting reaction are different from those of Types I and II. Type I enzymes require ATP, $Mg^{++}$ and s-adenosyl methionine besides the catalyst. Type II enzymes need only $Mg^{++}$. Type III enzymes require the same additional factors as Type I enzymes. The enzymes are also different in the three cases. Type II is a small monomeric protein. The type II enzyme is composed of two subunits, whereas the Type III is quite large with three subunits.

Restriction enzymes are obtained from bacterial species. Hence the name of the enzyme is an abbreviation of the first letter of the genus and the first two letters of the species name of the source bacterium; e.g., Eco RI, is from Escherichia coli. The last Roman numerical is the order in

which the enzyme of this type was discovered. An alphabet between the first three letters and the numerical is the strain designation, where specified.

Since Type II enzymes cut the DNA at a very specific bond within the recognition motifs, they are the only ones that are useful for use in recombinant DNA exercises. Type III enzymes also cleave in a site – specific manner, but the reaction mixture needs to be supplemented with S – methionine and ATP. The discussion here will be confined mainly to the features of the Type II enzymes.

The motifs for the Type II restriction enzymes are 4, 5, 6 or 7 bases long (figure 2.4). There is usually a rotational symmetry within this motif; that is, the sequence in each strand of the motif is palindromic. The motif may be cleaved asymmetrically, so that each cut end has a single – stranded extension, or may be cleaved at the same place on both strands. In this case the fragments are blunt ended. A few examples will illustrate the preceding text.

(i) The enzyme Eco RI and HindIII recognize motifs 5′GAATTC3′ and 5′AAGCTT3′, respectively, which are cut on the left side of the central axis, generating free 5′ – ended tails (Figure 2.5).
(ii) The enzyme Pst, on the other hand, cleaves its recognition motif to the left of the axis, generating 3′ – OH ended tails.
(iii) The enzymes HindII and Hae II produce blunt ended fragments by cleaving at the axis on both strands.

You may notice in the first two of the preceding examples that the 'tails' of the two generated fragments are complementary to each other; that is, if allowed to come near each other (in a solution) the fragment ends will align themselves by hydrogen bonding between the complementary bases. This apparently unspectacular event forms the foundation of genetic engineering. Of course even if the 'tails', which are referred to as 'cohesive' or sticky' ends, do align themselves to give the illusion of one continuous DNA instead of two fragments, the molecule is not truly one; there are gaps at the sites of cleavage, in each strand. The gaps could be closed only when the joining enzyme ligase was discovered.

By choosing the appropriate restriction enzymes, any two DNA molecules (or the opposite ends of the same duplex) can be made to align and become covalently joined with the addition of the enzyme ligase.

A few Type II restriction enzymes have been identified that cleave the DNA strands at a specific distance downstream from the 3′ – end of the recognition motif. One of them is Hga, which recognizes the penta – nucleotide 5′ G A C G C 3′, but cleaves 5 bases to the right in one strand (from the 3′ – end) and 10 bases to the right in the other (also counted from the 3′ – end) as

shown in figure 2.6. Also we have

5′ G A C G C N N N N N N 3′

3′ G T G C T N N N N N N N N N N 5′

Where N represents any nucleotide

| Enzyme | Motif |
|---|---|
| Eco RI | G ↓ A A T T C |
| Bam HI | G ↓ G A T C C |
| Sal I | G ↓ T C G A C |
| Hin dIII | A ↓ A G C T T |
| Acc I | G T ↓ A T A C |
| | G T C G A C |
| Eco RV | G A T ↓ A T C |
| Hin dII | G T C ↓ G A C |
| Hae I | T G G ↓ C C A |
| | A G G ↓ C C T |
| Hae III | G G ↓ C C |
| Sma I | C C C ↓ G G G |
| Pvu II | C A G ↓ C T G |
| Pvu I | C G A T ↓ C G |
| Hae II | G G C G C ↓ C |
| | A G C G C ↓ T |
| | C T G C A ↓ G |

**Fig. 2.4 Some restriction enzymes and the DNA motifs that they recognize. The arrows indicates the position at which, the endonuclease cleaves the DNA backbone.**

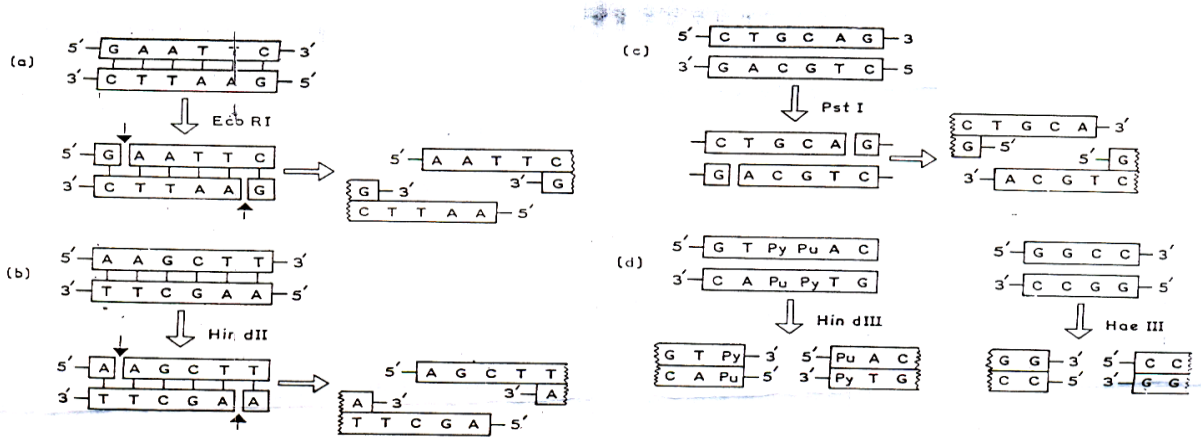
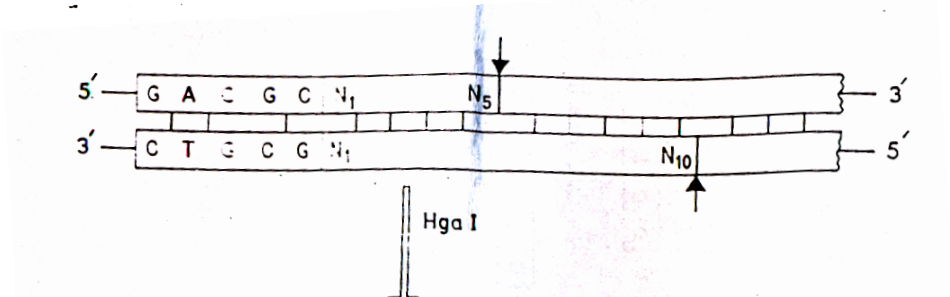



**Fig. 2.5 Some restriction enzymes generate single-stranded extensions at each end of the restriction fragment (e.g., *Eco* RI, *Hind*III and *Pst* I), while others create flush or blunt ended fragments (e.g., *Hind*III and *Hae* III).**



The sites of recognition of different restriction enzymes are inherent features of the DNA of a species. Hence, the exact pattern of distribution of the motifs in the total genome is theoretically identical in every member of a species. This allows one to cut different aliquotes of the same genome DNA with the same enzymes and recover identical arrays of fragments from each lot. Occasional deviations are observed in this restriction site map (hence the use of the term theoretically a little earlier). These deviations are due to mutations and have special significance for certain types of investigations and, so, are exploited for the particular goals.

The presence of recognition motifs for restriction enzymes in spatially defined locations in the genome has also been exploited to prepare what are referred to as 'restriction maps'. By cutting a length of DNA with one or more restriction enzymes, one can generate a 'physical' map of the DNA in terms of specific fragment lengths and the consecutive position pf each fragment. This fact has been of tremendous practical value; not only can the DNA be mapped in terms of physical distance but also specific restriction fragments may be identified with loci of particular genes. Restriction fragments are used also for charting the complete sequence of a DNA by what is known as 'chromosome walking'.

Restriction enzymes have to be extremely pure (not contaminated with any substance from the cell extract or reagents) to be able to mediate fragmenting of DNA. It is customary, therefore, to buy them from suppliers who guarantee their purity. In any event, before undertaking a 'cutting' job, the activity of the enzyme and the proportions of ingredients in the reaction mixture, which are optimum for the purpose, have to be assessed in a trial run.

Some kinetics of the restriction enzyme action is known, but not enough to be able to control such action with confidence. The manner in which the enzyme protein binds itself to the DNA motif has been recently indicated from X – ray diffraction and other studies of Eco RI. For the practical genetic engineer, however, lack – of sufficient knowledge of the physics of the systm has not been a bottleneck so far.

The large variety of restriction enzymes, some of which alter their specificities for

recognition at different temperatures, provides a very versatile tool for adding, removing, sequencing or otherwise engineering the DNA molecule.

**4. Write short notes on Enzyme Ligase.**

**Ligase**

Ends of DNA strands may be joined by the enzyme polynucleotide ligase. The enzyme catalyzes the formation of a phosphodiester bond between the 3′ – hydroxyl and the 5′ – phosphate terminals of two nucleotides. The enzyme is, thus able to join unrelated DNA, repair nicks in single strands of a DNA and join the sugar – phosphate backbones of the newly repaired and resident region of a DNA strand.

The reaction of ligation requires a source of energy. The ligase extracted from phage T4 infected cells (phage T4 lig. Gene or gene 30 encoded) requires ATP, and the E. coli ligase utilizes NAD for the purpose. The latter joins DNA fragments with sticky ends, and the T4 ligase manages to join flush – ended fragments. The T4 enzyme is obtained commercially from the T4 lig. Gene cloned in a phage lambda vector with which the E.coli K12 strain has been lysogenized.

The ligation reaction can be performed in a test – tube or inside a cell. It must be remembered that if the recombinant DNA is to be ligated in vivo, the ends of the DNA must possess cohesive ends. In practice in vitro ligation appeals to the genetic engineer, as fewer mishaps (loss of nucleotides in the ss tail due to cellular nucleases and so on) can occur outside a cell than within it. In addition, the type of ligated molecule to be produced (circularized or tandemly joined ones such as concatamers) can be controlled by in vitro ligation.

The ligation reaction is controlled by several factors, such as pH, temperature, concentration and kinds of sticky ends and so on. As the cohesive ends are held together only by hydrogen bonds, the temperature at which ligation is performed becomes very important. At temperatures above 15°C the single – stranded tails are liable to dissociate.

When a recombinant of, say, A and B DNA only (one each) is the goal, the fragments are made incompetent to curl up and join their own ends or form tandemly joined fragments of the same kind by removing the phosphate moiety from the ends of the A or B fragments. The A and B fragments will then get aligned at the cohesive ends and the ligase will be able to connect the 3′ – end of a strand with the 5′ – end of the other.

The optimum temperature for ligase action is 37°C. But in the case of joining of cohesive ended fragments, as the ends dissociate at such a high temperature, the reaction temperature is lower. This does not apply to ligating blunt ends.

In this case, the problem is to bring the blunt ends face to face long enough for the reaction

to take place. This is usually achieved by having a very large number of fragments, the ends of which are to be joined. In sticky end joining, therefore, temperatures lower than 15°C (preferably 12.5°C) are practical, while for blunt end ligation, one may go to a temperature as high as around 23°C.

As ligase uses the ends of DNA molecules as substrates, rather than the entire DNA, the kinetics of joining depend on the number of ends (concentration) available for joining. DNA molecules longer than 200 bp are liable to join end to end to form circular molecules. DNA molecules longer than 20,000 bp, on the other hand, do not circularize readily.

Joining of two ds DNAs takes place in two steps: two strands are linked by a phosphodiester bond, followed by linking of the opposite strands. It takes longer for the first step to occur, as only random collisions between the free 5′ and 3′ ends bring the moieties in the proper conjunction for the enzyme ligase to operate. Once one strand is ligated, the complementary one is joined rapidly, as they are already held immobilized, by hydrogen paining, with the other DNA strand.

A high concentration of joinable ends is required for ligation of blunt – end DNAs, as in this case too, only random encounters can clinch the issue. The initial joining of cohesive – ended molecules may be speeded up by extending the cohesive ends with homopolymer tailing. The ends to be joined are thus stabilized, or held in position by the base – paired extensions.

Many DNA engineering exercises do not require in vitro ligation. The hybrid, but unligated DNA, becomes ligated in vivo, by endogeneous ligase, in the cloning cell. Isolation procedures may introduce nicks in the DNA. It may be necessary to close these nicks with ligase before proceeding further.

The commercially available ligase may not always be upto the mark in terms of potency, due to any of a variety of environmental of handling parameters. It is wise, therefore, to test the ligating efficiency of the enzyme before utilizing it.

The optimum reaction mixture for cutting and ligating DNA has been developed empirically. In contains a reaction buffer, DTT, ATP or NAD, a restriction enzyme and ligase. One may test the efficiency of reaction mixtures in which the ratios between the above mentioned components are varied. The ligating ability is assessed by running control mixtures and ligation mixtures (after conclusion of the reaction) in agarose gels. The ratio of the ligated (single large DNA) and unligated (two or more smaller) fragments of DNA. DNAs may be determined from the corresponding bands in the gel. The reaction mixture (without the ligase) containing the DNA to be fragmented and subsequently joined is vortexed, spun briefly in an Eppendorf (microfuge) centrifuge and kept at 37°C for one hour. Reaction is stopped by holding the tube in a 65°C water

bath for about 10 minutes. This mixture is then supplemented with ligase, incubated at 14°C Covernight, diluted with the reaction buffer, heated to 60°C for 10 minutes (to inactivate the ligase and to dissociate DNA ends that have base – paired). Small aliquots (50 μl) of this sample are then run on a 0.8% agarose gel.

**5. Discuss the role of other enzymes modify the DNA molecules in gene cloning.**

**Enzymes to Modify Ends of DNA Molecules**

The central event in a gene – splicing project is the joining of ends of DNA molecules. The latter may be restriction fragments with blunt or sticky ends. In the latter, the protruding tail may have a 3′ – or a 5′ – end. The two ends to be joined may not be compatible, or may possess complementary single – stranded regions that are too short to hold the molecules together long enough for them to be ligated. It may be required to make an unevenly cut – end into a flush – ended molecule, or the latter made to acquire sticky tails. For these and other fine – tuning jobs, several enzymes are available which are widely utilized. They supplement the cutting and ligating enzymes to prepare the types of ends required for the DNA molecules participating in a splicing operation. Some of them are briefly discussed below.

***(1) Alkaline Phosphatase***

The enzyme alkaline phosphatase removes the phosphate moiety at the 5′ – end of a DNA strand, whether it is part of a blunt and single strand extension or a recessed end of a double – stranded DNA. The $PO_4$ at an RNA terminal is also removed by this enzyme. It is obtained commercially from two sources: bacterial and calf intestinal phosphatases (BIP and CIP, respectively). Alternatively, raising the pH to 8.3 after restriction enzyme digestion, also dephosphorylates the DNA strands.

***(2) Polynucleotide Kinase***

Polynucleotide Kinase is a phosphorylating enzyme that transfers the γ phosphate of ATP to a dephosphorylated end of a DNA or RNA. The enzyme is encoded by a gene of phage T4 and is extracted from E. coli cells infected with the phage. Kinasing is utilized to rephosphorylate DNA or RNA dephosphorylation with alkaline phosphatase, or for end labelling of DNA and RNA strands. The P label is in the γ phosphate of ATP. $Mg^{++}$ and dithiothreitol are used in the reaction.

***(3) Exonuclease III***

The enzyme exonuclease III is generally a phage lambda – encoded product, extracted from the phage – infected E. coli. Its main activity is that of removing nucleotides from a DNA strand, one at a time, in the 3′ – and 5′ – direction. A second activity is that of adding a phosphate to the 3′ – end of an ss DNA or a ds DNA. A third function is to disrupt a DNA at places that lack a purine base. Finally it is capable of acting as RNase H. Mg ions are needed in the reaction.

A exonuclease III is used to produce a recessed 3′ – end in a ds DNA. 3′ – Wu has utilized this method to degrade part of each strand in a ds DNA fragment to generate single – stranded DNA templates to be used for a sequencing procedure.

Removal of a terminal phosphate group becomes necessary for a variety of operations. One such operation is for end – labelling of a DNA strand for sequencing and another for restriction mapping. The phosphate is first removed by the phosphatase and a radio – labelled one added in its place by a phosphorylating enzyme.

The most common need for dephosphorylating DNA ends arises during the making of recombinant DNA molecules. Lack of a phosphate group at the end of one DNA strand prevents the formation of a phosphodiester bond with another DNA as both $PO_4$ and OH moieties are needed for the reaction. This lacuna prevents both circularization of fragments, (donor or vector) as well as the formation of concatamers of solely vector or solely donor DNAs. The procedure, therefore, when splicing a donor fragment with a vector DNA is to dephosphorylate one of these two sets and mix it with the other sets of fragments. The two types will be aligned by their sticky ends, and joined by one of the strands (Figure 2.7). The gaps left in the backbone due to each of the phosphates in one DNA become repaired in the cell into which the rDNA is introduced.

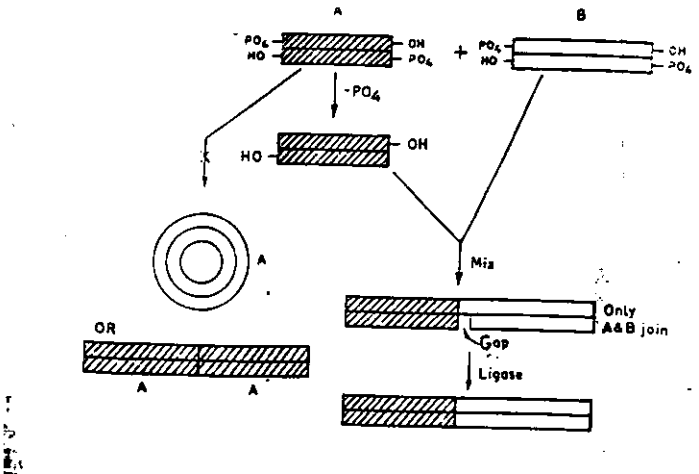



**Fig. 2.7 Dephosphorylation of ends of one DNA prevents ligation with the same DNA. This technique is utilized to ensure the joining of donor fragments only to vector ones.**

Calf intesunal phosphatase, (CIP) is preferred for most purposes, as it is easily denatured at 68°C

in the presence of SDS. Bacterial intestinal phosphatase (BIP) is not affected at this temperature, and, if used, requires several phenol – chloroform extractions to remove its traces from the DNA to be eventually ligated. BIP is utilized to dephosphorylate the end of an RNA molecule, the secondary structure of which hampers the removed of phosphate by CIP. The latter may be used for all DNA, single stranded of double – stranded with blunt, sticky tailed or recessed 5′ – ends. The reaction with CIP is usually carried out at 37°C but with BIP a much higher temperature (60°C) is required.

***(4) DNase I***

DNase I or deoxyribonuclease I is an endonuclease that generates single – stranded fragments having the phosphate at the 5′ – end of the strand or of the single released mononucleotide. It can act on both ss and ds DNA.

The enzyme mostly used is derived from bovine pancreas. The strands may be cleaved at random sites or at about the same place on both strands. Addition of $Mg^{++}$ and $Mn^{**}$, respectively, ensures the former and latter results.

***(5) Mung – Bean and SI Nucleases***

Both mung – bean and SI nucleases cleave ss DNA into single nucleotides or into short sequences of nucleotides. Excess enzyme is capable of degrading ds DNA. The mung – bean nuclease appears to require somewhat different conditions for its activity vis – a – vis SI nuclease. The latter can severe a ds DNA if there is a single nucleotide gap in one of the strands. The other enzyme operates only on larger deletions in the strand.

SI nuclease is used particularly for DNA footprinting to remove the ss DNA not protected by the bound protein. It is also used for removing ss regions of DNA not hybridized by another nucleic acid strand. The mung – bean enzyme is derived from the legume and the SI from the fungus Aspergillus oryzae.

***(6) DNA Polymerases and the Klenow Fragment***

The DNA polymerase that is generally utilized is either the DNA Pol I from E. coli or the T4 DNA polymerase encoded by the phage gene.

The E. coli enzyme is basically a proofreading and repair enzyme. It is composed of three subunits each with a specific activity. They are: 5′ → 3′ – polymerase 3′ – 5′ – exonuclease, and 5′ → 3′ – exonuclease. The enzyme is useful for synthesizing short lengths of a DNA strand, especially by the nick translation method. The 5′ → 3′ exonulease activity may be deleted. This

edited enzyme is referred to as the Klenow fragment.

The T4 DNA Pol possesses, like the Klenow fragment, only the polymerase and proofreading (3′ – 5′ – exonulcease) functions.

The choice of DNA Pol to be used depends on the nature of the substrate. For instance, the T4 DNA Pol can elongate both ss DNA and ds DNA, the latter from both exposed and recessed 3′ – OH ends. Pol I can extend a nicked DNA, starting from the exposed 3′ – OH of the nucleotide on one side of the nick. Pol I and the Klenow fragment repair mismatches at the ends and fill up gaps in either strand. In addition, the Klenow fragment can elongate a primer aligned to a template strand of DNA. The latter activity is exploited in the enzymatic method of DNA sequencing developed by F. Sanger and A. R. Coulson. The lack of 5′ → 3′ – exonuclease activity makes the Klenow fragment the most suitable of the DNA polymerases for certain jobs. One of them is filling up a recessed end where the latter is a 3′ – OH one.

***(7) Terminal Deoxynucleotidyl Transferase***

Terminal transferase is a DNA polymerase that can extend a strand (from the OH – end of a primer, hydrogen paired to a complementary strand) without using a template. Any nucleotide that is provided in the reaction mixture is utilized to elongate the DNA strand, of course, with a random sequence of bases. If only one kind of nucleotide is provided a mononucleotide polymer will be produced.

The preceding strategy is employed to add 100 – base – long 'tails' to the ends of restriction fragments. If the vector and donor fragments synthesize tails with complementary bases, only these two types of fragments join.

***(8) RNA Dependent DNA Polymerase***

RNA dependent DNA polymerase is reverse transcriptase. This enzyme synthesizes a single strand of DNA along an RNA template. It can also synthesize a second strand along the first one to make a ds complementary or cDNA.

Reverse transcriptase is usually utilized to copy mRNAs into ss or ds cDNA, and to make short labelled probes.

***(9) RNases***

Two RNases used in general are RNase A RNase T1. Both cleave the phosphodiester bond between adjacent ribonucleotides. However, RNase A cleaves next to uracils and cytosines such that the phosphate remains with these pyrimidines. The nucleotide on the other side of the phosphate in the DNA backbone is dephosphorylated. RNase A is obtained from the bovine

pancreas.

RNase T1, on the other hand, cleaves only next to a guanosine, again including the phosphate group at the 3′ – end of the nucleotide. This enzyme is derived from A. oryzae.

***(10) RNase H***

RNase H is an endoribonuclease that is useful for degrading the RNA strand from a DNA: RNA hybrid molecule. It cuts up the RNA into short fragments.

**6. What are the safety guidelines to be followed in recombinant DNA research?**

**Biosafety in Biotechnology:**

**Introduction:**

All the microorganisms used in biotechnology are non-pathogenic to humans and other animals. Biotechnology has made major advances in public health by the control of communicable diseases with vaccines and the improvement in the quality of the environment by the continued improvements of biological waste treatment processes.

Many microorganisms can infect humans, animals and plants and causes disease. Most microorganisms used by industry are harmless many are small number of potentially dangerous microorganisms have been used by industry in the manufacture of vaccines or diagnostic reagents, e.g. Bordetella pertussis (whopping cough), Mycobacterium tuberculosis (TB) and the virus that causes food and mouth disease. Stringent containment practices have been the norm.

In recent years, there have been many scientific advances permitting alterations to the genetic makeup of microorganisms. Recombinant DNA techniques have been the most successful but have also been the cause of much concern to the public.

**Classification of microorganisms according to pathogenicity:**

**Class 1**

Microorganisms that have never been identified as causative agents of disease in human beings and that offer no threat to the environment.

**Class 2**

Microorganisms that may cause human disease and might therefore offer a hazard to laboratory workers. They are unlikely to spread in the environment. Prophylactics are available and treatment is effective.

**Class 3**

Microorganisms that offer a severe threat to the health of laboratory workers but a comparatively small risk to the population at large. Prophylactics are available and treatment is effective.

**Class 4**

Microorganisms that cause severe illness in human beings and offer a serious hazard to laboratory workers and to people at large. In general, effective prophylactics are not available and no effective treatment is known.

**Class 5**

This group contains microorganisms that offer a more severe threat to the environment than to people. They may be responsible for heavy economic losses. National and international lists or regulations concerning these microorganisms are already in existence in contexts other than biotechnology (e.g. for phytosanitary purposes).

**Biosafety levels:**

There are four Biosafety levels that are described depending on the microorganism involved.

**Biosafety level 1:** This provides safety equipment and facility design and construction are appropriate in laboratory where viable microorganisms not known to cause disease in healthy adult human. Bacillus subtilis, Naegleria gruberi, infectious canine hepatitis virus are examples included in this level.

**Biosafety level 2:** This is applicable to laboratory in which broad spectrum of indigenous moderate risk agents that are present in the community and associated with human disease of varying severity. Hepatitis B virus, HIV, Salmonella and Toxoplasma sp. are represented in this group.

**Biosafety level 3:** This is applicable to laboratories in which work done with indigenous or exotic

agents with a potential for respiratory transmission and which may cause serious potentially lethal infection. Mycobacterium tuberculosis, St. Louis encephalitis and Coxiella burnetii are represented in this group. Hazards relate to autoinoculation, ingestion and exposure to infectious aerosols.

**Biosafety level 4:** laboratories in which done with dangerous and exotic agents that pose a high individual risk of life – threatening disease, which may be transmitted via the aero set route and for which there is no available vaccine or therapy.

**Biosafety cabinets (BSC)**

**Class I BSC:** Negative pressure, ventilated cabinet. Designed for general microbiological research work with how and moderate risk agents.

**Class II BSC:** Designed with inward air flow to protect personnel. HEPA filtered downward vertical laminar air flow for product protection. Class II BSC are classified into two types A and B based on construction, air flow velocities and paterns and exhaust systems.

Type A cabinets are suitable for microbiological research in the absence of volatile or toxic chemicals and radio-nucleotides, since air is re-circulated within the cabinet. Type B cabinets are sub typed into B1, B2 and B3. type B cabinets are connected to the building exhaust system and contain negative pressure. This allows working with toxic chemicals and radioisotopes.

**Class III BSC:** Totally enclosed, ventilated cabinet of gas tight construction and offers highest degree of personal and environmental protection. This cabinet is operated through negative pressure. Supply air is HEPA filtered and the cabinet exhaust air is filtered through two HEPA filters in series.

**Genetic engineering – safety, Social, moral and ethical considerations:**

Genetic modification involves the transfer of genetic material between different organisms by artificial methods and is now increasingly being used beneficially in the fields of healthcare, agriculture, food production, industrial enzyme production and environmental management. While there are undoubted benefits from the use of transgenic organisms, it is important to ensure that such systems do not cause problems of safety to people and environment or create unacceptable social, moral and ethical issues.

**Release of genetically manipulated organisms to the environment:**

Many people fear that genetically engineered microorganisms could escape from the laboratory into the environment, with unpredictable and perhaps catastrophic consequences. It was believed that such released microorganism could 'upset the balance of nature' or that foreign DNA in the new microorganisms could alter its metabolic activity in unpredictable and undesirable ways.

In response to these concerns, guidelines were established to ensure safe working practices and levels of containment based on potential hazards. Many important medical products such as insulin and human growth hormone and some industrial enzymes are manufactured in large-scale containment fermentation processes, involving specific GMOs. The final products from these processes are free from the genetically manipulated host organism and, therefore, do not constitute a release problem. Quite recently rennet (chymosin) for cheese manufacture has been produced from genetically manipulated microorganisms. Recombinant microorganisms are now being considered for deliberate release into the environment, where they cannot be contained, e.g. biological control, inoculants in agriculture, live vaccines, bioremediation, baker's and brewer's yeasts. Increased pathogenicity of microorganisms or microbial ability to destroy essential raw materials are often cited as potential problems of GMOs. Organisms with any possibility of unusual pathogenicity will never be permitted to be used. Where microorganisms are released to be used for biocontrol of, for example, insects, care must be taken that they will not influence other life forms. The use of a recombinant rabies vaccine in baits in a Belgium has significantly reduced the level of rabies in wild animals. The public were informed and in general approved this worthwhile use of the technology.

The overall aim of the food industry with respect to genetic engineering will be to improve the quantity and to increase the quality and properties of existing food productions, to produce new products and, of course, to improve financial returns. New biotechnology now offers a major opportunity to tailor food products to public demand.

While the public have readily accepted medical products produced from GMOs they are much less willing to accept such procedures with food. Genetic engineering is seen as 'unnatural' and unnecessary in food production.

Transgenesis is seen by some as a fundamental breach in natural breeding barriers that natural set up through the process of evolution to prevent genetic interplay between unlike species. In this way the species is seen as 'sacred'. In the viewpoint of many molecular biologists the gene has become the ultimate unit of life- the gene is merely a unique aggregation of organic molecules (common to all types of cell) available for manipulation. Consequently, they see no ethical problem in transferring genes between species and genera.

Risks associated with GM crops and foods have been identified (Stewart et al., 2000).

Ecological biosafety research has identified potential risks associated with certain crop-transgene combinations, such as intra- and interspecific transgene flow, persistence and the consequences of transgenes in unintended hosts. Resistance management strategies for insect resistance transgenes and non-target effects of these genes have also been studied. Food biosafety research has focussed on transgenic product toxicity and allergenicity.

Currently, the public interest in biosafety issues has focussed on the discussions surrounding the use of genetically modified organisms, very specifically on the use of transgenic plants in agriculture. Attention is now focussed on the problem of control of new reemerging infectious diseases, the need for new vaccines, control of transport and routes of dissemination, biosafety information exchange and networking, where research results are dearly needed. In the area of modern biotechnology new applications such as gene therapy and transgenic animals will be on the list of future priorities for biosafety related activities and research (Doblhoff-Dier and Collins, 2001).

**7. Discuss in detail on various steps involve in gene cloning techniques.**

**Gene Cloning:-**

**How to clone a gene**

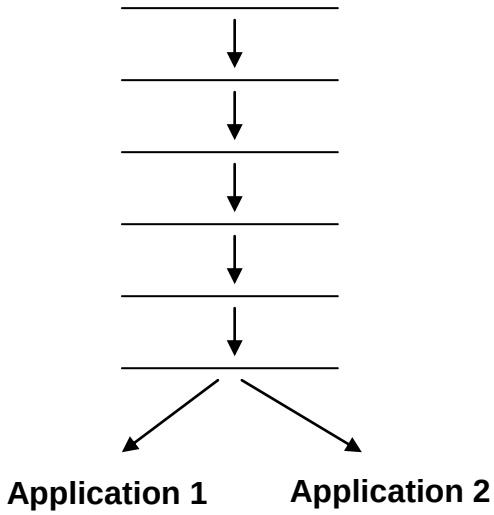
Gene cloning is a technique of recombinant DNA technology (rDNA tech) in which the foreign genes are introduced to other organisms through vectors carrying molecules (or) other gene transfer techniques. Finally recombinant identical copies (or) cells (or) organisms were obtained. It has more value than normal one.

Steps are;

(i) Cut the larger DNA (Donar) molecular with special enzymes called Restriction enzymes, Restriction Endonacleases.
(ii) Cut the vector DNA molecules with same special enzymes related to the above step and make opening (or) closing sites in vector molecules (cohesive ends).
(iii) Join the donar DNA fragment and vector DNA molecular with special enzymes called Ligase.
(iv) The combined foreign DNA and vector DNA molecules is called recombinant DNA molecule. One of the rDNA fragment has the desired gene (G).
(v) Amplify (produce clones) each rDNA molecules in organisms (or) cells like E.Coli or any other.
(vi) Screening the clones contains the desired gene 'G'
(vii) Selection of clones with gene 'G'.

(viii) Further production (or) increase the selected recombinant clones contains 'G'.
(ix) Extract rDNA from cells for isolation of 'G'
(x) Use rDNA for research application.
(xi) Use the organisms contain 'G' and its products.

**Explain with steps (Flow Chart)**




**Detailed steps in cloning:-**

**I. <u>Construction of rDNAs:-</u>**

1. Select vector molecules and foreign DNA to be cloned.
2. Cut each vector and foreign DNA molecule with R. enzymes.
3. Ensure the cut ends of Donar and vector DNA.
4. Modify the cut ends for better joining.
5. Mix the Donar and vector DNA with joining enzymes Ligase.

**<u>Result:-</u>**

Many rDNAs (hybrid, chimeni (or) recombinant) are obtained.

This contains desired foreign DNA fragment and vector DNA molecule.

**II. <u>Amplification of the rDNAs:-</u>**

(i) Introduce the rDNA molecule in to E. Coli. Cells by special techniques called transformation.
(ii) Allow the cells to multiply and
(iii) Screen the cells having rDNA.

**Result:-**

1. There will be several cells carrying donar and vector DNA molecules in transformed cells.
2. No fungus gene in non-transformed cells.

**III. Selection of rDNA clones:-**

1. Transformed cells are inoculated into a agar media plates.
2. Formation of colonies of transformed cells with rDNA.
3. Pure colony of transformed rDNA cells / non-transformed cells grow.
4. Selection of colonies with selection agent.
   Eg. Enzyme lactamase for the respective gene.
   Antibiotic, ampicillein for the respective gene.

5. **Selection of clones by screening:-**

   Based on contain visible parameters, the clones are screened and selected. The non-transformed (or) uncloned cells (or) colonies are eliminated.

6. **Amplification of clones with 'G'**

   The screened and selected clones are cultured in agar plates for large population the rDNA will be in a appreciable quantity.

7. **Extraction of rDNA**

   The cells are lysed by using chemical (or) enzyme. Low weight molecules are removed by lyase centrifugation technique.

   - The rDNA molecules are isolated and mixed in buffer solution.
   - Cutting (or) cleavage of rDNA molecules by using restriction enzymes.
   - Separation of vector DNA and donar 'G' fragment by agarose gel electrophoresis.

8. **Usage of isolated 'G' fragment (DNA)**

- The isolated 'G' fragment DNA can be used for many Genetic Engineering research (or) experiments.
- The rDNA is intact and ready for other. Set of cloning experiments with E.coli. cells for production of proteins (recombinant proteins)
  Eg. Insulin, Growth harvest, factor VIII
- Similarly may recombinant experiments can be conducted with the same isolated 'G' fragment.
- This is myriad and applications are planty.

9. **<u>Introducing cloned genes in higher organisms like plants & animals.</u>**

- Transgenic animals / Transgenic plants } – Various gene transfer techniques.
- Cell culture & Tissue culture } / Micropropagation methods.

## <u>UNIT – II</u>

## PART – A

**1. Define Restriction mapping. What are the types?**

Restriction mapping sketches a gross picture of a DNA on the basics of sites for restriction enzymes. These sites of cleavage are constant in a DNA from the same strain or spares, with allelic variations (polymorphism) as in the case of game sequences. The fractioning of fragments of DNA is according to size and sites.

Two types of restriction mapping as:

i) Indirect mapping – complete digestion of DNA
ii) Direct mapping – partial digestion of DNA.

**2. Write short notes on RFLP.**

RFLP in otherwise called as Restriction Fragment Length Polymorphism which is determination of restriction digestion of DNA through a application method. These are based on the polymorphisms assign due to base substitutions, inspection, deletion and translocation that might have occurred in the past in the specific regions of genomic DNA. These changes lead to differences in the size of restriction fragments obtained due to digestion with specific restriction enzymes.

**3. Describe direct mapping of DNA.**

Direct mapping is based on the analysis of partially digested DNA. In one protocol, the DNA end labeled selectively and after partial digestion, the fragments with the lable were examined. They formed a nested set of fragments ranging in size from the smallest to the largest. The length of each fragments represented the distance, from the labeled end of the intact DNA, occupied by a cleavage site for the enzyme employed.

**4. Describe indirect mapping of DNA.**

This method is based on the complete digestion of the DNA with two or more restriction enzymes, separately oz in double digestion reactions. The sub fragments of each initial fragment are identified. Mapping becomes complicated as the length of DNA increases. The systematic approach in followed such as the branch and bound technique. In this certain defined rules to assign sub fragments to the initial parent fragments; simultaneously eliminates the alternate fragments that could add up to give the size of the fragment in question. Pairs of single digestion fragments that share one double digestion fragments are identified, and the ones that appear to be

ambiguous are taken care of by the protocol of the technique.

**5. Defined chromosome walking.**

Chromosome walking is a technique by which mapping began in one DNA fragment of an overlapping genomic library can be continued in to the adjacent ones. Starting with any fragment one can walk to the proceeding as succeeding of the overlapping fragments. It the process is continued, the entire map is established by walking across consecutive fragments. Clusters of genes of genes have also been mapped in this manner.

**6. What is chromosome jumping?**

Chromosome jumping is an offshoot of the technique of chromosome walking. Where one can skip segments of the DNA represented by a d-done to reach a fragment of interest chromosome jumping is carried out in two ways as;

i) Junction sequences are closed and used as proxies to identify sequences several kilo bases away from the region hybridized by the probe.
ii) On second, the presence of chromosomal rearrangements (as inversion and translocation). The approach in identical to straight chromosome walking until one and of the rearranged region in reached. The probe for the overlap bat mean the terminal in the last fragment before the rearrangement now skips a fragment on trio before it hybridizes with another done.

**7. What are linkers? Write the important of it?**

Short lengths of DNA fragments are available as single stranded chains with different defined sequences. These are called linkers, which are used recombinant DNA technology. Linkers usually possess a set of restriction, sites, either singly, on in pairs, that are placed symmetrically on two sides of a central axis. A linker molecule is introduced into a vector at a restriction site; the vector now possessor several optional sites for restriction enzymes. The PVC and PMB series vectors possess such linker.

**8. What are adaptor molecules? Give the significance.**

Adaptors are a short DMA fragments which has double stranded or single stranded molecules. Adaptor molecules sane to join DMA stands with in compatible ends. Adaptor molecules are also chemically synthesized. The adaptor molecules have a preformed cohesive end. The adaptor molecules can be used for legation of foreign DNA which easily binds with cohesive end of adaptors. For example the Basmati adaptor has one blunt end being a 5′

phosphate group and a Bam HI cohesive end which is rot phosphorylated.

**9. Define connector molecules and its use.**

Connectors are two adaptors with an internal homology. They are used for joining (connecting) two fragments cleaved by different restriction enzymes. One connector, on legating to the ends of two strands (in the same orientation), completer the cleavage site at one terminus. The other single stand connector restores the cleavage site at the other terminus. The two fragments are now connected with are extra length of DNA between them.

**10. What are plasmids? Give the use of it.**

Plasmids are small, double stranded; covalently closed circular DNA molecules present mostly in bacteria cells. In year sachacorryle concussive, a small plasmid are additional are extrachromofomer in prokaryotic (or) bacteria cells. Same plasmids carry same special genes, which confer resistance to the cells to drops, antibiotics, toxim and toxic metals.

Plasmids are used as vector molecules to carry foreign sense in that through special characteristic genes in that through special characteristic features, framer to other organisms.

**11. Write the characteristic features of a plasmid to qualify as a vector.**

(i) Small in size
(ii) Presence of replication ori, in compatibility gene par sequences.
(iii) Absence of transfer or mobilizing gens.
(iv) Maker genes
(v) Unique restriction sites
(vi) Multiple closing sites
(vii) Multi copy nature.

**12. Mention some example of plasmids and it details.**

(i) PBR 332 – Most widely used vector 4.3 kb a enzyme present for cutting.
(ii) PCR 1 – 11.4 kg size. z enzymes for cutting.
(iii) PACYC – 177 – 3.8 kg, 8 enzymes
(iv) PACYC 184 – 4.0 kg, 4 enzymes
(v) PMBA – 4.4 kg, 6 enzymes
(vi) PAT 153 – 4.2 kg, 8 enzymes

**13. Write short notes on PBR 322.**

PBR 332 is the Bacterial plasmid vector. If in most widely exploited for recombinant DNA technology. It is a chimeric vector & source of sequences from col E1, PMBI and PSC101. It is 4.3 kb DNA carrying the B-lantanas (la) gene on Tn3 (that confers amplicillin resistance) and a tetracycline resistance was lifted from an R plasmid and the Tot R from PSC 101. The ori gene from col E1. It possesses the nick gene sequence, recognized by mob protein.

**14. Write short notes on phage vector. Give example.**

Use of Bacteria phage molecules have been reported as closing vehicles in genetic engineering. The phage vector is required for closing of large DNA fragment and therefore, the gene bank or genomic libraries can be constructor. The linear double stranded DNA molecule cyclists through the single stand of 12 nucleotides commonly known as cos sites cos sites are the key feature of the DNA. Phage infects cells and replicate with host DNA.

e.g. Phage
Phage M13.

**15. What are cosmid vectors?**

Cosmid vector are hybrid (or) chimeric vector molecules devolved from plasmid with cos sites of phage. i.e. small portion of & phage DNA incorporator with plasmid by in intro packaging cosmids lack genes encoding viral protein 4 no by sis.

Special feature of cosmids are,

(i) Origin of replication
(ii) A masker gene coding for antibiotic resistance.
(iii) A cleavage sit for the insertion of foreign DNA
(iv) It DNA small in size.

**16. Write brief note on phage lambda vector.**

DNA of phage is 50 kb in size. It has a single two extension at each end. The two extension are complementary and ear align to convert the linear molecule in to a circular one. They are referred to as strictly, or 'cohesive' ends. These cos ends are essential signal for packaging of DNA into a mature phage particle.

For gene manipulation, the by tic cycle is useful, as this provides large number of phases, therefore to amplify any insect DNA in the vector.

**17. Write brief note & gt WES vectors.**

These are & phage vector and these are safe one for closing techniques. There is a stop codon (amber codon) mutation each in genes W (Phage assembly), E (shall protein) and S (cell lysine), and one of 2 deletion (λ B (or) C) with in the non-essential region, flanked by Eco RI series. These tube mutants, λge WESB and λ gt-WESC are unable to grow on E-coli, unless the latter has suppressors for the W,E, and s genes.

**18. What are immunity vectors?**

Murray constructed a vector molecules with an Eco RI site with in the EI gene. This gene lies with in what is known as the immunity region. Such immunity insection vectors on λ gt ($imm^{434}$ 6527) can accommodate upto a maximum of around 7.6 kb of donor DNA.

**19. Mention briefly on g phage vectors.**

These are one of the replacement vector derived from generalized transuding (gt) and are referred as λ gt vectors. This was that was viable in spite of a loss of some sequences in the right arm. The lost region was that occupied in the wild-type phage DNA by the last two (rightmost) of the give Eco RI sites on the DNA. These mutation could be made in the vectors by removing the two fragments in the non-essential region, between the first three Eco RI sites. These fragments are the B (map position 2 to 26.6 kb) and C (between 26.6 and 32.14 kb) regions. A λ-gt with the B replaced in called λ-gt and C is λ-gt C.

**20. Write the use of animal viruses as vector with suitable example.**

The viruses vectors made from animal virus are packaged in viral shells and infect host cells with a very high efficiency. Some animal viruses are g node for a short time, expressing the vector-bonne gene(s) for a limited period until the host cell is killed. Other vectors become stable components of the host system and express the insert genes constitutively.

For e.g. SV40 a pap ova virus infects mammalian cells and produces a progeny of particles before killing it.

e.g. SV40 (simian virus 40)
Adenoviruses and
Retroviruses

**21. What are shuttle vectors? Give the significance.**

Shuttle vectors are plasmid vectors that contain replication origin sequences for two different host species. The hurt belong to divergent groups such as bacteria and year, monkey and E-coli and E-coli and human beings. These evection causes two selectable marker genes one each for the two hosts. The first shuttle vector used was for E. which yeast (sacharcorryes (exviscale). It contains the ori of troth species and a number of markers.

**22. Write short notes on expression vectors.**

Expressions vectors contain expression signals that best transcribe translate the foreign genes in a heterogamous system. Sometimes, these signals are promoters and terminators of E-coli or phage genes that are added at appropriate sites on either side of the gene to be expressed. A eukaryotic from an animal can be expressed in E.coli with promoters and terminators from E-coli genes.

**23. What are single stranded (ss) plasmid vectors?**

Single stranded plasmid vectors are derived from phage M13 DNA. M13 Vectors cannot accommodate large pieces of inserts stably, so, the plasmid DNA fragments in a single stranded form are hybridized with M13 and large M13 vectors called ss parried neutron obtained with other special features. These are known as EMBL vectors. For example from PVC by insect a 1300 by DNA from phage ft into the lacz gene. The spliced plasmid fragment in used.

**24. Write short notes on artificial mini chromosomes.**

Artificial mini chromosomes are plasmid vectors that contain DNA sequences that allow them to be segregated into daughter cells like chromosomes of eukaryotes. These sequences are ARS (Auto-noon replicating sequences) and CEN taken from year DNA and Tr from the protozoan Tetrahymena.

i) ARS (autonomous replicating sequences)

ii) CEN (Centro mere region of yeast chromosomes)

iii) Tr (Telemetric region sequences of yeast chromosome).

When this vector introduced into cell, the plasmid breaker between the two Trs, and the vector because eukaryotic chromosome. The advantages of such chromosomal mimics for closing eukaryotic genes one oblivious

**25. Write brief notes on λgt 11 phage vectors.**

λgt 11 is a 43.7 kb size double stranded λ phage for closing DNA segments, which are less than 6 kb in length (usually for cDNA). Foreign protein. Recombinant λgt 11 can be screened using either nucleic asides or antiphonary problems. The recombinant λgt 11 becomes gat -4 the non-recombinant for gat+, so that on appropriate E.coli host, with recombinant phage (gat) will form white clear colonies and that with non-recombinant with blue calories.

**26. What are phagemids? Give suitable example.**

Phagemids are chimeric vectors combined from plasmids and Bacterophages. They contain

i) Origin of replication from plasmid colEI

ii) Selectable maker for antibiotic resistance.

iii) Major intergenic (IG) region of a filamentous phage.

Necessary for mitigation and termination of viral DNA synthesis and for morphogenesis of phage particles.

e.s. phagemids PUC 119 and

These are devided PVC 119 with is acting events and phage M13 DNA.

**27. Briefly dry can on yeast bifacial chromosomes (YAC) and its significance.**

Yeast artificial chromosome (YAC) vectors are allow sequences for closing as several hundred kilo base (up to 1000 kb) long. These may represent chromosomes in lower eukaryotes with very small genomes can be closed in yeast by lighting them to vector sequences that allow their propagation as linear artificial chromosomes. These long DNA molecules can be generated and allow constructor of comprehensive libraucien in microbial horst YACs have 2 disadvantages;

i) Closing efficiency in low (1000 closes/μg)
ii) It is not possible to recover large amount of pure insert DNA from individual genes.

**28. How mammalian vectors (or) mammalian artificial chromosomes (MAC) are in will in recombinant DNA technology?**

With the isolation of mammalian artificial chromosomes (MACs) have been produced. MACs, are designed to be able is replicate, segregate and express I a mammalian cell like any other mammalian chromosome along with other chromosome. It contain all the elevenths as telomeres, origin of replication, Centro mere MAC will not be integrated into genome, and can be used as a vector maintaining single copy per cell. It carry large fragment of DNA representing intact

eukaryotic split gene with axons and intones permitting its normal expression regulated by the associated promoter sequences. In view of this, MACs are considered to be suitable for gene therapy, where the in selected DNA will be fully expressed and maintained without affecting hest genome.

**29. How promoter will function in vectors for expression of gene?**

Promoters of respective genes will express completely in related organism (or) genes devided.

For example promoters. From non-plant sources express poorly in plants, and non-animal sources express poorly in animals. To obtain correct expression, gave transferred to plants should be linked to plant specific promoters and those to animas to be linked to animal specific promoters. These promoters also can be interchangeable so as to confer on the transferred gene, a specific pattern of expression.

e.g. (i) Nopaline synthesize (nos) promoter from T-DNA.
(ii) 355 promoter of Camv

**30. Give an example of insert cell expression vectors.**

Development of baculovirus expression vector system in insert cells are the best example for insert cell expression vector. The method is as i) The virus particular are budded from infected cells and spread the virus in the early phase (12 sirs period) ii) In the late phase, occluded virus units accumulate in the host rucleses and get embedded in protein polyhedral. Iii) Insect is decomposed releasing polyhedral; iv) polyhedral are inverted by another in sect host; where polyhedral are dissolved in releasing virus for multiplication. The recombinant virus is multiplied and used for expression of hetrologous protein.

**31. Why cleaved plasmid molecules are treated with alkaline phosphatase in gene cloning experiment?**

When the plasmids used after cleaving in cloning are treated with alkaline phosphatase before the cloning with foreign genes. The reason behind is due to prevent the recircularization of plasmid when open with restriction enzymes which contain phosphate molecule which easily bonding with adjacent fragments. The alkaline phosphatase remove the phosphate by dephospharylation.

## PART – B

**1. Explain the types of restriction mapping and the techniques involve for mapping of genes.**

**Restriction Mapping**

Restriction mapping sketches a gross picture of a DNA on the basis of sites for restriction enzymes. These sites are constant in a DNA from the same strain or species, with allelic variations (polymorphism) as in the case of gene sequences. Any DNA from the same genetic background will, therefore, be cleaved into identical sets of fragments by a restriction enzyme. Making a restriction map is the first step in charactering a genome or a cloned DNA.

The procedure for restriction mapping involves the treatment of DNA with a restriction enzyme, fractionating the fragments according to size, and analyzing these fragments to ascertain the position of each in the intact DNA.

There are two methods for restriction mapping; direct and indirect. In direct methods, the DNA is digested to completion, and in the indirect ones only partial digestion is allowed.

One variation of restriction mapping that has found new applications is the determination of restriction fragment length polymorphism (RFLP). The essential features of direct and indirect approaches in restriction mapping, and one of the cases of RFLP determination, are described briefly.

As the size of fragments is the only criterion for restriction mapping, the quality of mapping depends on the accuracy of determined sizes. These are calculated from their nobilities in the gel, with reference to those of standard size markers.

Southern has developed a useful algorithm for calculating sizes from motilities of fragments. In practice two maps are made, starting with the two end of the DNA. The fragments that are of identical size in both gels are noted, and ambiguitities resolved by different means. Both maps should come out to be identical in the end. This operation can be carried out manually, but it is time-consuming and error-prone.

**(i) Direct Mapping**

**Direct mapping is based on the analysis of partially digested DNA.**

In one protocol, Smith and Birnstiesl (1976) end-labeled one end of the DNA selectively and after partial digestion (so that one cleavage per DNA was possible), the fragments with the label were examined. They formed a nested set of fragments ranging in size from the smallest to the largest. The length of each fragment represented the distance, from the labeled end of the intact

DNA, occupied by a cleavage site for the enzyme employed.

Another protocol end-labels ends of the DNA and cleaves the latter once to separate the two labeled fragments. Further, partial digestion of both sets of labeled fragments with another enzyme generates fragments, the analysis of which leads to the required map.

**(2) Indirect Mapping**

**Indirect mapping procedures are based on complete digestion of the DNA with two or more enzymes, separately or in double digestion reactions.**

The second digestion is often carried out in the gel itself, in a second dimension. The sub-fragments (if any) of each initial fragment are identified. Mapping becomes complicated as the length of DNA increases. To cope with the complications recourse is taken to a systematic approach, such as the use of the 'branch and bound' technique (Fitch and associates, 1983). This involves using certain defined rules to assign sub-fragments to the initial parents; the technique simultaneously eliminates the alternate (but incorrect) fragment that could also add up to give the size of the fragment in question. Pairs of single digestion fragments that share one double digestion product are identified, and the ones that appear to be ambiguous are takes care of by the protocol of the technique.

This procedure is very much shortened by using DNA labeled at both ends and incorporating any other known data for any part of the DNA under scrutiny. In some cases, there may be too many complications to allow an unambiguous solution based only on the data of the fragment size.

**Technique of Restriction Mapping**

Genetic maps are prepared using recombination frequencies. However, the recombination frequencies, which are the function of distances between genes, are not completely independent of (i) the nature of mutants used. (ii) the position of these mutants on chromosomes, (iii) the genetic background, (iv) the environmental conditions, and (v) a variety of other factors. Consequently, the distances between genes on a genetic map my not correspond to the distances between them on the DNA molecule of which they are a part at the genetic map due to non-availability of mutants in that region.

At the molecular level, the fine structure of a gene can be studied through determination of nucleotide sequence of the concerned DNA segment. Sometimes this is done through isolation of a DNA segment corresponding to a gene followed by sequencing of the DNA segment, which needs time and energy. Instead we can prepare a map of the DNA by its cleavage at specific sites

with the help of restriction end nucleases, which recognize very short specific DNA sequences and cleave the DNA at these specific sites. These sites of cleavage can be identified and mapped to give rise to a restriction map.

A restriction map thus consists of a linear sequence of sites, each for a specific enzyme and the distances between them are measured in terms of number of base pairs of DNA. This technique can be used both in prokaryotes and eukaryotes, although all regions of chromosomes can not be easily mapped in eukaryotes. Different steps involved in restriction mapping include the following.

**Restriction cleavage and gel electrophoresis**

If we take a particular DNA molecule or DNA sample, digest it with a specific restriction enzyme and then subject the sample to gel electrophoresis (a technique in which DNA digest is loaded on gel slab and the DNA fragments move under the influence of an electric current), we will notice a series of bands on the gel slab or cylinder. The bands can be observed under ultraviolet light after staining with ethidium bromide.

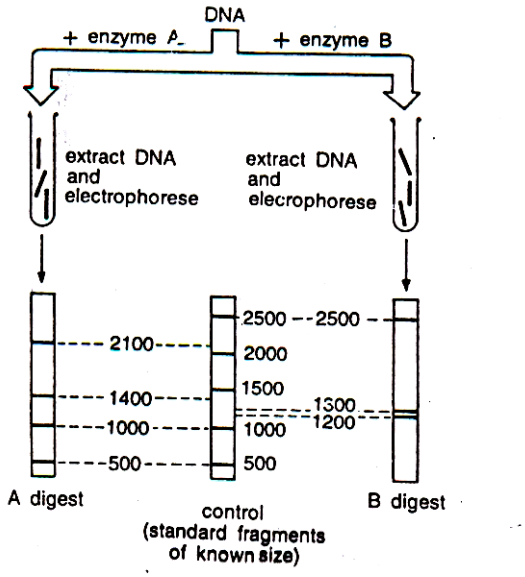



Fig. 3.1. Cleavage of DNA by two restriction endonucleases A and B into fragments which can be separated by agarose gel electrophoresis.

The position of different bands will depend on DNA fragment size; such that smaller the fragment, more rapidly will it move, and longer the fragment, more slowly will it move. It will mean that the fragment away form the loading site will be smaller and those close to the loading site will represent longer DNA fragments. The gel can be calibrated by using a mixture of DNA fragments of known lengths, so that the positions of the bands of known lengths on this standard

gel can be compared with the positions of bands in the experimental DNA digest. The fragment length for each band of DNA digest can thus be determined sometimes using a computer device. In Figure, we have shown the results of digestion of 5000 bp long hypothetical DNA molecule digested separately by two enzymes A and B. As shown in the figure, the enzyme A cleaves the DNA into four fragments of lengths 2100, 1400, 1000 and 500bp, while the enzyme B cleaves it into three fragments of lengths 2500, 1300, 1200bp. These data with some additional experiments can be used to generate a restriction map as shown in the next section.

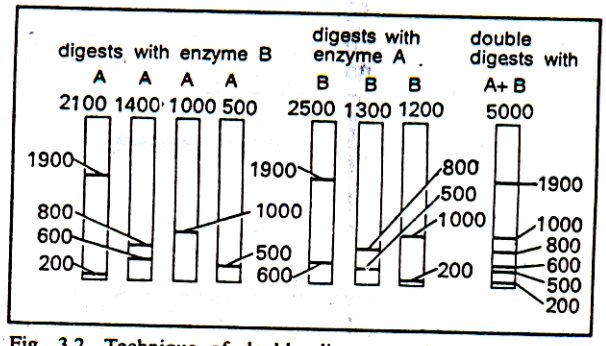



Fig. 3.2. Technique of double digests to determine cleavage positions of DNA due to one enzyme with respect to those due to another enzyme (the two enzymes are A and B); the four gels on the extreme left show the results of electrophoresis after digesting with enzyme B, each of the four fragments obtained after digestion with A; three central gels represent results of digesting with enzyme A, each of the three fragments earlier obtained with enzyme B and the solitary gel on the right shows the results of digesting the intact DNA with both the enzymes simultaneously.

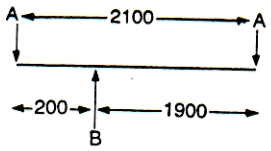



Fig. 3.3. The sites of cleavage by enzymes A and B in one of the four fragments, 2100 bp long, obtained due to cleavage by enzyme A; the fragment has a site for enzyme B at 200 bp length from one end.

**Construction of a restriction map**

The data on DNA digestion by more than one end nucleases as discussed above can be utilized to arrange the sites of cleavage in a defined order. This can be done by several methods and one of them is illustrated in Figure. This method involves successive digests with two individual enzymes, where we extract each fragment produced in the individual digests with either enzyme A or enzyme B and then cleave it with the other enzyme. The original DNA sample is also digested by a mixture of both the enzymes to confirm the results of individual successive digests. In Figure. we have shown results of such an exercise. When the fragment A-2100 is obtained and digested with enzyme B, it is cut into two fragments of 1900 bp and 200 bp. Fragment B-2500 (obtained from individual digest with enzyme B) is similarly cut by enzyme A

into two fragments of 1900 bp and 600 bp, suggesting overlap of A-2100 and B-2500 (Fig) in the region of fragment of 1900 bp which is obtained by one cut with enzyme A and the other by enzyme B. The DNA fragment of 1900 bp is also available, when DNA is digested with a mixture of both enzymes A and B (double digest). It may be seen in figure that in reciprocal digests (A followed by B and B followed by A) and double digests, same fragments are available. Using this information, we can find the overlapping regions in A and B. This will then allow us to prepare the restriction map as shown in Figure.

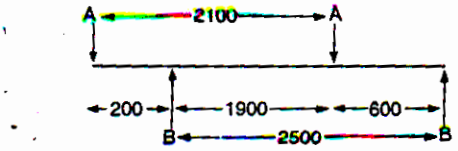



Fig. 3.4. Reconstruction of restriction map showing restriction sites of enzymes A and B in two overlapping fragments (2100 bp fragment obtained due to enzyme A and 2500 bp fragment obtained due to enzyme B).

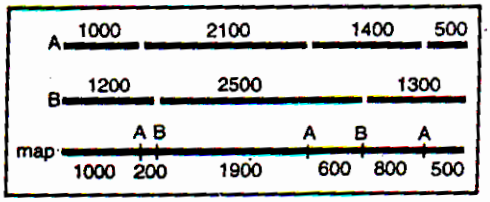



Fig. 3.5. A restriction map, in the form of linear arrangement of cleavage sites; the upper two figures show sites due to individual enzymes and the bottom figure shows arrangement of cleavage sites of both enzymes (see text for details); sites of more enzymes can be added to this map by same technique.

**Use of partial digests, end labeling and hybridization in restriction mapping**

The above technique of individual and double digests can be supplemented with other techniques for actual construction of maps. For instance, by permitting incomplete digestion, fragments longer than those obtained by complete digestion may be obtained. These will be called partial digests. In the example used in the last section, in partial digests with enzyme A, instead of getting A-1000, A-1400, A-500, we may get A-3100, A-1400 and A-500, which will suggest that A-1000 and A-2100 lie in adjacent regions. In another partial digest if we get A-1000, A-3500 and A-500, this will suggest that A-2100 and A-1400 are adjoining, which will mean that A-1000 and A-1400 are found on the two sides of A-2100. This technique will thus allow the arrangement f fragments in a linear order.

Another useful technique involves labeling of the ends (end labeling) of DNA molecule before digestion, so that thee fragments, containing these ends can be identified due to labeling, even after digestion. In the above example, using end labeling, if A-1000 and A-500 are found to b radioactively labeled, these will be present at the two ends of the restriction map. Some of the

features of a restriction map can also be confirmed by nucleic example, if A-2100 and B-2500 have an overlapping region of 1900bp, they should hybridize with each other, which will confirm overlapping.

The above technique will help in accurate completion of a restriction map, but will require that we have a complete set of restriction fragments which make the entire DNA region being mapped. Once the restriction map (which is a physical map) is ready, it can be compared with the genetic amp. Although large changes, already located on the genetic map, can be easily located on the restriction map, but point mutations can not always be easily located on the restriction map, since the restriction sites often do not change due to mutation. In such a situation, one may like to determine the nucleotide sequences of individual fragments and compare them in normal and mutant individuals. Since sequences of nucleotides is more laborious and can not be easily undertaken for the whole genome, another technique of using a variety of molecular genetic markers (e.g., RFLPs, RAPDs, STs, STMS, AFLP, etc.) has been utilized during the 1980s and 1990s to generate genetic linkage maps in human, mouse, rat, pig. Sheep, fruitfly (Drosophila), nematode (Coenorhabditis elegans), yeast, maize, wheat, rice, barely, tomato, potato, lettuce, rice, pepper, pea, etc. This technique will be discussed later.

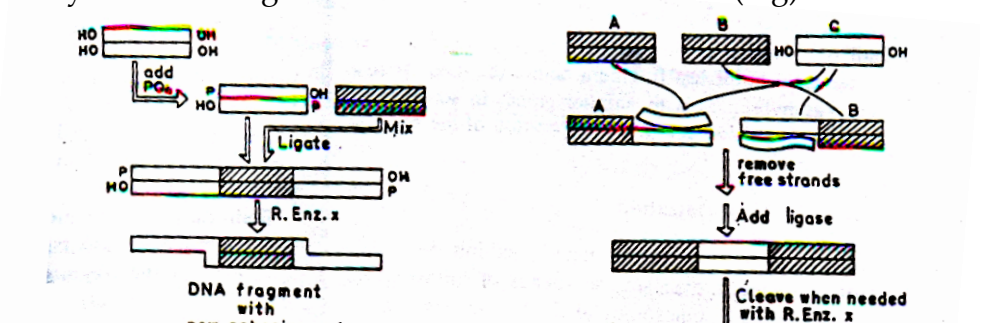
**2. Discuss the use of Linkers, adaptors and connectors in recombinant DNA technology and write how these are obtained.**

**Linkers, Adaptors and Connectors**

Short length of DNA are available as single-stranded chains with different defined sequences. They are called linkers, adaptors or connectors, depending on the use to which they are put.

Linkers usually posses a set of restriction sites, either singly, or in pairs, that are placed symmetrically on two sides of a central axis (Fig). A linker is introduced into a vector at a restriction site; the vector now possesses several optional sites for restriction enzymes. The pUC and pMB series vectors possess such linkers. A donor DNA cleaved with almost any restriction enzyme can be cloned into such vectors by using the appropriate cognate enzyme.

Adaptors, as the term implies, serve to join DNA strands with incompatible ends. Suppose one cut end has an extended 5 –end and the other an extended 3 end is the same orientation, an adaptor chain (at least 10 bases long) is used to first become intercalated between the recessed ends in one orientation. This leaves a gap where the two single-strand extensions fail to meet. The gap is usually filled and legated inside the transformed cell (Fig).



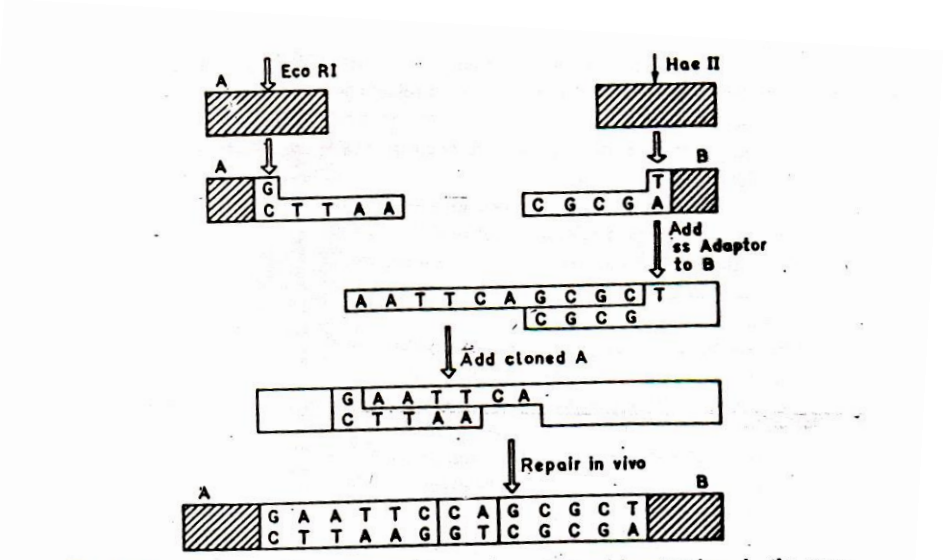
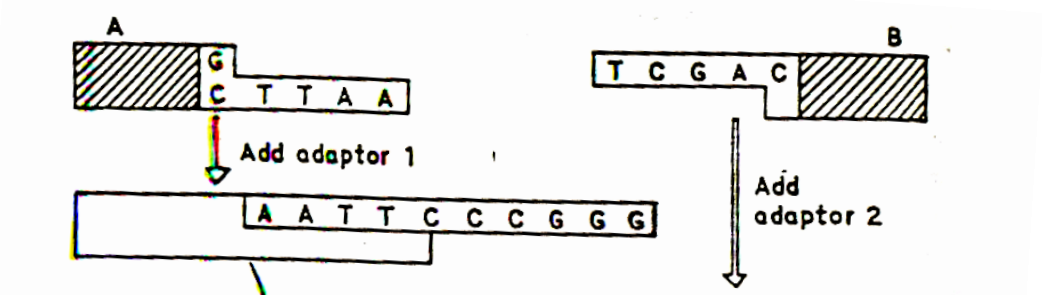



**Fig. 2.105 Adaptors. When two restriction fragments with extensions in the same strand are to be joined, use is made of an adaptor. This is a 10 bp synthetic oligonucleotide that is complementary at its terminal regions with two extensions, which on mixing with the two fragments, binds itself to the complementary tails. There is a gap in one of the strands between the joined fragments, which becomes repaired (filled and ligated) within the cloning cell by the appropriate enzymes.**

Connectors are two adaptors with an internal homology (Fig). They are used for joining (connecting) two fragments cleaved by different restriction enzymes. One connector, on ligating to the ends of two strands (in the same orientation), completes the cleavage site one terminus. The other ss connector restores the cleavage site at the other terminus. The two fragments are now connected with an extra length of DNA due to the connector) between them. The new DNA may be cleaved by either of the two enzymes, the recognition sites for which have been restored, as well as by a new site that may be present within one of the connector sequences.



Linkers may be utilized for a variety of goals. If a new cohesive end is to b created in a DNA, a linker containing the site for the new enzyme is attached to the DNA, If the latter already possesses and uneven end, it is blunted before ligating to the linker (using T4 ligase). On treating the DNA with the restriction enzyme, the linker is cleaved and the DNA now possesses the new cohesive end.

The linker may be unphosphorylated or phosphorylated. When it is unphosphorylated one of the strands of the linker will not be ligated. The free linker strand may be removed before using the DNA for cell transformation. The gap will be filled and the new strand ligated correctly in vivo.

**3. What is mutagenesis? Explain on oligonuclotide directed mutagenesis.**

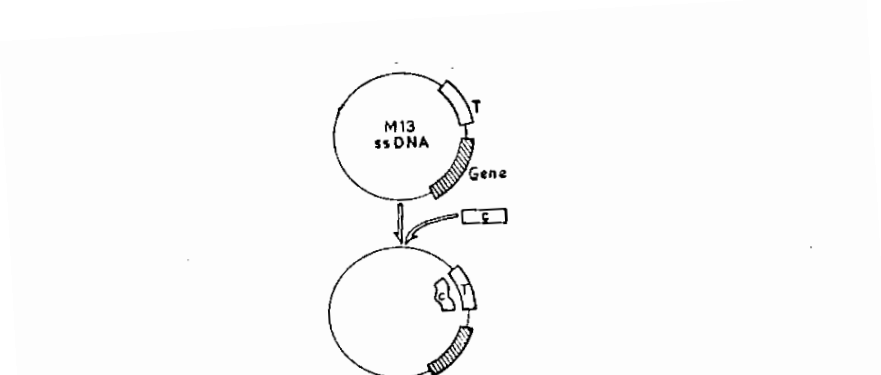
Mutagenesis is the process by which a lesion in the DNA becomes converted in a subsequent replication into a heritable genetic alternation. The alteration includes, substitution, addition or deletion of a single nucleotide or a set of nucleotides.

Agents that cause mutations are known as mutagens. Mutagens include physical, chemical and biological ones. Physical mutagens art h various ionizing and noniconizing radiations and

heat. Chemical mutagens cannot be so readily classified but include predominantly alkyl ting agents and others that induce modifications in the nucleotide, and alcidine dyes that bring about farm shift mutations due to the deletion or addition of bases. Substituting mutagens include nitrous acid, hydroxylamine, sodium bisulphate and alkylating agents such as ethyl-and methylmethane sulphonate (EMS, MMS) and N-methyl-N-nitroso-N-nitroguanidine (MNNG).

**(1) Oligonucleotide-directed Mutagenesis**

Oligonucleotide directed mutagenesis (Fig) can install a desired alteration in any site in a stretch of DNA. The technique is based on the use of a synthetic oligonucleosite (7-12 bases) that is identical to (complementary) the site to be mutated, except in one or more bases. This synthetic short length of DNA is hybridized with a ss DNA vector (usually form ϕ M13), and acts as the primer which elongates t make the M13 template duplex. The two strands of this circular DNA are separated and the new strand used as a template to synthesize its partner. This mismatched bases in the origianloligonucleoude are now matched with the correct complementary base(s). A mutation becomes essablished in this manner at the site of the bases that were different in the oligonuceoude. Almost all techniques based on this pioneering one (Hutchinson and associates. 1978) utilize a ss DNA as the template. Vectors from phage M13 and fd as well as plasmids (ss) of the pEMBL series are suitable for the purpose. All of these may be packaged as ss DNA-containing particles.



While the technique to be quite simple in principle, the strategy was found to encounter certain snags during practice. The chief among these obstacles was the propensity of cells to repair mismatched regions of a DNA. The second difficulty was the pushing out of the oligonucleotide by the advancing 3 end of the new DNA strand which had reached the 5-end of the oligonucleotide. Thirdly, the primer often hybridized to unintended regions of the template with which it had partial homology.

The first difficulty (of removal by repair or proofreading by DNA Pol I) is avoided by ensuring that the mutated strand (i.e., the one with the oligonucleotide) is methylated at appropriate bases. End nucleases do not attack fully methylated or even hemi ethylated (one strand) DNA. Except for a very few known restriction enzymes (e.g., Dbn I). The mutated strand is maintained by ligating the end of the new strand with the oligonucleotide. Hybridization at incorrect locations is avoided by allowing only a limited region of the template for attaching the oligonucleotide primer.

The troubleshooting jobs led to various improvements in the technique, so that it is now a reliable and routinely used one for site-specific mutagenesis. Kramer et al. (1984) developed the

following elegantly simple protocol (Fig). The template was a ss M13 DNA containing a short insert at the region to be mutated. This rDNA is an RF molecule synthesized while in a methylation-deficient (dam) E.coli host. A non-recombinant M13 DNA (ss) is obtained by infection of a methylation-competent strain)dam). The non-methylated RF recombinant DNA is dissociated and mixed with the methylted M113 DNA, which has been linearized. Double-stranded hybrid DNA is formed between the non-mehylated ss RF strand and the methylated ss M13 DNA. There is a gap in the latter opposite the insert in the former. The oligonucleotide is introduced to the left end of this gap and allowed to synthesize a new strand to fill the gap. The nicks between the new strand and the existing M13 DNA are ligated. By these tactics,

(i) The mutated strand is not vulnerable to attack by endonucleases (particularly the four GATC nucleosides in M13 DNA, which have been methylated in the $N^6$ of the adenine).
(ii) The primer does not hybridize at unwanted regions, and
(iii) The length of new strand to be synthesized is quite short.

This last feature prevents possible non-completion of strand synthesis due to incorrect primer attachment etc., and saves on time and material.

The preceding 'gapped duplex' approach has been further refined and modified both by Kramer and associates and others. Instead of having an insert in the template DNA, a gap may be generated by removing the short Eco R1-Hind fragment present in the M13 DNA. When this shortened DNA is hybridized with a full length DNA cleaved elsewhere, a gap occurs in one strand and in a single strand region opposite the gap in the other strand. Dalbadic-McFaland et al. (1982) produced a similar gap in one strand by nicking the latter at a site and degrading the nicked strand using exonuclease III, that had a 3′→5′ activity.

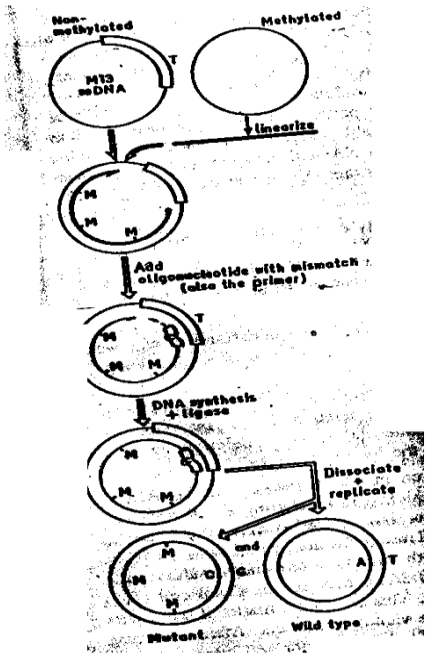



**Figure: The site-directed mutagenesis: An improved technique. DNA to be mutagenized is cloned in an M13 vector. The ss DNA of the vector, without the insert, and which has been methylated at the C residues, is linearized and allowed to hybridize with the vector containing the insert. There will be a gap in the linear DNA opposite for DNA. An oligonucleotide with the desired mismatched base and a primer for DNA symthesis are added to the incomplete M13 duplex. DNA synthesis from the 3'end fo the methylated strand generates the complementary strand for the insert DNA (except for the mismatch, C, in place of A). Ligase circularize the linear DNA. The ds DNA is dissociated and each strand allowed to replicate. Half the recovered circular DNAs will be of the wild-type, while the other half will be the mutants with the GC pair in place of the wild-type AT pair. This method limits DNA synthesis to a defined short region, a feature which makes it less vulnerable to various defects including non-specific annealing of the primer.**

The approaches mentioned above also reduced the number of steps in producing the ds mutant DNA. After the filling up and ligating in the gap region, it may not be necessary to dissociate the two strands and use the mutated one to acquire a new complementary strand. As the template strand is unmethylated, the DNA repair enzymes favour correcting the mismatch in this strand rather than in the one with the introduced oligonucleotide. Such repair would result in a ds DNA that was mutant and without a heteroduplex region. However, all mismatches are not corrected with equal frequency: in which even it is better to rely on other means of selection.

One such strategy is to use selection by complementation. The strand with the oligonucleotide could carry a lacZ gene, while its partner carries the same but with an amber mutation. So, the template DNA will be able to grow only on E. coli with a suppressor for the amber mutation. The mutated DNA will grow on a wild-type (non-suppressor) E. coli and will transform a lac host strain (Kramer et al., 1984).

**4. Discuss about plasmids characteristic features and role of plasmids in recombinant DNA technology with few examples.**

**Vectors from Plasmids**

Plasmids are small, double-stranded, covalently closed circular DNA molecules present mostly in prokaryotic cells. A small plasmid, 2 μ, has been identified in the yeast Sacharamyces cerevisiae.

Every plasmid carries one or more genes, one of which is required for DNA replication, and one for a quality referred to as 'plasmid incompatiblity'. Two plasmids that can survive and replicate in the same cell are said to belong to separate incompatibility groups. Those plasmids that do not coexist in the same cell belong to the same groups. There are about 30 incompatibility groups, named I, II, III, M, P, Q and so on.

Some plasmids carry, in addition, a gene or genes that confer resistance to the cell to drugs, toxins or toxic metals. Others carry genes that encode bacterial toxins. Still other possess a set of genes that are required for a very special function (e.g., nitrogen-fixing genes, nif, of Rhizobium and Klebsiella species; tumour-causing genes of Agrobacteria species).

Some plasmids carry genes that allow the plasmid to self-transfer to another cell via a conjugation tube (e.g., F and other conjugative plasmids). There are some that do not possess the transfer or tra genes of conjugative plasmids, but a couple of mobilizing genes (mob), one of which of which encodes a protein that on complexing with the second site, causes the super helically twisted plasmid DNA to relax and be nicked at a specific place on one strand. The free nicked end mobilizes the transfer of the plasmid to another cell. So, some plasmids are auto transmissible, while some are mobilizable. Still others mob protein in trans from another plasmid.

A natural plasmid is large and too unwieldy for manipulations, and also may possess several cleavage sites for the same enzyme. They are not suitable as vector material.

(i) Essential Features of a Plasmid vector

To be a vector, a plasmid must posses certain minimum qualifications. They include the following:

(i) Small size. Smaller DNA molecules pass through the cell membrane more easily than the larger ones. Linearized plasmids, or any linear DNA molecules pass more slowly than the circular ones. The efficiency of introduction into a cell depends, therefore, both on the size and the conformation of the molecule.

(ii) Replication Ori, incompatibility gene, par sequences. Presence of a replication origin (Ori), and incompatibility (Inc) and portioning (par) sequences are essential.

The Ori is the region where DNA replication is initiated. The par is required for distributing daughter plasmids stably to new cells. The Inc function determines the plasmid copy phenotype. Some plasmids replicate to several copies per cell, some only into 1-3 copies. The Inc function is somehow associated with this feature.

The reason underlying incompatibility has been partiality unveiled in the case of at least one plasmid, Col E1. In Col E1, an RNA primer (0.5 kb) is intiated upstream of the Ori. This primer is degraded at its 3 end if it overshoots the Ori region, by an enzyme RNase H. A second RNA is transcribed in the opposite strand, at the Ori region, ending at the initiation site of the first RNA. The second RNA is called RNA I, and the first is RNA II. Both these RNAs assume secondary structures somewhat like those of tRNA molecules; a loop in each possesses bases that are complementary to each other, like the codon-anticodon pairing between tRNA and mRNA. RNA I prevents further primer (RNA II) formation after the first bout of DNA synthesis, by engaging the RNA II at the coupled loops. A mutation in the pairing bases of RNA I was found to have lost the power to inhibit further RNA II synthesis, so that DNA replication continued for several rounds.

Perhaps plasmids that are in the same incompatibility group posses the same or very similar RNA I and RNA II pairing regions, which compete with each other, while those that coexist are independent from the point of view of requirements of DNA replication.

When using different vectors in the same cell, one has, therefore, to make certain that they belong to separate Inc groups. Plasmids F, pSC101 and RP4 belong to different groups and may be used together, while pMB1 and Co1 E1 may not, as they are members of the same Inc group.

**(iii) Absence of transfer or mobilizing genes.** Some plasmids are auto transmissible due to the presence of tra genes; others are mobilizable by virtue of the mob functions. A vector cannot afford to posses genes that allow it to dispense wantonly the cloned DNA, which has been acquired at a considerable cost of time and effort.

**(iv) Marker genes.** One more marker genes that allow easy detection of the recombinant clones must be present in the plasmid vector. The most commonly used markers and drug-resistance genes borne on transposes. E coli vectors carry one of the following markers: resistance to tetracycline, ampicillin (β-lactamase gene on Tn3), kanamycin (aph on Tn5), streptomycin, and chloramphenicol.

**(v) Unique restriction sites.** There must be one each unique site of cleavage for several restriction enzymes, with a few in each marker gene. Use of any of these enzymes would linearize the plasmid, by cutting only at one place. The cutting sites on a vector also allow the insertion and removal of a passenger DNA (gene or otherwise).

**(vi) Multicopy nature.** Some plasmids occur in only a few (1-3) copies per cell. These copies replicate only once during the synthesis of the bacterial DNA. These plasmids are said to exhibit a stringent mode of replication. There are other plasmids, the replication of which is not linked with that of the chromosomal DNA, and are not inhibited by the presence of chloramphenicol which interferes with protein synthesis and stops chromosomal DNA synthesis. Such plasmids exhibit the relaxed mode of replication. Treating a cell containing relaxed plasmids with chloramphenicol stop cell division, but not plasmid replication. This allows the plasmid to form as many as 1000 copies per cell.

Since the aim of gene coining is to recover amplified copies of the cloned DNA, the plasmid vector must be of the relaxed type.

It goes without saying that no naturally occurring plasmid is a paragon of all these virtues. Hence, for use as vectors several plasmids have been tailored by adding deleting, and substituting specific regions of natural and tailored plasmids. Even the copy number trait of a plasmid has been altered where needed. A low copy number plasmid (Stringent) is made amplifiable by joining it to a relaxed plasmid. The stringent pDF41 was spliced to the relaxed mode pMK16 to yield a larger replicon that can be increased in number by chloramphenicol treatment.

Various specialized plasmid vectors have been constructed that have insertions of sequences required for specific functions. Some of these will be described under the section on 'special plasmid vectors'.

(2) Some Common Plasmid Vectors

The parents for a large number of plasmid vectors are pSC101 (stringent), Co1 E1 (relaxed), pMB1, R6 and p16A. The last one is a cryptic mini-plasmid that has no genes.

A short list of some of the plasmid vectors in vogue is given in Table 1.

| | | **Size** | **Markers** | **Cutting site (one each)** |
|---|---|---|---|---|
| 1. | pBR322 | 4.3 kb | $Tet^R$, $Amp^R$ | 9 enzymes |
| 2. | pCR1 | 11.4 kb | $Co1^R$, $Kan^R$ | 2 enzymes |
| 3. | pACYC177 | 3.8 kb | $Amp^R$, $Kan^R$ | 8 enzymes |
| 4. | pACYC184 | 4.0 kb | $Ch^R1$, $Kan^R$ | 4 enzymes |
| 5. | pMB9 | 4.4 kb | $Col^R$, $Tet^R$ | 6 enzymes |
| 6. | pAT153 | 4.2 kb | $Tet^r$, $Amp^R$ | 8 enzymes |
| 7. | pUC | | $Amp^R$, lacZ | |

Four of the often-utilized vectors are now described briefly.

(i) pBR322. Perhaps the most exploited vector is pBR322. It is source of a large number of uniquely designed vectors.

pBR322 is a composite of sequences from Co1 E1, pMB1 and pSC101. Its geneology is an excellent example of the power of the art of cutting and pasting DNA (Fig).

pBR322 is a 4.3 kb DNA (Fig). carrying the β-lactanase (bla) gene on Tn3 (that confers ampicillin resistance) and a tetracycline resistance gene (with four open reading frames). The $Amp^R$ was lifted from an R plasmid and the $Tet^R$ from pSC101. The ori parent. It possesses the nic sequence, which is recognized by mob proteins that mediate plasmid mobilization. If there is another plasmid in the cell with pB322 that expresses the mob gene, the vector could be co-mobilized into another cell. To avoid this altogether the plasmid pAT153 was developed with a deletion in the nic region (Twigg and Sherrat).

(iii) pACYC177.pACYC177 is a plasmid vector (Fig) with the replicon of p15A, the cyptc small natural plasmid, that has been joined to a portion of the kanamycin resistance gene (from a transposon on plasmid R6-5) and the amplicillin resistance gene (Tn3). It is a relaxed mode plasmid, 3.45 kb in length (Change and Cohen, 1978), and pACYC177 and r vectors derived from it are compatible with Co1 E1 based plasmids. Both may be employed in the same cell, a strategy often required for housing two sets of genes on separate plasmids.

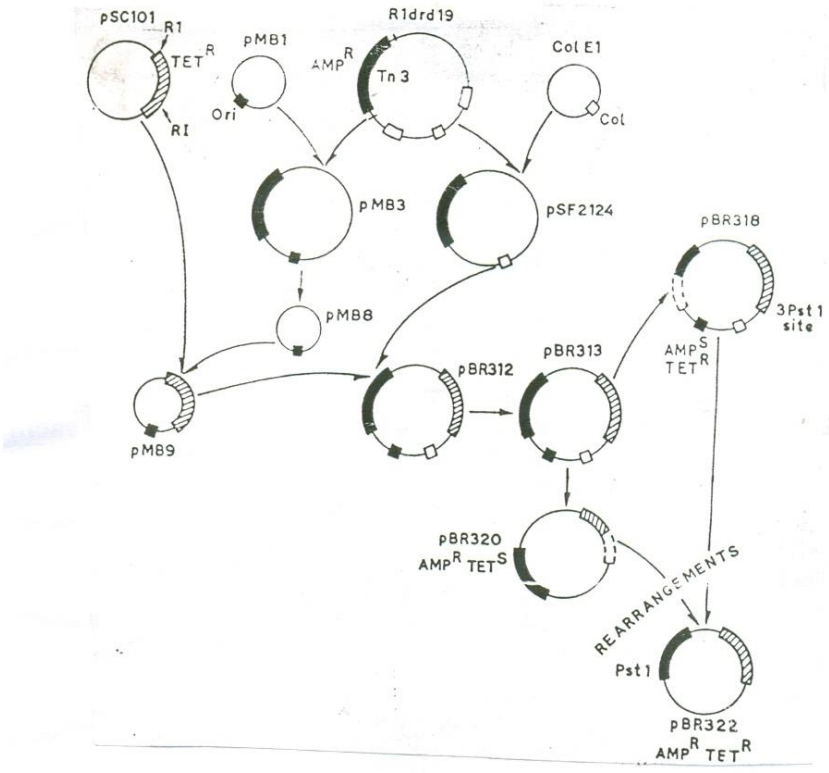



**Figure: Ancestry of pBR322. Judicious additions, deletions and rearrangements of fragments of plasmids R1, pSC101, pMB and pColE1 generator pBR313 which, after further combinations and rearrangements with regions**

**from pBR318 and pBR320 resulted in pBR322.**

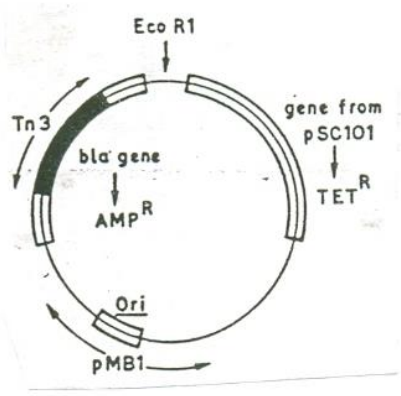



**Figure: The origins of regions of pBR322: a schematic representation. The bla gene encode β-lactamase and is taken from the transposon Tn3. This gene confers ampicillin resistance to the cell carrying it. The Tet$^R$ gene confers resistance to the antibiotic tetracycline.**

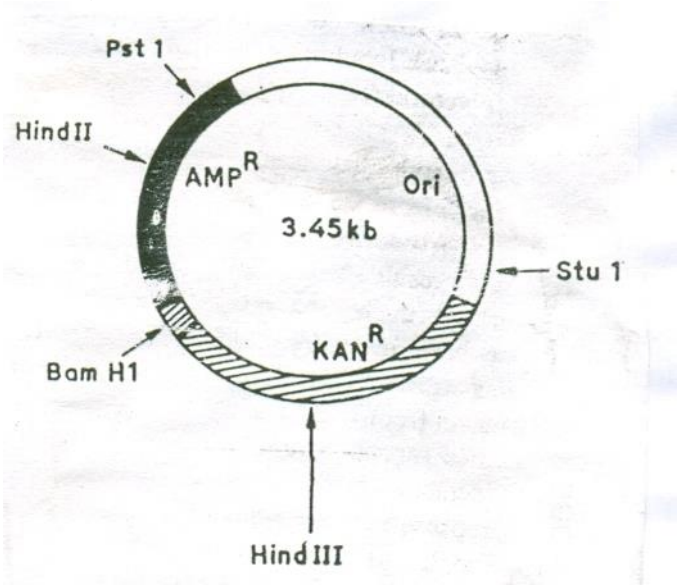



**Figure: pACYC177 is a plasmid with a replicon of the cryptic plasmid p15A from E.coli strain 15.**

(iii) πVX. This is a 902 bp Col E1 derived plasmid, into which a sup F gene and a polylinker have been added (Fig).

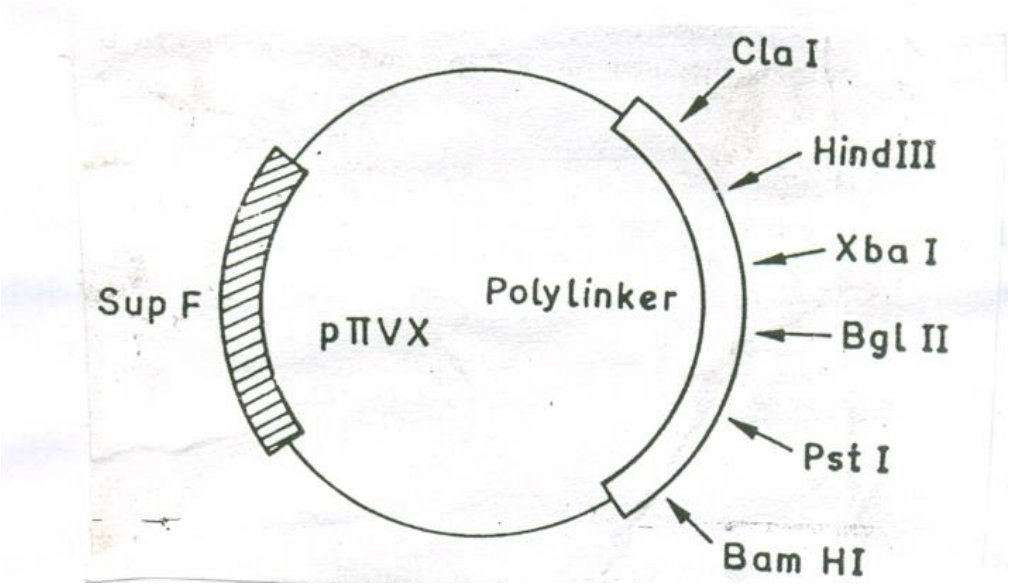



**Figure: πVX is a microplasmid carrying a polylinker and a sup F gene that suppresses amber mutation (mutation that alters the codon for an amino acid residue to a stop codon).**

(iv) pUC. pUC plasmids (Fig) contain polylinkers, which are a set of cutting sites on a synthetic DNA for different restriction enzymes which are used as cloning sites. Also pUC8 contains the lacZ and the $Amp^R$ genes.

**5. Write short notes on**

**(i) Chromosome walking**
**(ii) Chromosome jumping**

**(i) Chromosome Walking**

Chromosome walking is a technique by which mapping begun in one DNA fragment of an overlapping genomic library can be continued into the adjacent ones. Starting with any fragment one can 'walk' to the preceding or succeeding sequences of the overlapping fragments. If the process is continued, the entire map is established by 'walking' across consecutive fragments.

The walk is begun with a probe for one end of a fragment. The probe is the used to screen the library of overlapping fragments. The fragment to which the prob hybridizes a placed in tandem with the initial fragment with the probed regions overlapping each other. The two fragments thus unite to form a larger fragment. Probes from both ends of the latter screen other fragments the collection and meld the positive ones as before. One, therefore, 'walks' forward or backwards starting from any one fragment.

Probes for the ends of the fragments are prepared in the following manner. The fragment is

cloned in a vector that carries a specific strong promoter (T3,T7,SP6) just upstream of the cloning site. Replication begun at the promoter, and in the presence of two or more radioactively labeled nucleotides, yields specific probes. A more arduous method is to identify ends of restriction fragments and then isolate the fragments for preparing the probes.

Chromosome walks are useful not only for melding the sequences of large fragments of DNA into a single sequence but also for identifying the sequences on either side of a sequence of interest. Quite often, the latter is a gene that is too large to have been included in the phage or even cosmid vector. The entire gene, incliding the 5 and 3 making regions may be mapped by identifying clones that represent overlapping regions of the DNA. Clusters of genes have also been mapped in this manner. Gitschier et al. (1984) mapped the gene for the human clotting factor VIII using this technique.

**Chromosome Jumping**

Chromosome jumping is an offshoot of the technique of chromosome walking, where one can skip segments of the DNA represented by a λ-clone to reach a fragment of interest.

Chromosome jumping is carried out basically in two ways. In one approach, 'junction' sequences are cloned and used as probes to identify sequences several kilobases (up to 100 kb) away from the region hybridized by the prob. The technique involves the following steps:

(i) The DNA is cleaved into large fragments (100 kb or more).
(ii) The fragments are spliced to plasmid vectors and hybrid DNA circularized.
(iii) The hybrid DNA are cleaved with an enzyme that does not cut within the vector.

Usually, a large insert has more than one cleavage site of an enzyme. The insert region after cleaving may circularize into a smaller molecule, due to the loss of one or more internal fragments. The vector now possesses a shortened insert representing the two ends of the latter. A probe is made from this 'junction' fragment and used to screen the fragment library. A probe from one end of the junction is thus sufficient to identify a very large segment of the DNA. One thus 'jumps' over long distances of the DNA and maps the two extremities of such a large fragment (Collins and Weiss man, 1989).

A second technique of chromosome jumping utilizes the presence of chromosomal arrangements (such as an inversion or a translocation). The approach is identical to straight chromosome walking until one end of rearranged region is reached. The probe for overlap bet the terminal in the lass fragment before arrangement now skips a fragment or two before it hybridizes with another crone. In some cases, the is due to an inversion in the region. In other due to a translocations, of the segments next to another chromosome.

**6. How the mapping of genes done by using somatic cell hybridization?**

**Mapping Genes by Somatic Cell Hybridization**

Somatic cell hybrids between different mammalian species usually exhibit the phenomenon of loss of chromosomes of one species over several rounds of mitoses. This feature has been exploited to assign genes to chromosomes of the human genome.

Human cells (primary or cultured, normal or diseased), are fused to rodent cells (usually mouse or hamster that is mutant for an enzyme or other phenotype that is cells. The hybrid cells are transferred to limiting medium in which only those hybrids survive and proliferate which posses the gene that allows overcoming of the mutation in the rodent partner. Let us say in a man-mouse hybrid the mous cell is tk. In is a safe bet that the TK gene is one this chromosome.

This fundamental approach is combine with other data and genes assigned not just vaguely to a chromosome but also specifically to regions in either arm of a chromosome. The human genome has been mapped t an exponential rate in this manner. Retention of a chromosome is correlated with the presence of a particular gene. It is confirmed by the loss of function of that gene in cells that lack the particular chromosome (Creagan and Ruddle, 1975; Shows et al.1982.)

Chromosomal abnormalities are also pressed into service for assigning genes to human chromosomes. The procedure is the same; the function is now correlated with the presence or absence of the chromosomal segment in hybrid cell. The genes hprt, good, pgk (phosphoglycerate kinase) and the β-galctosidase gene have been mapped to the human X chromosome using this technique.

It is particularly easy to distinguish the activity of human genes in a man-mouse hybrid. In some cases, the mouse marker gene is experimentally inactivated. In others, distinct difference between the two allow identification of expression of a human marker gene. Many of the markers are isozymes that are distinguishable by their electrophoresis mobility, antigen city or resuscitation kin tics.

The site of a gene on the human chromosome in a man-mouse somatic hybrid cell case is assigned unambiguously by using a probe for the wanted gene. The probe may be from another species, provided there is sufficient homology between it and the wanted gene. The probe is used on Southern blots of restriction fragments of human genes. These case be distinguished from the fragments of the mouse DNA generated by the same enzyme.

Single copy gene sequences are currently mapped by in situ hybridization of meta-phase chromosomes with an appropriate probe. The chromosomes are banded by a high-resolution banding technique (produces more than 1000 bands per chromosome).This allows assigning the

probed gene to exact location in the chromosome (Zabel of al.1985). Mortion et al. (1986) have employed high-resolution banding with in sits hybridization of human chromosomes in man-mouse cell hybrids to assign the genes for insulin-like growth factor (IGFF) I and II to chromosomes 12 and 11, respectively.

**7. Write short note on yeast expression vectors.**

**From Yeasts**

Sacharamyces cerevisiae possesses a natural plasmid in many of its strains. This is a 6.318 kb (2 μm) long sequence in a covalently closed circular form (Fig.). This plasmid, called the 2 μ or scp (Sacharamyces cerevisiae plasmid), is a multicopy variety with up to 100 copies per cell (Hollinger, 1982).

There are two 599-base-inverted regions in the plasmids that divide the DNA into two unequal regions. Recombination between these similar sequences results in plasmids that fall into two classes, A and B, differing only in the orientation of the inverted regions with respect to each other. There are several restriction enzyme recognition sites on this plasmid.

There are three ORFs (open reading frames, or putative gene sequences) in the plasmid; they are A, REP1 (or B) and REP2 (or C). The product of A is involved in recombination, and the others are involved in the stable but random portioning of the plasmid copies into daughter cells.

Some strains of yeasts possess endogenous 2 μ plasmids. They are the cir strains. Those without the plasmid are called the cir strains. It has been observed that it is more difficult to transform cir cell with a 2 μ vector than $cir^+$ cells. Vectors that contain the entire 2 μm DNA are transferred more easily than those that carry pars of the plasmid lacking the replication retgion.

Yeast plasmids posses ARS (Autonomous Replicating Sequences) and CEN (centromere-like) that are needed for replication of the plasmid and its portioning to daughter cells. These sequences have been exploited to construct what are known as Yep, YRp, YCp vectors, as well as ones known as artificial minichromosomes.

YEps are yeast episomal plasmids derived from the 2 μ plasmid. They occur as multicopies of free circular DNA. Hybrid plasmids containing sequences of 2 μ and MB9 (with $Tet^R$) have been further modified by the addition of sequences from yeast chromosomal DNA (Beggs, 1981). Some of them are the yeast integrating plasmids (YIP) utilized for integrating a donor DNA into yeast chromosomes. LEU2 is one of the yeast markers utilized in these vectors: it allows recognition of transformed leu cells among untransformed leu cells.

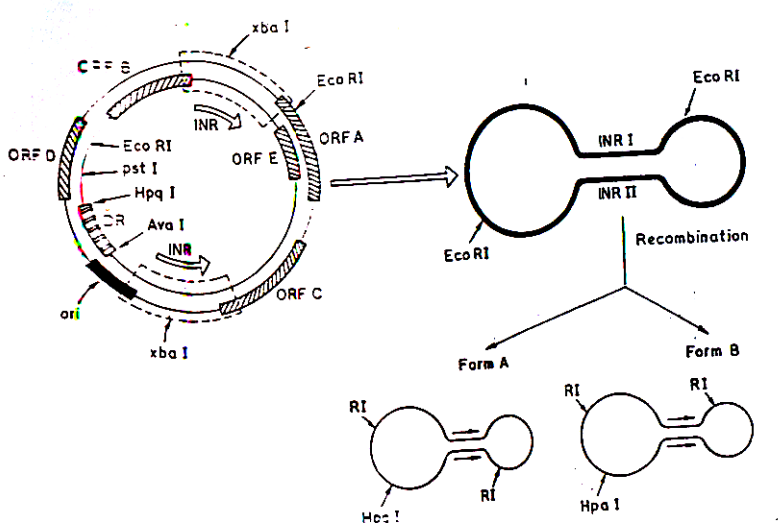



**Fig. 2.56 The 2 µm yeast plasmid. Strains of *Saccharomyces cerevisiae* possessing this plasmid are said to be cir$^+$; those lacking it are cir$^-$. There is a pair of 6000 bp long repeated sequences that are in opposite orientations (inverted repeats or INR) which, on pairing, divide the plasmid into a central stem with unequal sized loops at each end. Homologous recombination between the INRs results in types A and B plasmids that differ in the orientation of the sequences in the smaller loop. This is evident from the position of the single *Eco* RI site in this loop.**

A YRp contans, besides the E. coli plasmid sequences, some sequences from yeast DNA that include the ARS segment. These yeast replicating plasmids replicate in yeast cells. Struhl et al. (1979) were the first to utilize these vectors.

A YCp is a yeast vector containing the CEN sequences. The latter regulate, portioning of the plasmids during cell division.

The ARS, CENT r (telomere) sequences from another source have been combined in a vector known as an 'artificial minichromosome'.

**8. Briefly discuss on Artificial Minichromosomes.**

**Artificial Minichromosomes**

Artificial chromosomes are plasmid vectors that contain DNA sequences that allow then to be segregated into daughter cells like chromosomes of eukaryotes. These sequences are ARS and CEN taken from yeast DNA and Tr from the protozoon Tetrahymena (Fig.).

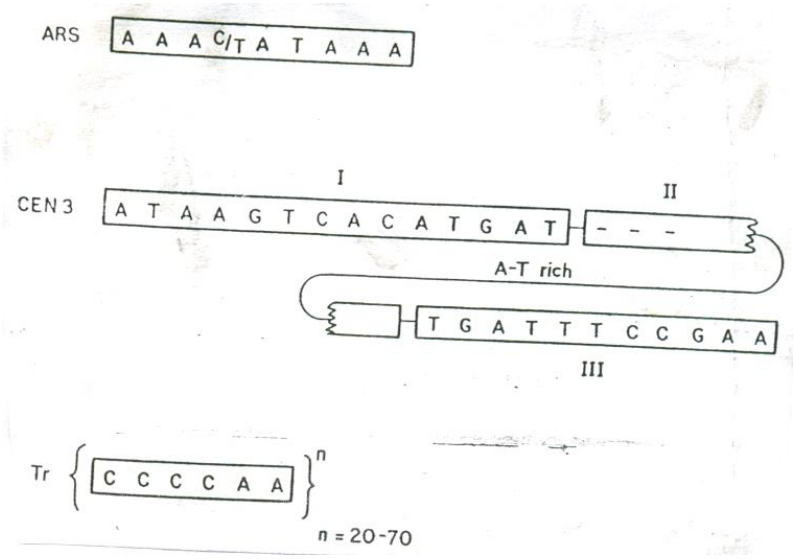



**Figure: The ARS, CEN and Tr sequences. ARS (autonomous replicating sequences) are required for DNA replication. CEN is an A-T-rich centromeric sequence involved in an equitable distribution of daughter chromosomes to progeny cells. Tr represents primitive eukaryotic telomeres (terminal regions of chromosomes).**

(i) ARS. These are automous replicating sequences that function as replication origins in the plasmid. It is not certain whether they have the same function in their original settings. The ARS were first cloned from yeast chromosomes and from the 2 μ yeast plasmid.

Chromosomes of other species (e.g., slime mold, maize, fruit fly, nematode, yeast) contain a common consensus sequence

AAAC/TATAAA

Which is the ARS motif.

(ii) CEN. These are sequences taken from the Centro meter regions of yeast chromosomes number 3.4 or 11. The CENs from different chromosomes.

Addition of the CEN sequences associates the vectors with mitotic apparatus, and the copies of the plasmid are transmitted to daughter cells. This is a danger faced by most is not guaranteed for equal segregation to the new cells. This is a danger faced by most plasmid vectors that are used to transform yeast cells. In fact, the plasmid is often lost completely in sectors of the population.

Segregation of CEN-containing vectors during meiosis depends on a sequence that is longer (6-10 kb) than that which is sufficient for segregation at mitosis.

The ARS- and CEN-containing pBR322 vectors are also provided with a marker selectable in yeast. One marker utilized popularly for use in yeast is LEU2 (for the enzyme β-isopropy1 malate dehydrogenase) required for the biosynthesis of leucine.
(iii) Tr.Ends of DNA molecules (telomeres) in yeast chromosomes, and in those of the ribosomal DNA of the ciliate Tetrahymena, have been found to be hairpin loops with a characteristic composition.

The ends of the rDNA of Tetrahymena contain 20 to 70 repeats of a sequence

*C C C C A A*
*I I I I I I*
*G G G G T T*

which a closed loop where the two strands end. (Fig).

Two telomeric regions cannot join. This is possibly the reason why eukaryotic chromosomes are able to remain linear. When these telomeric (Tr) sequences are added to an artificial chromosome, the vector becomes linear, and stays so. In fact to make the circular DNA linear, two Tr sequences are added, one from yeast and the other from Tetrahymena, in a head-to-head orientation. When this vector is introduced into a cell, the plasmid breaks between the two Trs, and the vector becomes linear. The linear vector acts effectively like a minieukaryotic chromosome (Fig). This advantages of such chromosomal mimics for cloning eukaryotic genes are obvious.

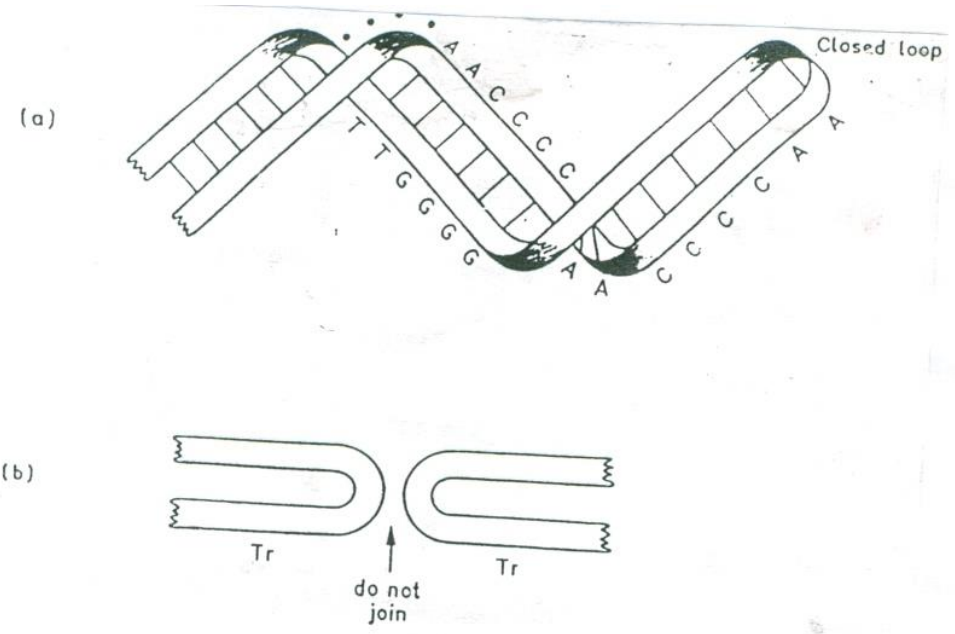



**Figure: The Tr sequences of Tetrahymena ribosomal gene DNA (rDNA) are about 700 bp long.**

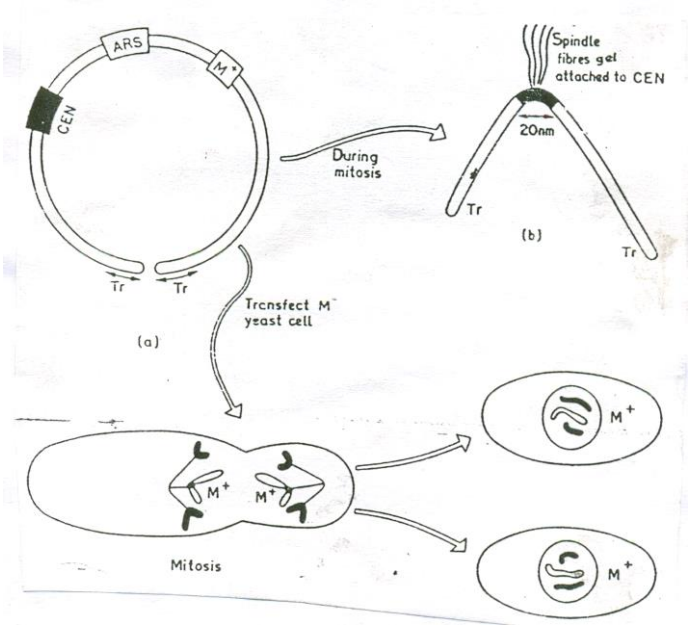



**Figure: A minichromosome with ARS, CEN, and Tr becomes linear inside a cell and is dispensed to daughter cells in a stable manner, especially if the length of the artificial chromosome is greater than a certain minimum length.**

**9. Write short note on**

**(i) BAC – Bacterial artificial chromosome**

**(ii) YAC – Yeast artificial chromosomes**

**(iii) MAC – Mammalian artificial chromosomes**

**Bacterial artificial chromosomes (BACs).** In order to overcome the above difficulties associated with YAC vector, a bacterial cloning system based on E. coli F factor was designed which was capable of cloning fragments of up to 300-350kb. These were described as bacterial artificial chromosomes (BACs) and are user friendly' being a bacterial system. BAC vectors are superior to other bacterial systems, based on high to medium copy number of replicons, since they show structural instability of inserts, deleting or rearranging portions of cloned DNA. However, the F factor has regulatory genes that regulate its own replication and controls its copy number. These regulatory genes include (i) oriS and repE which mediate unidirectional replication and (ii) parA and parB, which maintain the copy number to 1 or 2 per E. coli genome. These essential genes of F factor are incorporated in every BAC vector (pBAC), which also has a chloromphenicol resistance gene as a marker and a cloning segment (Fig). Cloning segment includes the following sequences: (i) phage lambda cosN site (providing a fixed position for specific cleavage with lambda terminase) and loxP site (providing a position for cleavage due to P1 Cre protein in presence of loxP oligonucleotide); these two sites allow generation of ends that can be used for restriction site mapping to arrange the clones in an ordered array; (ii) two cloning sites (HindIII and BamHI) and (iii) several C+G rich restriction sites (NotI, EagI, XmaI, Smal, Bg/I cloning site is flanked by T7 and SP6 promoters for generating RNA probes for chromosome walking and for DNA sequencing. BAC libraries have already been prepared in humans, mouse, rice, wheat, Lotus, etc.

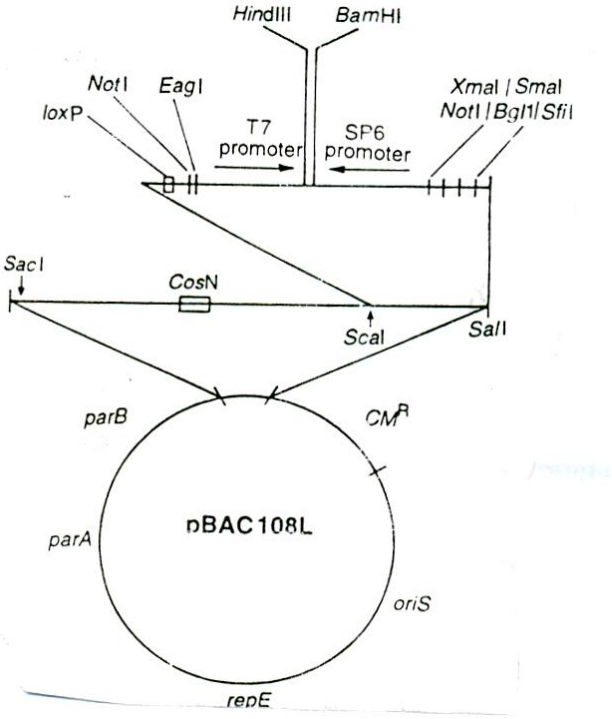



**Figure: A BAC (bacterial artificial chromosome) vector showing its essential elements for cloning.**

## YAC and MAC vectors fo cloning very large DNA segments (1000 kb)

Yeast artificial chromosomes (YACS). We known that in plasmid vectors, sequences upto 10-15 kbp, in lambda (λ) phage vectors sequences upto 22kbp and in cosmid vectors sequences upto 40kbp can be cloned. Yeast artificial chromosome (YAC) vectors, mentioned earlier allow cloning of sequences that and several hundred kilobase pairs (upto 1000 kbp of DNA which may represent whole chromosomes in lower eukayotes with very small genomes can be clone in yeast by ligating them to vector sequences that allow their propagation as linear artificial be generated and allow construction of comprehensive libraries (with large DNA segments) in microbial hosts. With the isolation mammalian artificial chromosomes (MACs have also been produced.

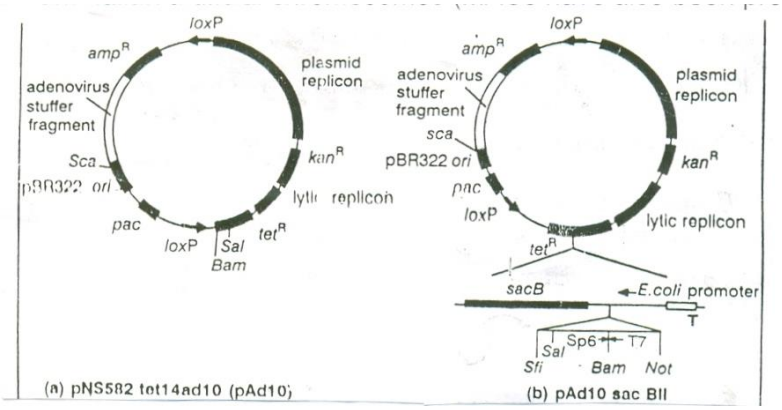



**Figure: An artificial yeast chromosome vector, which may carry a long DNA segment to be cloned in yeast cells.**

YACs have two disadvantages. (i) Cloning against $10^6$ – $10^7$ clones/μg DNA as thus making

it impractical to generate complete genomic libraries through the use of YACs. (ii) It is not possible to recover large amount of pure insert DNA from individual clones; selective amplification of YAC's DNA has recently allowed this problem to be overcome. Both these problems have been overcome in BACs and PACs described earlier.

Mammalian artificial chromosomes (MACs). Mammalian artificial chromosome (MAC) vectors are designed to be able to replicate, segregate and express in a mammalian cell like any other mammalian chromosome along with other chromosomes. Since it will be an independent chromosome, with all the functional elements (telemres, origins of replication, cntromere, etc.,) MAC will not be integrate with the genome and can be used as a vector maintaining a single copy per cell. it could carry large fragments of DNA representing an intact eukaryotic split gene with exons and introns permitting its normal expression regulated by the associated promoter sequences. In view of this, MACs are considered to be suitable for gene therapy, where the inserted DNA will be fully expressed, yet stably maintained without affecting the host genome.

**10. Write short notes on Transposan vectors.**

**From Drosophila**

Drosophila melanogaster possess two classes of transposable elements that are potential cloning vectors. One class is represented by the P elements and the other by retrotransposons such as copia.

**(i) P Elements**

Drosphila strains that always carry the P element (Fig) are known as the P strains. There are others named M strains that rarely posses P elements. When a P strain male mates with an M strain female, the offspring exhibit a variety of defects, collectively referred to as the P-M dysgenesis syndrome. The aberrations include mutations, chromosomal aberrations, and germ line defects that lead to sterility. The syndrome is never exhibited in the progeny of the reciprocal cross.

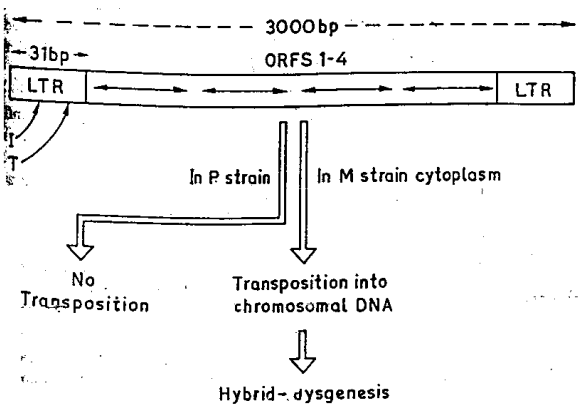


**Figure: A schematic representation of the transposable P element (2.9 kb) from Drosophila melanogaster. Four open reading frames (ORFs) have been identified in the DNA between the two 31 bp long terminal repeated sequences. Transcription signals are within the LTRS.**

Such a one-sided affair has been diagnosed a due to the propensity of the P element to undergo transposition in the cytoplasm of the M strain, but not in that of the P strain (which perhaps harbourssome repressor mechanism). In addition to this bias, transposition is also tissue-specific; it occurs only in reproductive cells or their precursors. Somatic cells are not affected.

Many naturally occurring strains are P strains, some having a many as 50 copies of the element per cell. the element varies in length from 0.5to2.9 kb. In fact, one class consists only of the 2.9 kb elements, and another one of the smaller ones. It appears that the latter are derived from the former as a result of deletions. The 2.9 kb is the complete element and transposes automously with the aid of a transposase enzyme encoded in the element. The smaller elements are transposition defective.

The transposase gene is a spilt one with four exons. The mRNA is splicd differently is somatic and germ-line cells. Only the mRNAs from the latter ar translated into the enzyme. The gene is flanked by 31 pb inverted repeats a regular characteristic of all transposable elements (IS or insertion elements without genes, Tn or transposons containing genes, and giant transposed if it is supplied in trans with the transposae.

The frequency of P element transposition depends on the nature of the P strain and is not quite understood. Some genes are more vulnerable to insertion of the P element than others. The locus singed is one of the hot spots for P insert ional mutations.

Rubin and Spradling (1982) developed a strategy for the stable insertion of cloned DNA sequences into the germ line cells of Drosophila, using vectors based on P elements. The sequences of the rosy ($ry^+$) gene, that provides a component of the wild type eye colour, was used for the purpose.

Two plasmids were constructed by Rubin and Spradling. One contained the inverted repeats of the P element flanking the $ry^+$ gene and the other had a P element lacking the terminal repeated sequences at one end. The latter was transposition defective but carried the transposes gene.

Both types of plasmids were injected into the polar plasm of the ry D. melanogaster embryos. The polar plasm becomes organized into pole cells that differentiate eventually in the adult into gamets. The $ry^+$ gene is transposed into a Drosophila chromosome and remains integrated stably in the offspring derived from the manipulated individual. The presence of the $ry^+$ locus in the progeny files. Among the offspring were some with the $ry^+$ phenotype. The fruit

fly mutant for the rosy allele would have, if untampered with, produced only ry gamete that would yield only homozygous recessive offspring. But the introduction of the $ry^+$ gene rescued some of the progeny from the mutant stats.

Rescue of the mutant phenotype by the insertion of cloned DNA in P elements has been utilized successfully for defining the actual limits of a gene. Different lengths of DNA, including the 5 and 3 flanking regions, are used to transform files that carry the mutant allele for the gene. The minimum length of the inserted gene that restored the wild phenotype is the actual length of the gene. Smaller genes of Drosophila (43 kb to 54 kb or less) have been defined in this manner. They include the above mentioned ry (encoding the enzyme xanthine dehydrogenase) and the genes for alcohol dehydrogenase and dopa decarboxylase.

P element vectors have also been useful in studies of gene regulation during development. The gene of interest is fused to the E. coli lacZ gene and successful insertion assessed visually in situ by monitoring the expression the E. coli gene. Cell and tissue-specific expressions have thus been located, using the promoter for hasp 70 (the major heat shock protein) that functions in almost all tissues during development.

P element-mediated gene transfer promises to b a valuable addition to the gimmicks available to a genetic engineer.

It may be mentioned here that gene transfer into offspring via genetically engineered reproductive cells in Drosophila is no different from the gene transfer system that produces transgenic mammals. In the latter (see Section) the vector used to integrated the desired gene in the germ line DNA is predominantly based on retroviruses. The story of the 'transgenic' fruit files is inserted here to emphasize the use of its own special vector or P element.

**(2) Retrotransposons**

The second group of mobile Drosophila elements is similar to the integrated proviral sequences of vertebrate retroviruses. The elements possess long terminal repeats and open reading frames in the intervening DNA. The best characterized elements of the group are copia and gypsy.

Copia has been utilized for delineating genes by the technique of transposon tagging (see Section). It is not clear whether retrotransposons can be exploited as vectors as well.

**Transposons as vectors**

Transposons of higher plants (Ac/Ds) or Mul of maize). Ac and Ds are popular transposons in corn, and were earlier known to represent activator-dissociation (Ac-Ds) system. Each represents a transposon with short terminal repeats enclosing a long DNA segment, which

measures more than 4500 bp in Ac and about 400 bp in Ds. Each possesses genes including the gene for transpose enzyme responsible for transposition. Part of this region can be deleted and the transposon can be used for cloning of foreign DNA segment in much the same way a in other cases.

**Transposons of Drosophila (P elements).**

P element of Drosophila consists mainly of 31 bp (base pairs) terminal inverted repeats enclosing a 3 kilobase protein coding region, coding for transposase and a repressor of transposition.

Many P elements carrying internal deletions are also found, where the transposase derived from other complete P elements help their mobilization. In these deleted P elements, foreign gene can be inserted to give recombinant P element, which can be microinjected into the fertilized egg, along with normal P element (helper P to supply transposes). The inserted gene is transposed on an embryonic chromosome.

**11. Briefly discuss on yeast is used as a cloning vector.**

**From Yeasts**

Sacharomyces cerevisiae possesses a natural plasmid in many of its strains. This is a 6.318 kb (2 μm) long sequence in a covalently closed circular form (Fig.). This plasmid, called the 2 μ or scp (Saccharomyces cerevisiae plasmid), is a multicopy variety with up to 100 copies per cell (Hollenberg, 1982).

There are two 599-base-inverted regions in the plasmids that divide the DNA into two unequal regions. Recombination between these similar sequences results in plasmids that fall into two classes, A and B, differing only in the orientation of the inverted regions with respect to each other. There are several restriction enzyme recognition sites on this plasmid.

There are three ORFs (open reading frames, or putative gene sequences) in the plasmid; they are A, REP1 (or B) and REP2 (or C). The product of A is involved in recombination, and the others are involved in the stable but random portioning of the plasmid copies into daughter cells.

Some strains of yeasts possess endogenous 2 μ plasmids. They are the cir strains. Those without the plasmid are called the cir strains. It has been observed that it is more difficult to transform cir cell with a 2 μ vector than $cir^+$ cells. Vectors that contain the entire 2 μm DNA are transferred more easily than those that carry pars of the plasmid lacking the replication retgion.

Yeast plasmids posses ARS (Autonomous Replicating Sequences) and CEN (centromere-like) that are needed for replication of the plasmid and its portioning to daughter cells. These

sequences have been exploited to construct what are known as Yep, YRp, YCp vectors, as well as ones known as artificial minichromosomes.

YEps are yeast episomal plasmids derived from the 2 μ plasmid. They occur as multicopies of free circular DNA. Hybrid plasmids containing sequences of 2 μ and MB9 (with $Tet^R$) have been further modified by the addition of sequences from yeast chromosomal DNA (Beggs, 1981). Some of them are the yeast integrating plasmids (YIP) utilized for integrating a donor DNA into yeast chromosomes. LEU2 is one of the yeast markers utilized in these vectors: it allows recognition of transformed leu cells among untransformed leu cells.

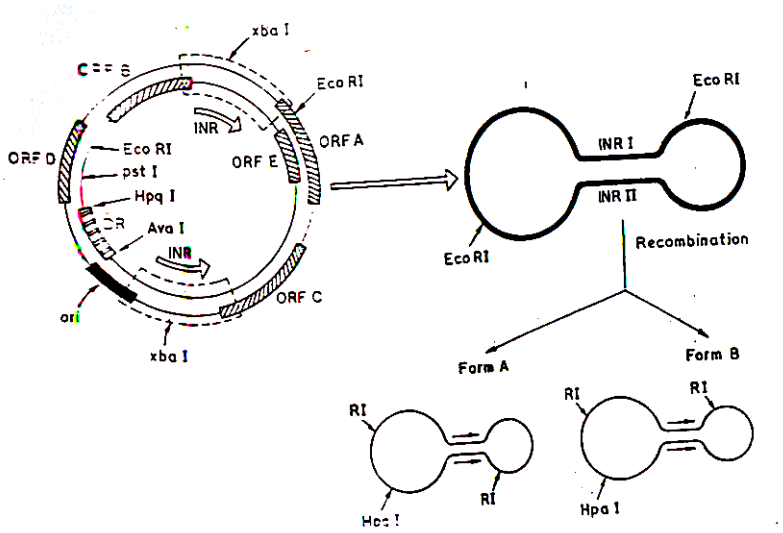



**Fig. 2.56 The 2 μm yeast plasmid. Strains of *Saccharomyces cerevisiae* possessing this plasmid are said to be cir$^+$; those lacking it are cir$^-$. There is a pair of 6000 bp long repeated sequences that are in opposite orientations (inverted repeats or INR) which, on pairing, divide the plasmid into a central stem with unequal sized loops at each end. Homologous recombination between the INRs results in types A and B plasmids that differ in the orientation of the sequences in the smaller loop. This is evident from the position of the single *Eco* RI site in this loop.**

A YRp contans, besides the E. coli plasmid sequences, some sequences from yeast DNA that include the ARS segment. These yeast replicating plasmids replicate in yeast cells. Struhl et al. (1979) were the first to utilize these vectors.

A YCp is a yeast vector containing the CEN sequences. The latter regulate, portioning of the plasmids during cell division.

The ARS, CENT r (telomere) sequences from another source have been combined in a vector known as an 'artificial minichromosome'.

## UNIT – III

## PART – A

**1. What are clones? Give an example.**

Clones are the production of identical multiple copies of the recombinant DNA molecules or the production of identical multiple numbers of recombinant organisms which are produced by recombinant DNA technology. The clones are identified by screening or selection methods. The recombinant clones are identified by special features and screened by specific technique.

**2. What is genomic Library?**

In order to isolate one or more related genes from a genome, we like is prepare at mixture of clones each carrying DNA derived either from the genomic DNA or from CDNA C derived from MRNA isolated from a specific metabolically active tissue of an organism. This mixture may contains thousands of clones, which when derive directly from the genomic DNA are collectively called a genomic library.

**3. Give any four uses of Genomic Library.**

Genomic Library is the source of collection of gene fragments of genome. The uses of genomic library is one can understand the genome as how much of clones can be derived from one genome. This genomic library is used for fish out a desired gene for chromosome walking. This is mainly used for preparation of clones made form the nature DNA.

**4. What are isoshizomers? Give examples..**

Occasionally enzymes with noval DNA sequence specificities are found but most prove to have the same specificity an enzymes already known.

Isoshizomers are the restriction enzymes with the same sequence specificity and cut site.

Enzymes that recognize the same sequence but leave at different points, are known as neoshizomers. E.g. SmaI(CCC/GGG)
XmaI(C/CCGGG).

**5. What are the specific methods used to select a specific clone?**

1. Direct selection

i. Complementation or supporessor mutation
ii. Selection of recombinant deficient phages
iii. The spi phenotype: Recombinant proficiency
iv. Selection for the correct promoter sequence
v. Tetracyclin resistance due tot inactivation of CI
vi. Selection of the size of an E.coli colony.
vii. The TK/HAT system
viii. The gpt system.

**6. Partial digestion of DNA is preferred for constructing genomic Libraries – why?**

The nature DNA or complete genomic DNA is used to digest to get various length of fragments which are consist of many genes together in the fragment or individually in the fragment. So, it is more convenient to have smaller DNA fragments by partial digestion with

digestion enzymes before preparing the genomic Library. It complete digestion is done, larger number of gene fragments are obtained which is cumbusome to handle genomic Library.

**7. Give any 4 screening methods used to screen a clone.**

i) Direct Screening

a) Insertional inactivation of a marker gene
b) Visual screening methods
c) Plague phenotype.

ii) Indirect Screening

a) Immunochemical Technique
b) Hybrid –arrested translation
c) Hybrid – selected translation

**8. Write short notes on Southern Blotting.**

Southern blotting is otherwise known as Nucleic acid Hybridization. This is used for fragmentation of DNA or RNA which is fractioned on an agarose gel electrophoresis and the bands are separated and identified. The separated bands are transferred ( other wise blotted) to nitrocellulose strip/filter paper to which nucleic acids adhere firmly. This blot is them flooded with a radioactive nucleic acid probe for the gene to be searched for in the bands. The auto radio graphed printed sheet is used for characterization of hybridized Nucleic acid probes.

**9. What is the use of colony hybridization? (Hogness and Grunestein)**

The principles of the colony hrbridization is more or less similar to southern hybridization method. This is developed by hogness and Grunestein in 1975. there, the colonies are allowed to grow on nitrocellulose paper placed in contract with the nutrient agar. When the colonies developed about 1mm dia, then it is lysed with detergent and the DNA is denatured. Then the nitrocellulose strip is auto graphed and the DNA probe in characterized. The identified calories are amplified separately from the master plate and the DNA utilized as required.

**10. What are the nucleic acid probes? How it is prepared?**

A nucleic acid probe is a short or long length of single – stranded RNA or DNA that is complementary to a portion of the nucleic acid to be identified. There are thus RNA, CDNA, DNA and rick – translated SS DNA probes. A general procedure fore preparing a nucleic acid probe is to have a cell – free reaction system containing the template, the polymerizing enzyme, the four NTPs, one or more of which are radio actively labeled, and other factors needed for the reaction to

occur. The reaction mixture is periodically assayed for the length of the nucleic acid polymers.

**11. How will you prepare RNA probes and identify the RNA probes?**

The RNA pobes are generally prepared from mRNAs of gene that are abundantly expressed in a cell. For examples, cells such as erythrocytes (RBCs) are primarily filled with globin mRNAs. The total mRNA may be extracted by gel electrophoresis. The mRNA of the size expected for the DNA (gene) to be probed is isolated and then end – labeled, by first removing the 5'-terminal phosphate using alkaline phosphate, and adding $^{32}P$- labeled phosphate with the help of kinase.

**12. How CDNA probes are prepared?**

CDNA probes are prepared from mRNA of suitable to produced a complementary. The mRNA is copied to produced a complementary DNA with the help of reverse transcriptase (RNA – dependant – RNA polymerase). The enzyme used routinely is from the AMV (Avion mycloblastosis virus) , which does not posses the exonuclease activity. The polymerase requires a primer. In enkanyotic mRNA, the poly adenglated tail may be utilized as one. A poly dT oligo nucleotide primer (about 12 Ts) is synthesized chemically.

**13. Give two methods of hybridization of nucleic acids.**

**i) Southern blotting:** Southern blotting is generally used for characterization of DNA probes after separation by agar gel electrophoresis. Nitrocellulose strip is used for transfer of DNA bands and compared with labeled markers.

**ii) Northern blotting:** Northern blotting generally used for identification of RNA probes. The RNA bands are transferred to Nitrocellulose or equal solid substrate after separation by agar electrophoresis.

**14. Write short notes on Western blotting.**

Western blotting is generally used for hybridization of protein bands / molecules. The technique of transferring protein fractionated on a gel to a solid support inevitably became dupped as western blotting. Here, the protein blotting of filter paper is pretreated with specific reagents appropriate to the protein bands or probes identified. The methods used are i) immunodetection, ii) binding of proteins to ligands immobilized on the filter paper and iii) binding of tagged molecules to proteins immobilized on the filter paper. Protein are fractionated on polyacrylamide gels and transferred to nitrocellulose paper.

**15. How the immunodiagosntic probes are prepared and utilized?**

Antibodies or immunoglobulins constitutes the main ammunition of the defence system of the animal body. Antibodies are an excellent biomolecular reagents that may be used to identify and isolate even small quantity of antigen from a mixture. The probe may the protein itself, or the antibody it provokes in a foreign body. The serum containing the relevant antibody in usually called as antiserum is used for hybridization.

**16. What are the immunological tests used for identification of immunodiagnostic probes used for hybridization technique?**

The antigen – antibody reaction and complex formation is a main principle used for estimation of amount of antigen (or antibody) present in the system. Following are the fue methods used.

i. The precipitin test
ii. Immuno diffusion technique
iii. The immuno electrophoretic technique
iv. Radio immunoassay (RIA)
v. Enzyme linked Immuno adsorbent assay (ELISA)
vi. Immuno affinity chromatography

**17. Define (DNA Library) Give its significance.**

CDNA is otherwise complementary DNA is prepared or synthesized from RNA template using the enzyme reverse transcriptase. A CDNA Library or alone bank / gene bank contain CDNA rather than genomic DNA. The CDNA fragments are choosed for splicing to a vector in recombinant genetic engineering technology. A CDNA bank is useful when one is particularly interested in recovering a specific gene.

**18. What are the advantages of having cDNA Library?**

1) For cloning genomes of RNA viruses. CDNA copies are the only possible inserts applied to vector.
2) One can get idea about the base level house – keeping genes comparing CDNA clone banks, from cells of different stages and tissues.
3) The expression of eukaryotic split gene in prokaryotic cell can be achieve.
4) CDNA is useful for delineating the exon and intron regions of a split gene.

**19. How will you prepare cDNA library for a particular gene?**

i) The CDNA bank (or) library is prepared by extracting the total MRNA responsible for the gene. The mRNA is extracted and purified by using special techniques.
ii) The mRNA is next copied into single stranded CDNA using a poly dT – oligonuceotide as primer and the AMV reverse transcriptase is an enzyme.
iii) Then synthesis of second strand of CDNA and obtain ds CDNA.
iv) Providing a suitable joining ends to CDNA.

**20. How suitable joining ends are provided in cDNA or made for joining with vector DNA?**

If the ends of CDNA are not suitable to join with vector DNA molecule, then suitable ends are provided. These are made by using SL nuclease. Flush ended CDNA are joined with flush ended vector ends using $T_4$ ligase. It staggered end, specific for a particular restriction enzyme, may be created at the CDNA terminally by first joining the restriction enzyme linker, which is subsequently cut with the restriction enzyme.

**21. Write short notes on cosmids and phage λ gene Libraries.**

Cosmids carry maximum 45 Kb of insert DNA, but phage λ vector bear a maximum of 25 Kb of the donar DNA. These larger insert DNA can encompass long genes, particularly eukaryotic split genes. The long DNA of cosmid gene is less easy to handle and the events reduce the size of the cloned fragment. Therefore, larger number of clones is required to screen.
On phage λ libraries, the techniques for screening are simple and large number of clones for screening is not a problem.

**22. What is dot blots and slot blots?**

Dot blots and slot blots are otherwise the techniques of southern blotting and Northern blotting on dot blots, coned or pure extracted DNAs are slotted adjacent to each other on a nitro cellulose membrane. DNA blots thus produced are immobilized and denatured so that they are bound or the membrane as ss DNA blots. The membrane is then hybridized with radio active labeled Nucleic acid probe. The identity of the dot will indicate the probe in the DNA sample used for particular Dot.

**23. What is concatemer? Write about concatemerization.**

Concatemerization results in the formation of heterodimers and transcription across the junction yields a mRNA that can be processed to splice out the terminal repeats of the vector. In this way, CDNAs of up to 10 Kb can be expressed. This is known, as concatemers.

## PART – B

**1. Explain in detail about four methods that can be used in the selection/screening of recombinant clones By Direct Screening / election method.**

**Direct Screening**

The most popular direct screening methods are the ones in which the desired clone can be distinguished by the activation of a gene, by the biochemical reaction of a cell product with a chemical with a chemical in the growth medium, or by the appearance of plaques due to bacteriophages.

**(1) Insertional Inactivation of a Marker Gene**

Cells carrying the hybrid DNA can be identified by the mutant phenotype of a marker gene. The mutation is due to the cloning of the donor DNA within the marker gene. The disrupted gene, therefore, exhibits the mutant phenotype. The most common example of this technique is the cloning of an insert in the $Tet^R$ or $Amp^R$ gene of pBR 332. In the former case the hybrid DNA containing cells will be $Tet^s$ (unable to survive in tetracycline – supplemented media). Similarly, the cells with a disrupted β-lactamase gene ($Amp^R$) will not survive in the presence of ampicillin.

Of course, the above mentioned screening tests are not carried out on the valuable master plates. Replicas are made of the latter in several fresh Petri plates, and the replicas are utilized for screening. If a alone is not evident in a replica plate, the one in the corresponding position in the master plate is identified as the one that did not grow under selection pressure. The identified colonies or plaques are then amplified in separate plates to recover the clones carrying the desired DNA.

Another 'trick' is to use vectors that carry part of a bio or trp operon of E. coli. These handicapped phages can only grow in E. coli mutants that possess mutation in some other region of these operons. When an intact bio-vector is introduced into the E coli mutant for another part of the same operon, the two defects complement each other, so that the cells grow without added biotin in the medium. However, if a donor DNA is inserted within the bio sequence of the vector, it can no longer complement the mutation in the cell; the result is an inability of the cells to grow unless biotin is supplied to them.

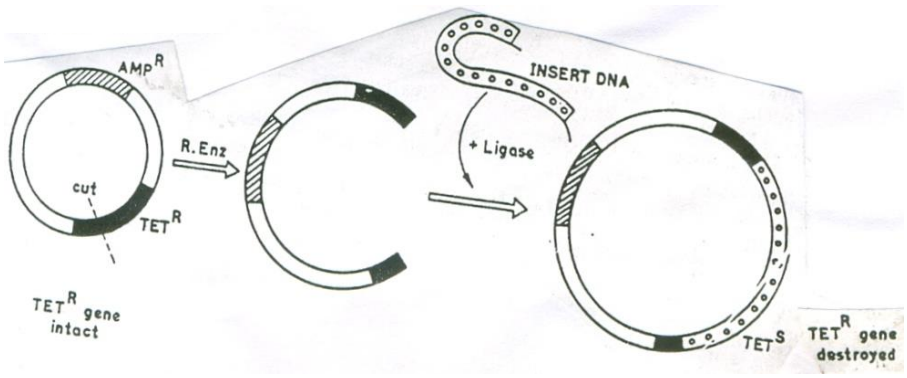



**Figure: Insertional inactivation of the $TET^R$ gene Cells containing a hybrid vector with the foreign gene, inserted**

**within the $TET^R$ gene, do not survive in the presence of tetracycline. Cells with the intact $TET^R$ gene (and, therefore, non – hybrid vector) grow in the presence of the antibiotic.**

Hence, cloning in such a vector will readily allow screening of cells carrying recombinant DNA; the latter will only grow on medium with added biotin or tryptophan (in the case of the vector with trp sequences).

Insertinal inactivation of the lamba – repressor gene, CI, has been mentioned earlier. A DNA cloned with in CI prevents lysogency; hence, recombinant plaques are always clear, while non- recombinant ones are turbid in appearance (Fig.)

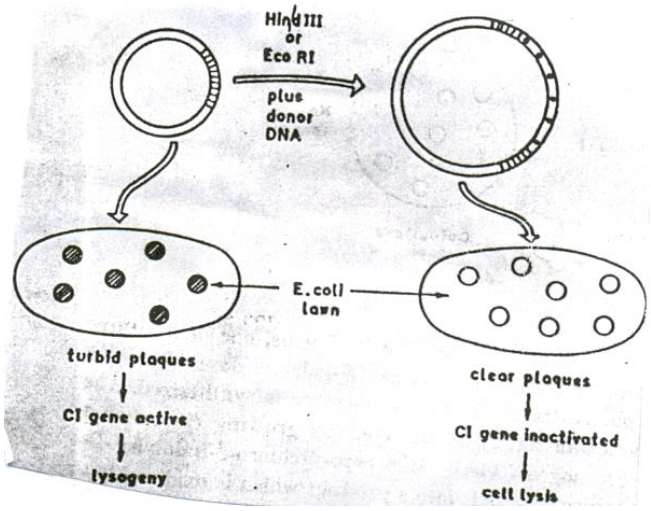



**Figure: Insertional inactivation of the CI gene. Disruption of the CI gene, by cloning a foreign DNA within it, results in the absence of the lambda – reprocessor and the establishment of the lytic phase of the phage infection. Hence, clear plaques represent clones of recombinant phages.**

**(2) Visual Screening Methods**

The commonest: Visual screening strategy uses the action of a gene product on a chromogenic substance to distinguish recombinant and non – recombinant clones. The gene product is the enzyme β-galactosidase that converts a colourless substance, XGAL (5-bromo-4-chloe-3-indolyl-β-D-galactopyranoside) into a derivative that oxidizes into a blue non-diffusible dye (5,5′-dibromo-4,4′-dichloro indigo).

β - galactosidase is encoded by the lacZ gene of the E. coli lac operon. Several vectors carry the promoter and the first 145-150 codons of the lacZ, which represent the β-poly peptide of the enzyme. Cloning sites are available to introduce inserts into the lacZ sequences. A donor DNA inserted within the lacZ prevents enzyme synthesis. Cells grown in a medium to which XGAL has

been added will be blue in colour or colorless. Depending on whether or not the cell is expressing the gene for the enzyme. Recombinant cells are colourless, while non- recombinants are blue (fig.).

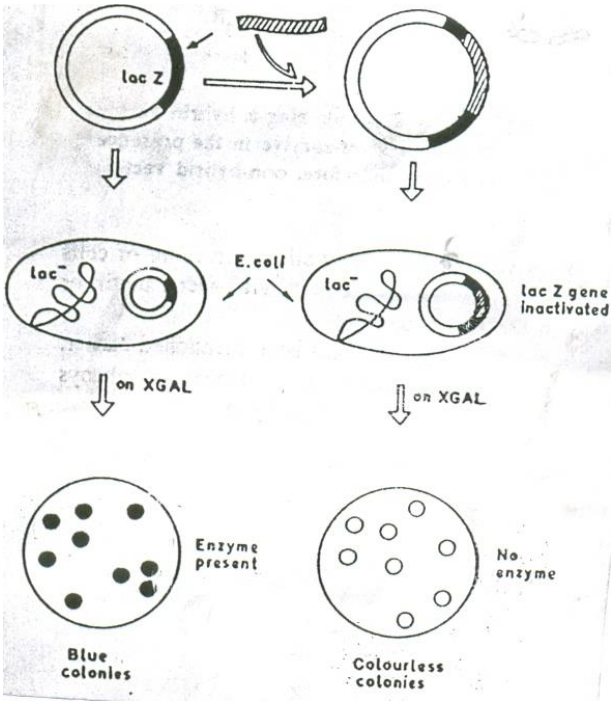



**Figure: Insertional inactivation of the lacZ gene. The lacZ gene, one of the three genes of the E. coli lactose operon, expresses the enzyme β - galactosidase. Cloning a foreign DNA within this gene inactivates the latter; no enzyme is synthesized. The presence or absence of β-galactosidase may be detected by growing E. coli in a medium supplemented with a chromogen XGAL into a product which is oxidized to a dark blue coloured product (5,5′-dibromo-4,4′-dichloro-indigo). Colonies synthesizing β-galactosidase are dark blue in colour. Colonies harbouring disrupted lacZ sequences (hybrid vectors) are colourless on XGAL.**

Messing has exploited the lacZ XGAL system in several screening strategies. In one of them the β-peptide region of the lacZ and the promoter of an M13 vector is present. The E. coli in which the vector will be introduced carries a deletion in the aminoterminal region of the β-galactosidase which is complemented by the carboxyl end – deleted lacZ product from the vector. Together they produce functional β-galactosidase. If a donor DNA is cloned in a site with in the lacZ region in the vectgor, this disrupted gene will no longer complement the truncated one in the cell. So, once again, the recombinant DNA containing cells will be colourless.

A second vector constructed by Missing is a pUC (a derivative of pBR322) that carries the lacZ gene within which there is an inserted polylinker. The presence of the extraneous poly linker does not affect the expression of the enzyme. However, if the donor DNA is cloned within one of the restriction sites in the polylinder, no enzyme is synthesized.

**(3) Plaque Phenotype**

The repressor protein, encoded by the CI gene establishes lysogeny and prevents the formation of free phage (λ) in E. coli. Vectors are available (immunity vectors) that have restriction sites within the CI (or immunity) region; imm 434 is one such vector with sites for Eco RI and Hin dIII. A DNA fragment cloned in any one of these sites destroys the CI gene. Hence recombinants are picked out as clear plaques.

**Direct Selection**

A dominant selectable market is used to announce the presence of recombinant clones. The marker may be the insert DNA itself, or included in the system when the cloned DNA does not proffer a selectable phenotype. A few of the more commonly used systems are given below.

**(1) Complementation or Suppression of Mutation**

The phenotype after transformation of the cell could be due to a complementation (fig. or a suppression (fig.) of a mutuant phenotype in the cloning cell. If the transfected gene is $his^+$ and the E. coli a $his^-$, transformation of the latter will make it a wild – type for the his locus. Such a cell will grow in minimal media without added histidine, unlike this $^-$ cells that require histidine supplement for growth. In other words, the transfected gene complemented the lesion in the cell. K Struhl (1976) utilized this method to isolate E. coli clones containing $his^+$ carrying fragments of yeast DNA. Fragments of yeast DNA were spliced to plasmids that were used to transform E. coli cells.

**(2) Selection of Recombinant – deficient Phages**

Lambda vectors, with cloning sites within the redA, and redB recombination genes, are available. Intact vectors can grow in $lig^-$ and $polA^-$ E. coli cells (incapable of recombining DNA) due to the presence of the phage red gene. If the latter is cut asunder by an inserted DNA, the phage no longer mediates recombination in the lig, $polA^-$ E coli. The rDNA containing phages are recognized by plaque morphology.

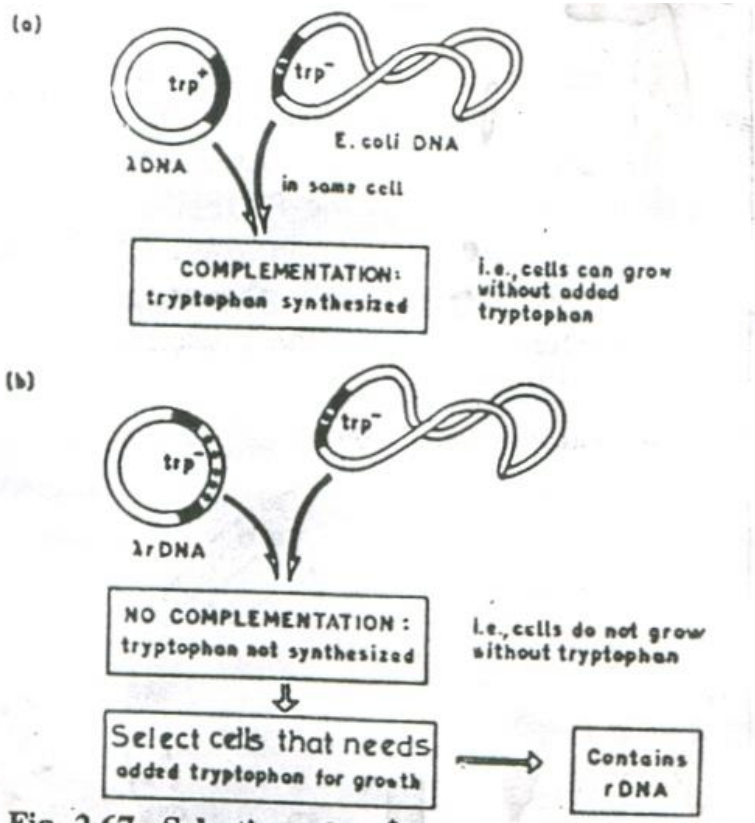



Fig. 2.67 Selection of *trp*$^+$ by complementation

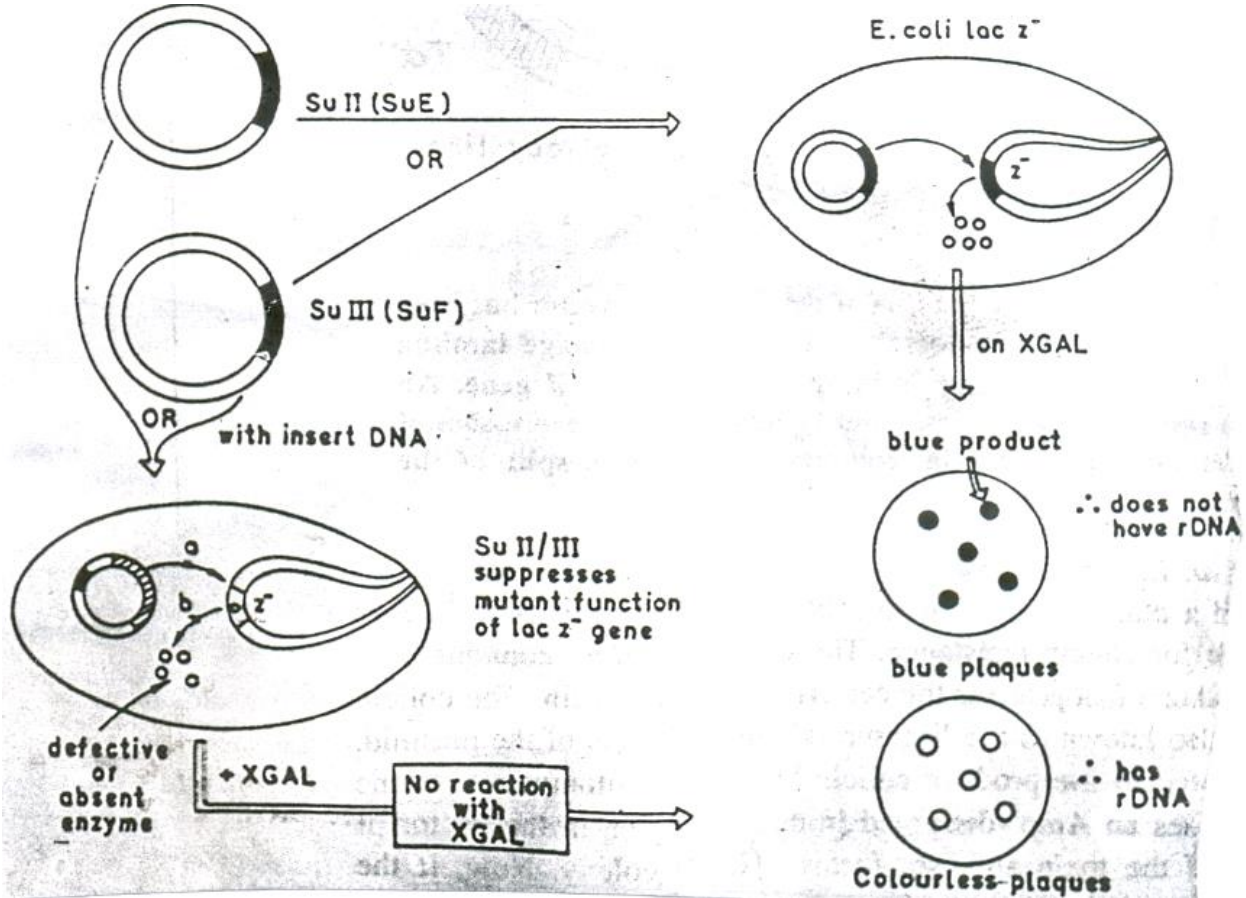


**Figure: Selection of recombinant clones by suppression of mutations. The mutant tRNA, su II (or SupE) and Su III ( or SupF), genes suppress amber mutations (UAG) by reading the amber stop codon as anb amino acid, SupF, a tryrosine – specific tRNA, reads UAG as a codon for tyrosine. Translation, therefore, continues with only onle deviant amino acid residue in the polypeptide, which restores the wild-type phenotype of the mutated locus. This feature of suppression is exploited in many ways. In this illustration a foreign gene is cloned within a Sup F gene inactivating the latter. That is, it is no longer able to reverse the activity (non- activity) of the mutation present in the lacZ gene. Recombinant colonies, therefore, remain colourless on an XGAL medium.**

### (3) The Spi Phenotype: Recombinant proficiency

A phage lambda containing the recombination genes red and gam does not grow on E. coli (P2). The phage is said to be sensitive to P2 prophage instigation or interference ($spi^+$). A cell with a replacement vector that is $gam^-$, $red^-$, and which has the non essential region replaced with the insert DNA, with the aid of the recA product; only multimeric DNA can be the substrate for packaging into lambda capsids.

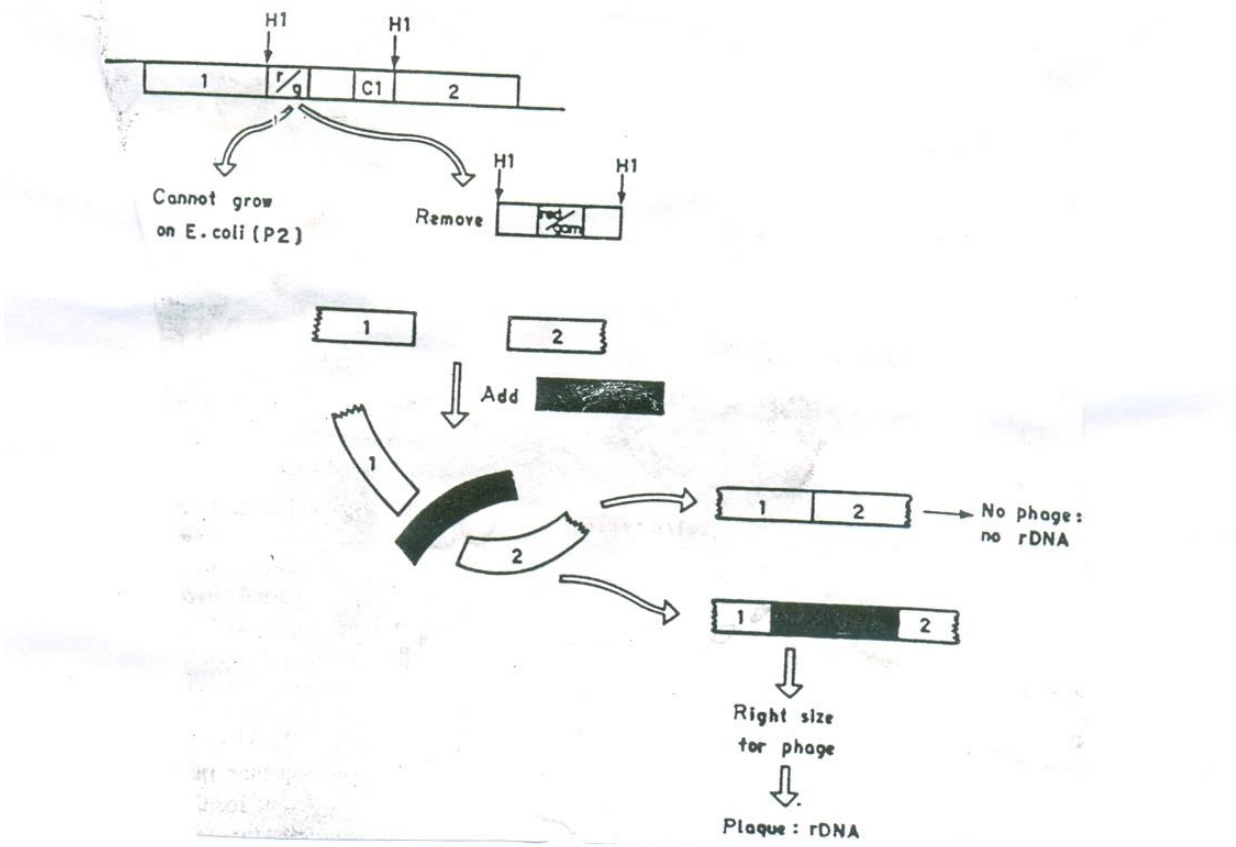



**Fig. Spi phontype selection. The phenotype spi $^{+}$ is possessed by phage lambda (red$^{+}$ gam$^{+}$) that are unable to grow in E. coli (P2); the phage is said to exhibit 'sensitivity' to phage interaction. Spi$^{-}$(red$^{-}$gam$^{-}$) phages grow on the same lysogens. Recombinants in which the red, gam region has been replaced are, therefore, spi$^{-}$ and can be picked up from clear plaques on lawns of E. coli (P2).**

### (4) Selection for the Correct Promoter Sequence

The DNA fragment to be cloned is not necessarily only the coding region of a gene. Non-coding regions are cloned and studied for an understanding of their role, if any, in the expression of a gene.

Different regions of a promoter sequence, as well as different promoters, have been examined by using 'promoter probe plasmids that have been derived from pBR 322 (fig.). The promoter of the Tet$^{R}$ gene of this plasmid is inactivated by an 8- base insertion within in it. The octanucleotide is an artificially synthesized one having an Eco RI site with in it. A promoter sequence, from a promoter probe plasmid, may be inserted at the Eco RI site. The Tet$^{R}$ gene thus acquires a new promoter. If the latter has the correct sequence of bases. The Tet$^{R}$ gene is expressed. Degrees of efficiency of different versions of the inserted promoter may be assessed quantitatively by the activity of the Tet$^{R}$ gene.

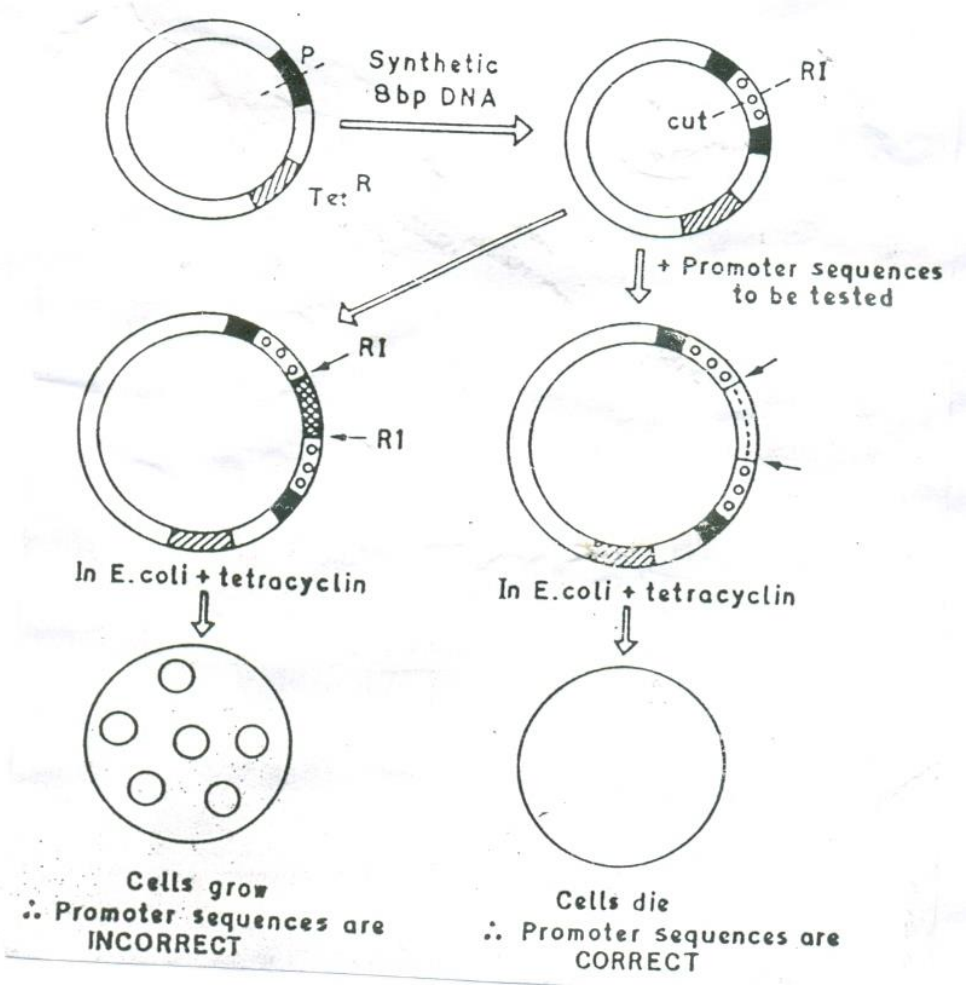



**Fig. Selection of correct promoter sequences. Any part of the DNA, genic or non- coding may be examined by rDNA techniques. In this illustration we see a system in which different sequences of a promoter are tested to discover the one. Promoters to be tested are cloned in 'portable' vectors, which are inserted within the promoter of the $Tet^R$ gene of pBR322. If the 8 bp long sequence with in the promoter is a correct one the $Tet^R$ gene will be expressed; as the test resistant to the drug. Incorrect promoters will fail to initiate transcription of the gene, so that cells carrying them will remain tetracycline sensitive.**

### (5) Tetracycline Resistance due to Inactivation of CI

A derivative of pBR322 has been developed by Roberts and associates (1980) in which the promoter of the $Tet^R$ gene is replaced by a piece of DNA carrying the CI gene and the λ $P_R$, which are in cis with the $Tet^R$ locus. There is a cloning site with in the CI sequences. When the latter are intact the CI protein binds itself to the $P_R$ region and prevents transcription of the $Tet^R$ gene. If the CI gene is interrupted by an insert DNA, there is no repressor to block expression from the $P_R$ and the $Tet^R$ phenotype of expressed. Recombinant DNA in a cell makes the latter survive in the presence of tetracycline, but not intact vectors (Fig.).

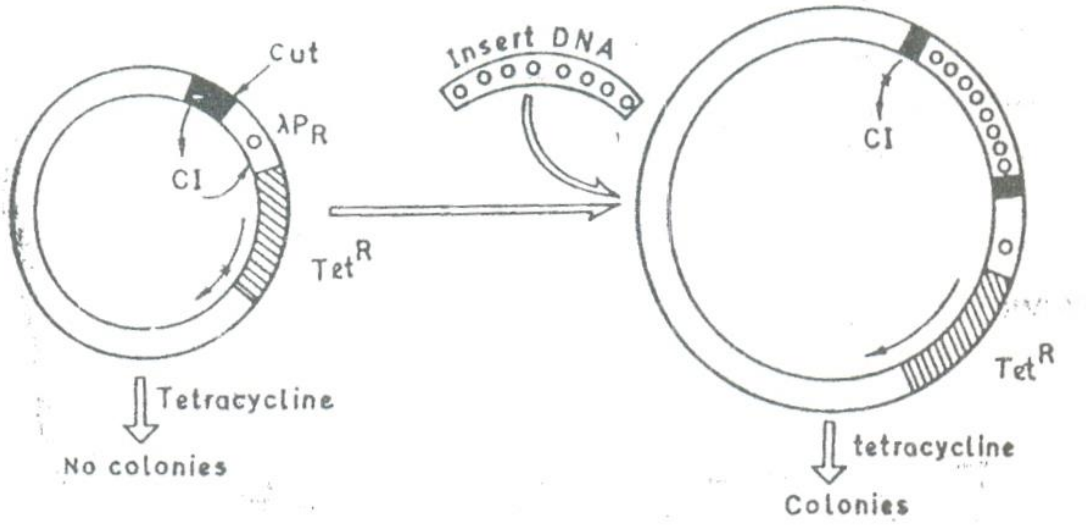



**Fig. Tetracycline resistance due to inactivation of the CI gene. A vector has been constructed in which the $Tet^R$ gene is under the control of the phage lambda repressor (CI) gene. The DNA to be cloned is inserted with in the CI gene. An inactivated CI gene fails to repress transcription from $P_g$ and so allows expression of the $Tet^R$ gene. Recombinations are, therefore, colonies that grow in spite of the presence of the antibiotics.**

### (6) Selection of the Size of an E. coli Colony

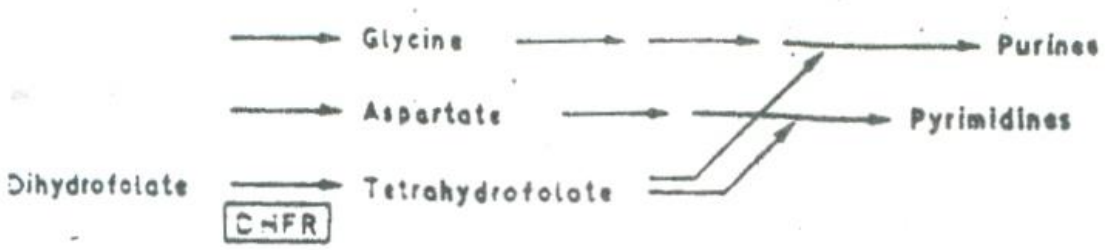
Okazaki (1980) has designed a plasmid vector with a bla (β-lactamase or $Amp^R$ gene) sequence inserted in the gene for colicin resistance. The col E1 plasmid contains both a gene for the toxin colicin and one that protects the cell from its own colicin. The colicin resistance gene is, therefore, also known as the 'immunity' (imm) region of the plasmid.

E. coli cells can be stimulated to overproduce colicin by adding mitomycin C to the medium. If such a cell possesses an $Amp^R$ – disrupted immunity region in the vector, it cannot withstand the effect of the toxin and, so, forms a small colony. Now, if the foreign gene is cloned within the colicin EI gene, the toxin is not synthesized even when provoked by mitomycin C. Such cells grow into normal sized colonies. Recombinants are thus picked off as normal colonies on mitomycin C plates.

### (7) The TK / HAT System

One of the first selectable markers for cloning in eukaryotic ells is based on the availability of the proper enzyme to synthesize thymidine monophosphate (TMP), a precursor of DNA. There are two pathways in higher eukaryotes for the synthesis of TMP (fig.). The regular one is synthesized from dUMP (deoxyuridine monophosphate) with the mediation of the enzyme thymidylae synthetas which requires tetrahydrofolic acis as well.

**(a) Regular biosynthesis**




**(b) Salvage pathways:**

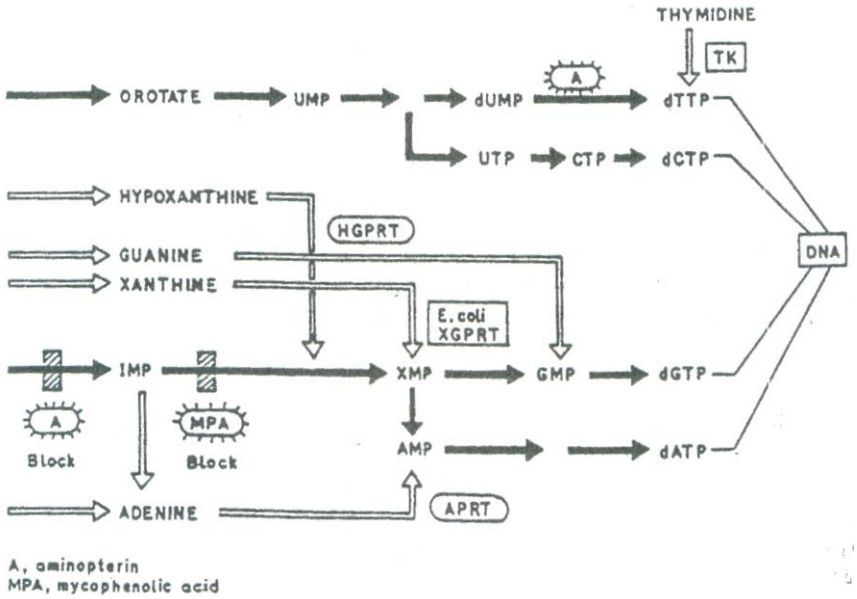



**Fig. Regular and slavage pathways for purine and pyrimidine synthesis and sites for metabolic blocks by the inhibitors aminoperin and mycophenolic acid. There are two pathways each for the synthesis of dATP, dGTP and dTTP. If one of them is blocked, the alternate or salvage pathway is utilized. Blocking with aminopterin allows the cell to convert hypoxanthine or guanine into GMP provided the genes hgprt or xgprt are available, together with hypoxanthine, similarly, instead of using the pathway that begins with aspirate, thymidine is used to synthesize dTMP with the help of the $tk^+$ gene. The E. coli xgprt gene is analogous to the animal gpt gene. Aminopterin blocks the action of genes hgprt required for the maintenance of the dihydrofolate – tetrahydrofolate – methyltetrahydrofolate cycle and the action of thethymidylate synthetase (ts) gene that is required both for the above cycle and for the synthesis of TMP.**

This enzyme can be stymied by analogues of folic acid such as aminopterin and methotrexate. However, there is a second or slavate pathway via which TMP can still be synthesized. This depends on an enzyme thymidine kinase (TK) that can utilize thymidine if supplied exogenously. If both pathways blocked, the cell does not make any more DNA and so fails to survive. The salvage route is blocked when the cell is $tk^-$.

A selection system has been extensively in use for eukaryotic cells in which the above mentioned facts have been exploited (Litchfield 1964). If the media contain the inhibitor of the de novo pathway as well as exogenous thymidine, cells that contain the $tk^+$ allele manage to survive, courtesy of the salvage pathway. Such a medium is the HAT which contains hypoxanthine, aminopterin and thymidine. The hypoxanthine is added to allow purine synthesis (adenine and guanine) which is also inhibited by aminopterin. The vector carries a $tk^+$ gene.

The TK/HAT system is used to monitor transformation of $tk^-$ cells. Such a strain is the L- strain of cultured mouse cells. The recombinant and non – recombinant vectors are mixed with L- cells and first plated on a rich medium. This allows every cell to grow into substantial sized colonies. The medium is then replaced by the HAT one. Only rDNA – containing cells survive in this medium (fig.).

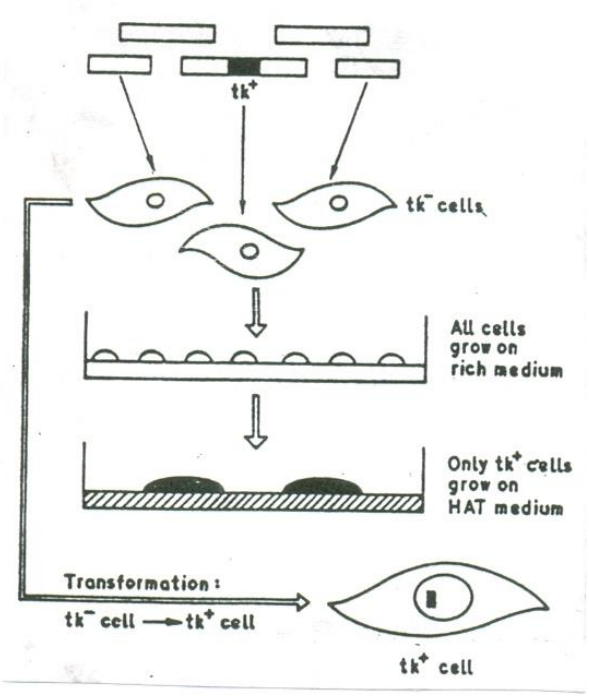



**Fig. HAT (hypoxanthine – aminopterin – thymidine) selection of $TK^+$ cells. Mouse cells of strain L (TK) are transformed with DNA fragments some of which may posses the $tk^+$ gene. All the treated cells are first allowed to proliferate on a rich medium into sizeable colonies. They are then transferred to a HAT medium. Aminopterin blocks the regular biosynthetic pathway for thymidine. However, the tk gene can utilize exogenously supplied thymidine in an alternate pathway. Hence, cells that are $TK^+$ will grow on HAT, while TK cells will not survive on it.**

Not many genes possess selectable phenotypes. Such gene is selected by cloning them in vectors carrying the $tk^+$ gene. The HAT positive cells are further searched for the presence of the gene of interest. Now, there are times where it becomes necessary to distinguish between the vector – specified tk gene product and that from the tk gene of the cloning cell Marker. It can be

discriminated from the host cell gene in a number of ways. Wide et al. (1979) have cloned the HSV -1 gene on pBR322. The gene is on a 3.4 kb Ban HI fragment (Wigler. 1977).

The TK/HAT system is very much in demand for cloning in eukaryotic cells. It suffers from a common handicap of most selection systems in eukaryotes in being limited for use only with the special L- strain cells.

The above strategy has been used successfully by Mantedi (1979) to clone rabbit β-globin genes in L cells. Perucho and associates (1980) utilized the plasmid rescue method to isolate the chicken $tk^+$ gene from total chicken DNA, in the following manner. Fragments of total chicken DNA were used to transform L-cells. The $tk^+$ transformants were isolated, and the DNA of the recombinant vector extracted and spliced to pBR322. These were used to transform cells to the $tk^-$ phenotype. The DNA from the transformed cells was treated with a restriction enzyme that cleaves the chicken tk gene and the pBR322 on the same fragment. These restriction fragments were used to transform $Amp^s$ or $Tet^s$ E. coli cells. The $tk^+$ gene was finally delinked form the BR322 DNA.

The TK/HAT system cannot be utilized for studying the expression of all eukaryotic genes in the L strain of mouse of cells, as all genes are not expressed either at the same developmental stage of the individual or in the cells of every tissue. If the $tk^+$ marker is to be a selection criterion for the broader range of genes, a proper cloning cell that is also $ti^-$ has to be identified for each on e of the genes to be cloned this is still at tall order. There has, therefore, been a search for other direct selection methods in which a mutation or a development stage does not become an essential feature of the cloning cell. One of them is described in the next subsection.

**(8) The gpt System**

As mentioned in the previous paragraph, there is a need for a selection system that is not tied down to aspecial mutant cloning cell. The gpt system satisfies this need.

E. coli possesses a gene for XGPRT – (xanthine guanine phosphoribosyn tranferase) that converts xanthine to XMP (xanthine monphosphate) which is one of the alternate prescursors of GMP (guanine monophosphate). XGPRT also mediates the formation IMP (ionosine monophosphate) from hypoxanthne and GMP from guanosine by condensation with phosphoribosyl pyrro phosphate. Eukaryotic cells do not possess XGPRT and hence cannot utilize xanthine in case the normal route for GMP in eukaryotic cells is blocked.

A vector with a gpt (XGPRT) marker can, therefore, be utilized in any cell, and not in one that needs to be a mutant for the marker gene. Cells with or without gpt can be distinguished easily by growing them on HAT as well as on XAT (xanthine – aminopterin thymidine) media. Whereas $gpt^+$ cells will grow on both these media, gpt non transformants will not (Mulligan and Berg. 1981).

The purein and pyrimidine pathways and inhibitors that block one or the other routes have

been further exploited by using mycophenolic acid as the block for the conversion of IMP into XMP. A $gpt^+$ cell survives in a XAT or HAT medium supplemented with mycophenoilic acid. A $gpt^-$ cell will grow only if adenine and guanine are supplied exogenously (see fig.).

**(9) The dhfr as a Selectable Marker**

The dhfr (dihyrofolate reductase) gene is required for the synthesis of tetrahydrofolic acid. Which is an essential precursor of purines, gylcine and thymidine. Methotrexate and aminopterin inhibit the action of dhfr. Vectors carrying the $dhfr^+$ gene allow dhfr cells to be transformed and the latter may be isolated by their lack of resistance to added methotrexate. The concentration of the inhibitor added is sufficient toblock dhfr expression in the cloning cell. The $dhfr^-$ mutated product has less affinity for the mthotrexate; hence, the $dhfr^-$ transformants are resistance to the inhibitor.

Resistance to methotrexate may also be due to a greater level of the dhfr product brought about by amplification of the dhfr locus.

Subramani and associates (1981) have inserted the dhfr gene from mouse DNA into several SV 40 vectors, and SV40 – derived plasmid vectors.

**(10) A Phosphotranferae Selection System**

The genes for aminoglycoside 3′ – phosphotransferase [aph (3′) I, aph (3′) II] confer resistance to the antibiotics kanamycin and neomycin in bacterial cells and to the antibiotic G418 (2-dexoystreptamine) in yeast, plant, Drosophial and vertebrae cells. This neomycin resistance gene (neo) is therefore a useful selectable marker that may be shuttled between prokaryotic and eukaryotic cells. The aph I and aph II genes are derived from Tn601(Tn903) and Tn5, respectively. They do not posses any similarities in their sequences.

**(11) The CAT System**

The product of the gene for CAT (chloramphenicol acetyl transferase) mediates the conversion of chloramphenicol into acetylated derivatives. There is no CAT in mammalian cells. But the latter can express the gene. The gene, derived form Tn9, has been added to a plasmid vector containing the promoter of the SV40 early (T) gene.

Chloramphenicol poisons the eukaryotic mitochondrial system, so that the cells die within a very short time after treatment. A cell carrying a CAT gene can withstand the effect of this toxin, as the latter is made ineffective by the enzyme. Foreign DNA on CAT-containing vectors (e.g., pSV2-cat) can be thus utilized as dominant selectable marker for transformed cells.

**(12) Alu Markers for Human Genes**

The eukaryotic DNA contains many repeated sequences, many of which are similar enough to be grouped into families. One such ubiquitous highly repeated family is the alu (130-300 bp). The name is derived form the presence in the sequence of the recognition sequence (AGCT) for the restriction enzyme Alu I.

The alu sequences occur more that $30 \times 10^4$ times in the human genome; they are positioned at short intervals throughout the DNA, both within and outside the regions occupied by genes, as well as in exons and introns. There are indications that these sequences are results of several transposition events.

Because of their presence in almost any short length of the human DNA, alu sequences are useful markers for human DNA inserts in a transformed cell. With due apology to Shelley: if Alu comes, the human gene cannot be far behind.

**2. Discuss various screening / Selection methods involved in recombinant clones by Indirect screening techniques?**

A some what indirect – but a surer way of detecting the presence of the transferred DNA is to identify the DNA itself or the product synthesized due to the inserted DNA. The DNA that leads to the synthesis of an mRNA or protein product need not be the coding sequences alone of a gene. The insertion of a promoter or other regulatory moufs will also result in gene expression if the insert is accompanied with or placed in the proper context of expression signals and coding sequences.

Proteins expressed by the cloned fragment may be identified by very sensitive immunochemical techniques. The DNA insert may be identified by hybridization with a probe (mRNA, cDNA etc.) or by procedures that pinpoint the presence of the desired DNA. Such procedures include techniques known as 'hybrid – arrested and hybrid – selected translations'.

**(1) Immunochemical Technique**

Immunoglobulins (antibodies) that match the gene product in question are the basis of several tests that identify as well as isolate the protein products of the inserted DNA. Some of these tests are described in Section.

A crude method of identifying the clones synthesizing the desired protein is to lyse bacterial colonies in situ on discs of filter paper, and treat the discs with the antibody probe. The latter bind themselves only to the desired protein. In a subsequent step the antibody – antigen (protein) complexes are identified with a labeled antibody probe that matches the same protein (Skalka and Shapiro 1976; Ehrlich et al., 1978; Carbon et al., 1978; and Broome and Gilbert 1978).

Broome and Gilbert's method consists of lowering an antibody – coated plastic disc on the surface of the Petri dish containing bacterial colonies (lysed) or phage – caused plaques. The antibody

molecules will bind themselves to the antigen only form clones that are producing it. The disc with the attached proteins is now treated with the radioactively – labeled antibody. The disc, after appropriate washes to remove non attached probes, is screened for radioactivity. By comparing with the plate of clones, the ones that are positive for the protein are identified and if needed isolated and amplified. Young and Davies refined this method for detection of clones with a foreign gene inserted in a lacZ sequence in a gt vector (Figs.).

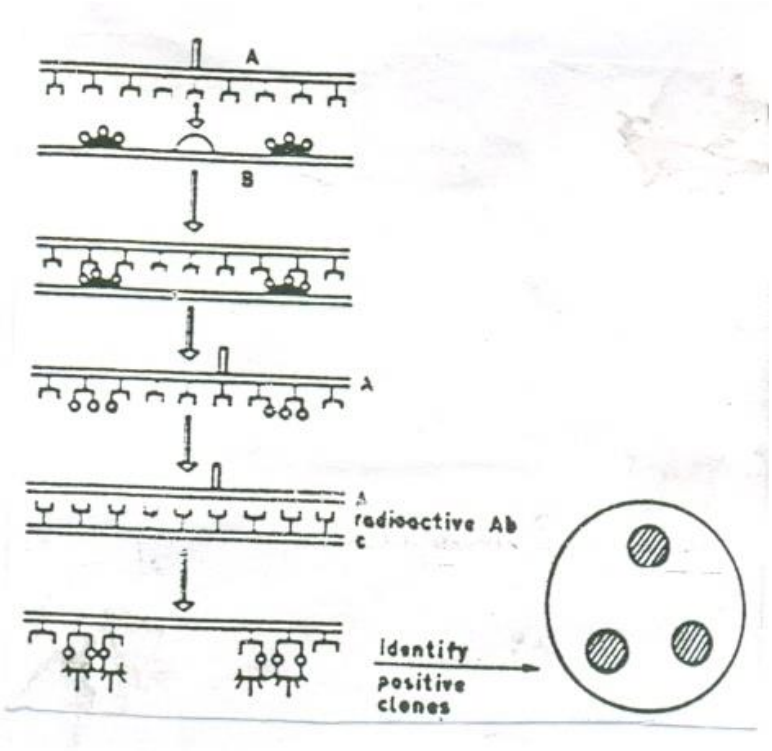



**Fig. Immunochemical detection of a cloned gene product. A plastic disc is coated with the antibody matching the gene product to be identified in colonies growing on a Petri dish. By placing the antibody – coated surface against the colonies, complexes are allowed to form between the AB and An of interest. The disc is thin placed over a surface coated with the same antibody, but one that is radioactively labeled. The antigen in the initial Ab-An complexes binds itself also to the labeled Ab. The regions of the disc that are radioactive are determined and used to identify the locations of the colonies expressing the gene product of concern.**

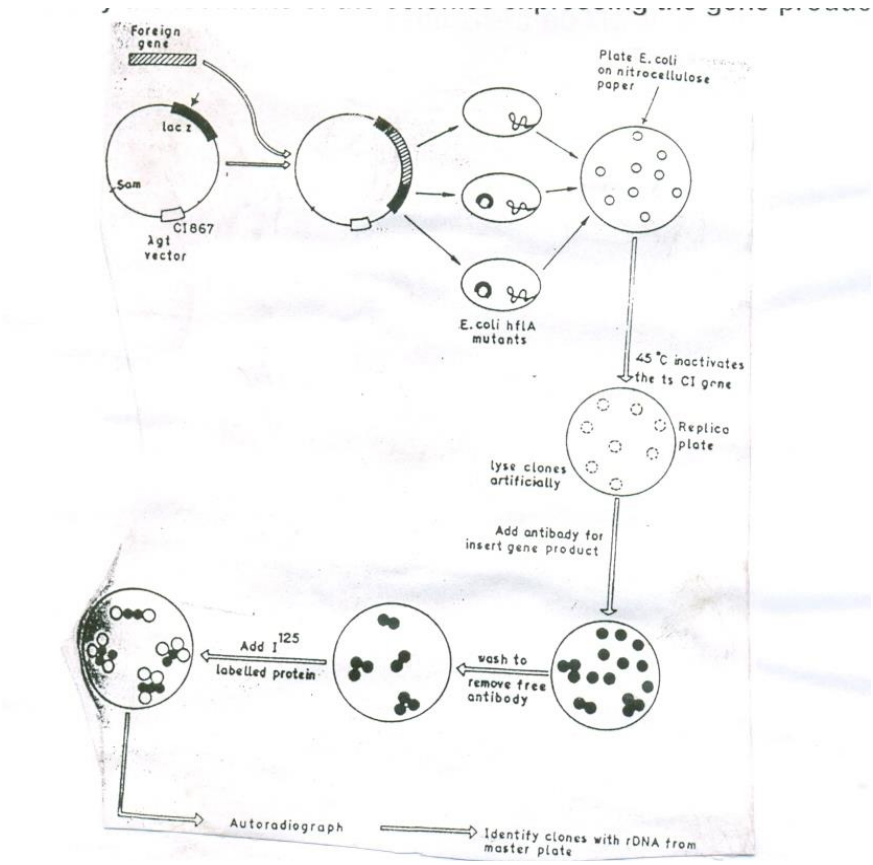



**Fig. An improved immunochemical detection technique (Young and Davies). A lambda gt vector with a ts CI gene (C1857) and Sam is used to clone the foreign gene E. coli cells transformed by this – hybrid vector are plated either (i) directly on agarsolidified medium or (ii) on nitrocellulose paper placed over the medium. In the former case, a nitrocellulose paper (disc) is utilized to 'blot' loose cells on to it. Several replicas may be made in this way. In the latter event, the paper with the colonies is treated further. The colonies on nitrocellulose paper are lysed. They are treated to a temperature of 45° C and the colonies lysed. The clones producing the desired proteins are detected with antibody probes.**

### (2) Hybrid – arrested Translation

When the mRNA transcribed from the inserted DNA forms a small proportion of the heterogeneous mRNA population in the cloning cell, a strategy is employed by which the presence of the cDNA of the gene sought is established (fig.). The technique, designed by B. Roberts, includes the following basic steps:

i) The insert is a cDNA copy of the mRNA of the gene in question. This rDNA is isolated and purified from the selected clones.

ii) The rDNa is dissociated by heating

iii) The total mRNA of the cloning cell is extracted and the dissociated rDNA added to it. The cDNA will hybridize to its matching mRNA.

iv) The above mixture is added to a 'cell – free translation system' that contains radioactively labeled amino acids and the paraphernalia needed for protein synthesis in vitro. Two results can be expected:

1. The cDNA has tied up the desired mRNA. In this case the latter cannot be translated.
2. If the mRNA is not hybridized, it will be translated.

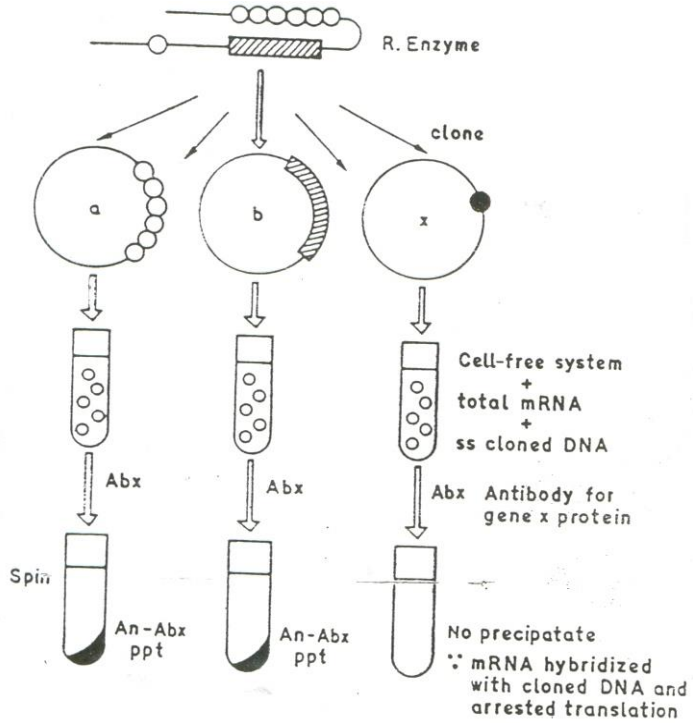



**Fig. Hybrid – arrested translation (HART). The mRNA of the gene of interest hybridizes with the sense strand of dissociated cloned DNA fragments and prevents (or arrests) its translation into a polypeptide. Antibodies are used as probes for the product of interest. Lack of precipitates in this system is the basis of selection.**

(v) Antibody matching the desired protein is added to the reaction mixtures.

(a) The mixtures in which proteins have not been synthesized will have no antibody – antigen precipitates. This indicates that the cDNA has found the complementary mRNA.
(b) Antibody –antigen precipitates, on the other hand, indicate that the mRNA are free and are not complementary to the cDNA. The clones from which the reaction mixtures were made, and which showed no precipitates are the ones containing the insert DNA being searched. In short, the arrest of translation by hybrid RNA-cDNA formation indicates the clones that have recombinant DNA.

**(3) Hybrid – selected Translation**

This technique differs marginally from the previous one (fig.). The rDNA from recombinant clones are hybridized with the total mRNA from the cells. The hybridized mRNAs are isolated and purified, and the mRNA released from the hybrid molecule.

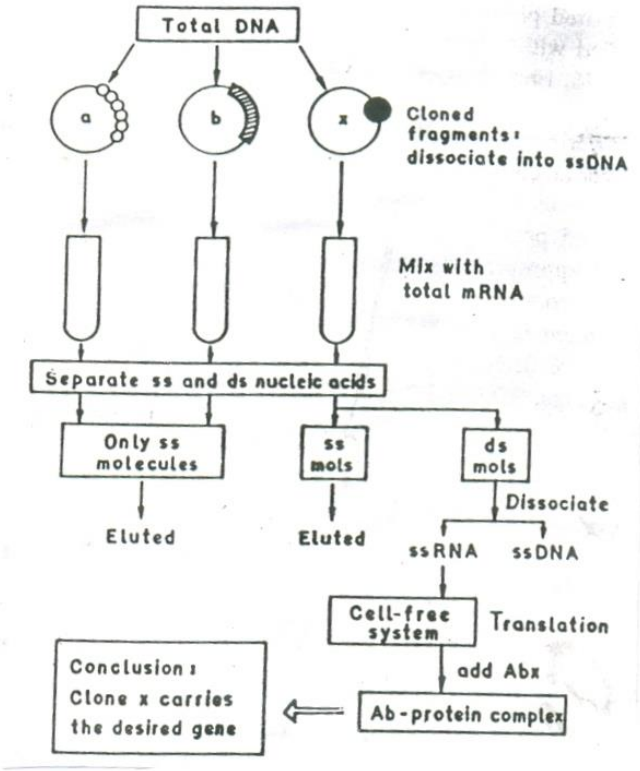



**Fig. Hybrid – selected translation (HST). Cloned fragments of DNA to be searched for the gene of interest are dissociated and mixed with the total RNA of the cell type from which the DNA has been isolated. The DNA of concern will hybridize into DNA – RNA duplexes. The reaction mixture is passed through a column which will bind only the duplex molecules. The ss RNAs and unhybridized DNAs will be eluted. After washing the column, the ds molecules are released and dissociated. The mRNA is translated in a cell – free system. if it is the correct one, the antibody match in the protein product will precipitate the latter. The presence of Ab- An precipitates is the basis of HST selection.**

The cleaned mRNAs are now used for translation. The reaction mixtures contain the correct proteins translated from the mRNA transcribed from the desired gene. The proteins are identified by immunochemical or other methods.

Sobel and associates (1978) and Nagata et al. (1980) immobilized the DNA to be hybridzed. While Woolford and Rosbash (1979) allowed the hybridization to occur in solution. This is a very sensitive method and can be employed to pick out DNA sequences that are expressed at very low levels (0.1 percent) in the cell.

**(4) Rescue Techniques: Plasmid and Others**

Rescue techniques both identify and isolated the gene of interest, the techniques involve splicing fragments of genomic DNA to a DNA handle that can be used to advantage either for recombination by hybridization or to supply some other advantage for detecting the DNA fragments joined to the handle.

The first of these methods is the plasmid rescue technique, which as employed by Perucho et al. (1980) to isolate the chicken $tk^+$ gene from the whole chicken DNA. The technique can be described best by following the steps utilized in this project (fig.).

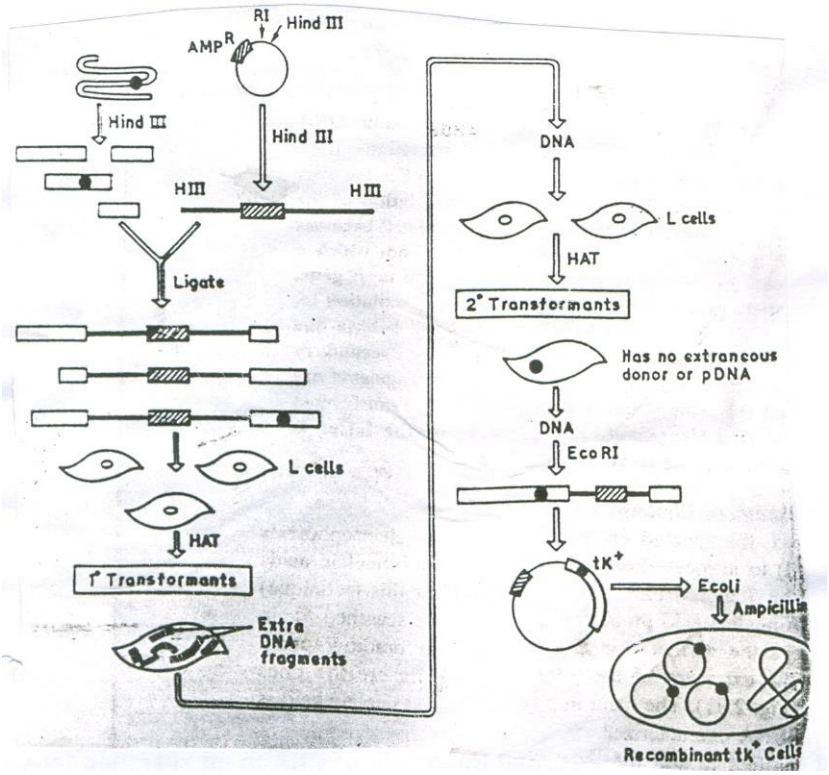



**Fig. Plasmid rescue technique. Fragments of DNA, one of which carries the gene of interest (in this case the $th^+$ gene) are ligated to a plasmid (pBR322), and the hybrid molecules mixed with L- strain cells ($tk^-$) for transformation. Transformed $tk^+$ cells are selected on HAT medium. These primary transformants may possess more than one fragment. It is, therefore, customary to obtain secondary transformants using the DNA from the primary ones. DNA from the last transformant is introduced into E. coli cells. Those which are $Amp^R$ contain both the pBR322 and the required sequences.**

The gene to be isolated, in this case, was the $tk^+$ gene, which is itself a selectable marker. Several restriction enzymes were used to find some that did not cut through the tk gene but could lineariz the plasmid pBR322. Hin dIII was one of the. Total DNA chicken was isolated and treated with Hin dIII. The hybrid molecules were transfected into mouse L-cells. Those which grew on the HAT medium were amplified into mass colonies. The DNA from these colonies were extracted and Southern blotted. The blots were hybridized with pBR322 probes. The autoradiograms of the probed bands revealed the ones which hand both the pBR322 and tk gene sequences ( the latter because all of these grew on HAT). The DNA from these bands (from the clones form which the band was derived) was used once more to transform a second batch of L- cells. The DNA from the secondary transformants were cut with an enzyme that does not cut through the resistance genes in pBR322 (Eco RI) and the tk gene (Bam H1). These fragments were used to transform E. coli cells and cells that grew on ampicillin – supplemented plates were identified. These were then selected

on the HAT medium. Cells that grew in HAT possess the tk gene as well as the pBR322 sequences. The DNA from these last transformants was analyzed. The tk gene together with flanking chicken DNA was found linked to the plasmid DNA.

The preceding technique has been refined by other, especially to increase the capacity of the vector for the donor DNA. It may not always be possible to splice a full gene to a plasmid. Cosmids are better, as up to 45kb insert DNA may be accommodated. Also, cosmids may be packaged into infectious particles. Lan and Kan (1984) used a coasmid library to look for the human tk gene. The DNA from the transformants was packaged with the packaging mix and the particles tested for their ability to transform Lcells of mouse into $tk^+$ phenotypes.

A modification of the above strategy is the marker rescue technique employed by pellicer et al. (1980) and Low et al. (1980) and Low et al. (1980) to isolate the aprt (adenine phosphoribosyl transferase) gene from the hamster (fig.). Also aprt cells do not survive on a selection medium containing adenine and azaserine; aprt transformants are, therefore, selected as colonies on this medium. The scheme for this technique is similar to the previous one up to the second round of transformation. The DNA from the secondary transformants was cut up into restriction fragments and a bank of fragments was made in Charon 4 vectors. These fragments were screened with a pBR322 probe. The clones that were positive were seen to contain part of the plasmid and some of the hamster DNA. This DNA was used to transform $aprt^-$ cells. Those cells which grew on the selection medium had DNA that complemented the defective $apart^-$ in the cell. The intact aprt gene was discovered in the transforming DNA.

A third variation on the rescue theme is the tRNA rescue technique, in which the recognizable nucleic acid spliced to the genomic DNA fragments is a tRNA gene with a suppressor (sup F) for amber mutations (fig.). In the last round of transformation the DNA from the short listed clones is used to transform an $su^-$ strain of E. coli. Fragments that suppress the amber mutation in the E. coli are the ones carrying the desired gene. Goldfarb et al. (1982) and Chimizn et al. (1983) in Wigler's team followed this method to isolated fragments that contained the oncogenes c-Ha-ras and N-ras, respectively.

The principles underlying the aforementioned techniques have been utilized in various modification to isolate and delineate regulatory and other functional regions of a gene.

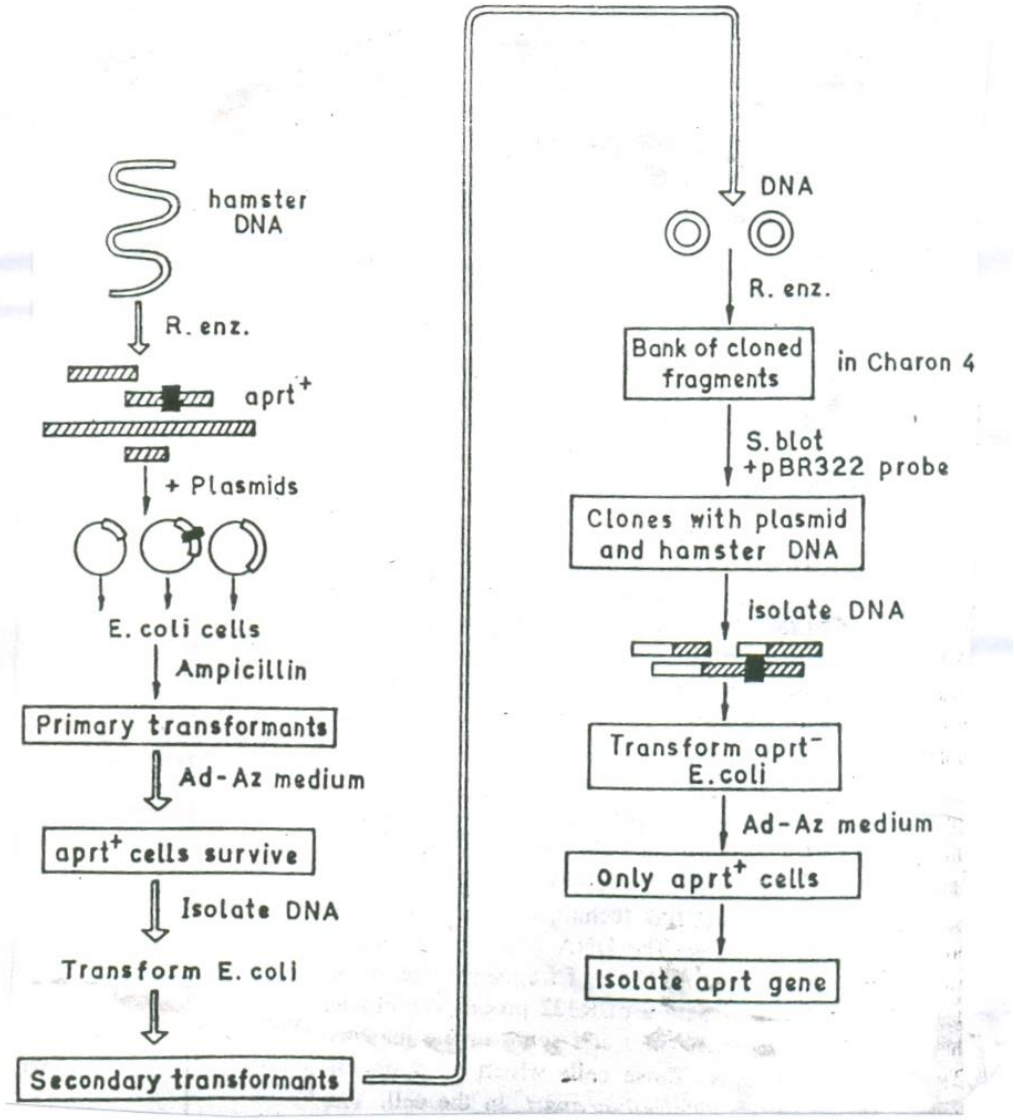



**Fig. Marker rescue technique. This technique isolates DNA by virtue of a particular marker gene present on it. In this illustration the marker aprt gene is rescued form DNA fragments, some of which only possess this marker gene. Also, aprt encodes the enzyme adenine phosphoribosyl transferase which mediates the synthesis of AMP from adenine, by a salvage pathway of biosynthesis. The regular pathway for the biosynthsis of purines may be blocked by amopterin (see caption of fig.) or azaserine. A medium containing azaserine and adenine will support cell growth if the gene aprt is present as well. Cells that are aprt$^-$ cannot, conversely, grow in the presence of adenine and azaserine. In this technique, cells transformed with DNA fragments ligated to a plasmid are selected for the presence of the plasmid vector with an appropriate probe. The clones thus identified contain the aprt gene.**

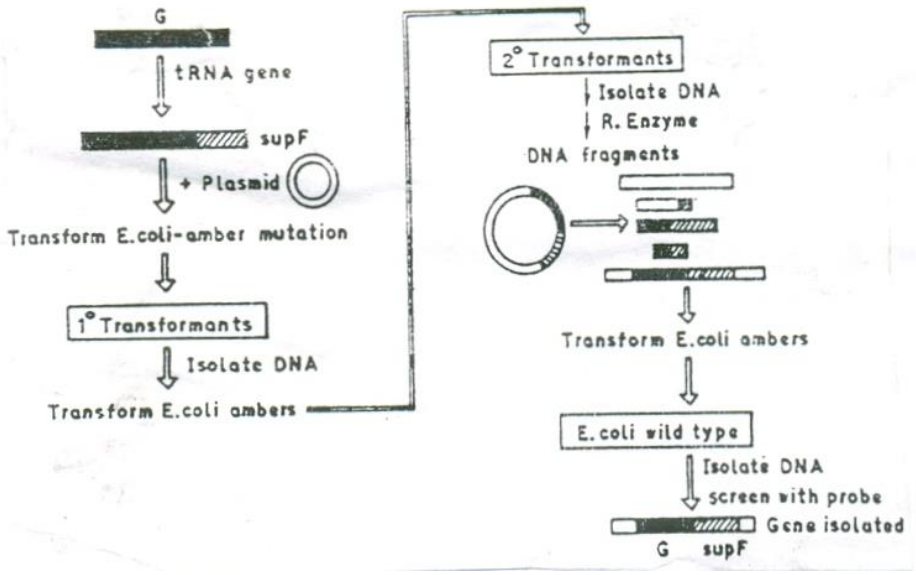



**Fig. The tRNA rescue technique. This technique of isolating a particular DNA fragment exploits the ability of suppressor tRNAs to ignore amber mutations. Her supF is the gene for a tyrosine – specific tRNA with a mutation in its anticodon that incorporates a tyrosine residue at an amber (UAG) stop codon. Translation of the mutated mRNA is not prematurely terminated and the phenotype of the cell becomes that of the wild-type allele of the mutated gene. Fragments of DNA, from which a desired sequence is to be fished out, are spliced to plasmids bearing the sup F gene. These hybrid plasmids are replicated in an E. coli strain with an amber mutatin in, say, a nutritional (auxotrophic) gene. Cells with the hybrid plasmid will behave like wild – type cells and grow in minimal medium. DNA is extracted from secondary transformants and cleaved with a restriction enzyme. The restriction fragments are used to transform a third batch of the same mutated strain of E. coli Transformed cells should possess the desired DNA fragment. The presence of the latter is confirmed by a suitable radioactive screening technique.**

### (5) Nucleic Acid Hybridization: Southern Blotting

DNA or RNA may be fragmented, fractionated on an agarose gel by electrophoresis and the band transferred (blotted) to nitrocellulose filter paper to which nucleic acids adhere firmly. This blot (called the Southern blot, after the pioneer for this technique) is then flooded with a radioactive nucleic acid probe for the gene to be searched for in the bands. After appropriate steps the washed and dried paper is autoradiographed. Bands on the print that show the exposure of the photographic film are the ones containing the hybridized probe (fig.). The band in a refractionated nucleic acid is eluted and the desired gene desired gene or mRNA characterized.

The above procedure was modified to suit the hybridization of RNA and protein molecules. These techniques are described elsewhere as Northern and Western blotting, respectively.

It is sufficient to emphasize here that hybridization with a complementary nucleic acid or antibody (in the case of proteins) probe is a very sensitive technique that pinpoints even a small amount of the desired molecule in a given sample.

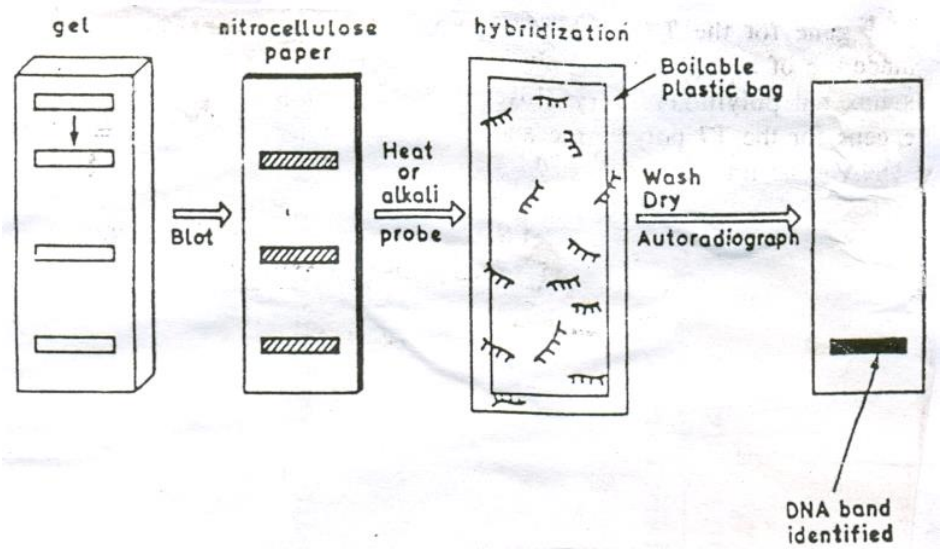



**Fig. Southern blotting principle. DNA from an electrophoretic gel is transferred without displacement in position to the nitrocellulose paper. This 'blot' is first treated to denature the ds DNA. The paper is then treated with an RNA (or DNA) probe for the DNA to be detected. Unhybridized probes are removed, and the 'blot' autoradiographed. Only the bands containing DNA hybridized to the radioactive probe will expose the photographic film. The latter is developed. The exposed band will indicate the band in the gel that contains the desired DNA. This technique, developed by E.M. Southern, introduced a new dimension in investigations based on recombinant DNA techniques.**

### (6) Colony Hybridization: Hogness and Grunestein

In principle, this method is similar to the Southern blotting technique. In the colony hybridization method (fig.) first developed by Hogness and grunestein, (1975) the colonies are allowed to grow on nitrocellulose paper placed in contact with the nutrient agar. When the colonies are about 1mm in diameter, replicas are made in other paper covered dishes. The nitrocellulose papers are removed, washed, treated with a detergent to lyse the cells, and heated to 80$^{o}$C. The exposed DNA bound to the paper is denatured with 50 percent formamide, treated with the probe and autoradiographed. Colonies that possess the hybridized DNA expose the films. The identified colonies are amplified separately from the master plate and the DNA utilized as required.

### (7) Dot Blot hybridization

Small samples of RNA may be assayed by the 'dot blot' hybridization technique. This technique is useful for both tittering the concentration of RNA viruses and for screening a sample for particular species of RNAs.

The Dot Blot Hybridization technique consist of placing drops of the RNA ample (viral genomic or transcripts) on nitrocellulose or diazotized paper, fixing the RNAs on it by baking, denaturing the secondary structure of the RNAs and hybridizing the 'dots' with given probes. The desired spots are identified as usual by autoradiography. This is a very rapid method for screening RNAs.

Quantitative estimates of the wanted species may be made by comparing degrees of hybridization with known or control samples as little as 1-5 µ1 may be used per 'dot'. Several commercial suppliers now carry apparatus that can be used to blot a larger amount (100 µ1) of the sample uniformly and simultaneously.

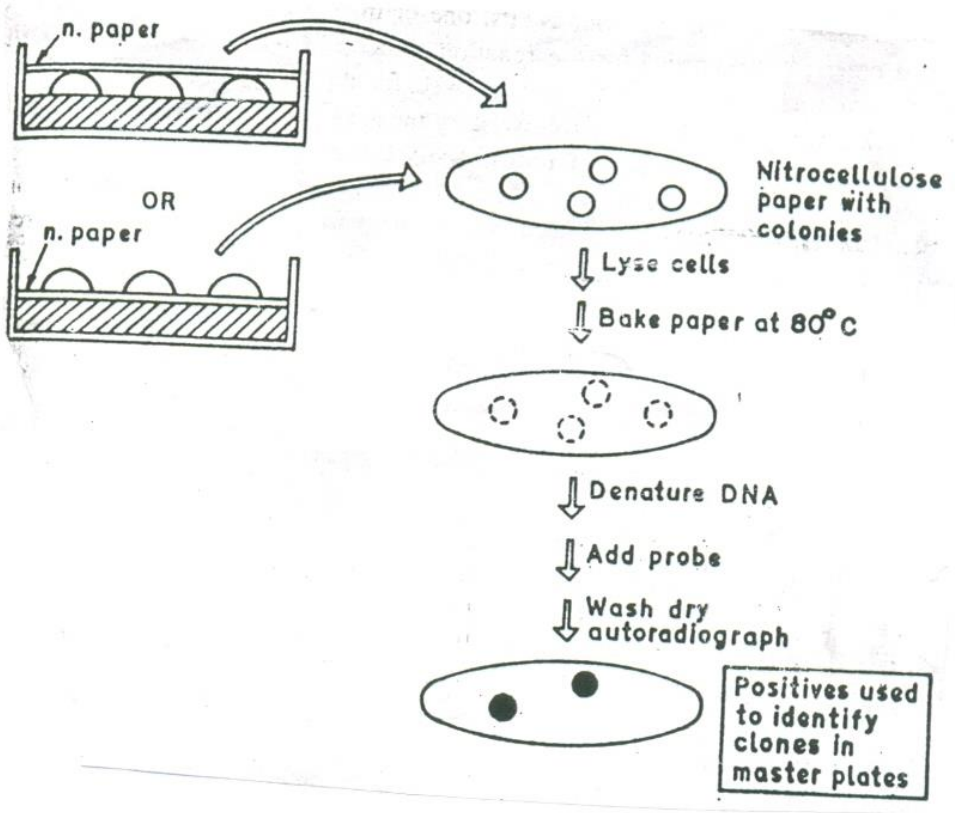



**Fig. Hogness and grunestein: colony hybridization technique. This is a variation of the Southern blotting technique that blots DNA from cells in situ, and identifies colonies harbouring a desired gene. Cells are plated on an agar –solidified medium and 'blotted' with a disc of nitrocellulose paper. The paper is now treated, first to lyse the cells, next to dissociate the DNA to be hybridized with the probe and finally to be autoradiographed. The exposed regions on the developed film indicate the colonies in the master plate possessing the DNA matching the probe.**

### 3. How will you prepare the Nucleic acid probes? Explain the tests involve in study of Nucleic acid probes.

**PROBES AND TESTS**

The exact pinpointing of a particular nucleic acid or protein is one of the important objectives is gene cloning ventures. This is achieved by employing appropriate 'probes' that match the sought – for molecule, and by a radio active or other label declare the successful hybridization or complexing of the probe and the nucleic acid or protein.

Procedures have been also developed that have revolutionized the manner in which these probes my be utilized, particularly when one is looking for a needly in a haystack. Prominent among

these procedures are the Southern blotting technique for probing fractionated DNA and allied ones for RNA and protein, and sophisticated immunodiagnostic tests that are also valuable for the recovery of minute quantities of a unique protein from a heterogeneous mixture.

**Nucleic Acid Probes**

A nucleic acid probe is a short or long length of single – stranded RNA or DNA that is complementary to a portion of the nucleic acid to be identified. There are thus RNA, cDNA, DNA and nick-translated ss DNA probes.

A general procedure for preparing a nucleic acid probe is to have a cell – free reaction system containing the template, the polymerizing enzyme, the four NTPs, one or more of which are radioactively labeled, and other factors needed for the reaction to occur. Aliquots of the reaction mixture are removed at regular intervals and assayed for the length of the synthesized strand. This check involves a TCA assay followed by measurement of the degree of radioactivity in the sample. The amount of radioactivity counts is plotted against time. A linear relationship exists between them. The end point is reached when there is no further increase in radioactivity in an aliquot with an increase in the time of reaction. The reaction is stopped and the probe isolated. A TCA (trichloroacetic acid) assay consists of mixing the TCA with a reaction mixture chilled at $0^{o}C$, and allowing the mixture to pass through a special filter that retains the new nucleic acid stands. The filter papers are counted in a Geiger-Muller counter.

**(1) RNA Probes**

The first RNA probes were m RNAs of a gene that are abundantly expressed in a cell; these were end – labeled with $^{32}PO_4$.

Cells such as erythrocytes (RBCs) are primarily filled with globin mRNAs. The total mRNA may be extracted by gel electophoresis. The mRNA of the size expected for the DNA (gene) to be probed is isolated and then end – labeled, by first removing the 5′ – terminal phosphate using alkaline phosphatase, and adding a $^{32}$ P- labeled phosphate with the help of a kinase.

If the proportional of the mRNA to be made into a probe is very low, other strategies are utilized to identify and isolate them for end – labeling. One strategy relies on a differential hybridization preliminary step. This consists of preparing a cDNA bank from the total RNA from one type of cell. These cDNAs are then used to hybridize the RNAs in a cell from another tissue or developmental stage, which differs in some unique gene product from the first. The hybridized mixture is run through a hydroxyapalite column which binds itself to double – stranded unhybridized RNA is eluted. This is, hopefully, the mRNA that is wanted. This strategy was successfully used to isolate a protein that is present in the liver cells of male rates but not in those

of female rats.

In both the above strategies, there is the possibility of false identifications. In the former method, although the predominant mRNA is expected to form the bulk of the RNA that is isolated for labeling, there is little guarantee that it dos not include other RNAs that may be present in the heterogeneous pool of cellular RNAs.

A far more efficient way of preparing RNA probes involves transcription from a template that is cloned in a plasmid. This transcription is initiated from a promoter that is specifically recognized by an RNA polymerase. Now, RNA polymerases of E. coli are rather unspecific. They may initiate transcription from different unrelated promoters. On the other hand, a promoter of a bacteriophage is very specific for the phage polymerase. Green, Maniatis and melton (1983) decided to utilize one of the phage promoters and the corresponding enzyme to trtanscribe a DNA cloned in a plasmid vector. And a cloning site for the template for transcription introduced downstream of the promoter.

The first promoter – polymerase system was taken from phage SP6 that infects salmonella typhimurium. Later, use of the phage T7 (of E. coli) promoter-enzyme system came into vogue. The T7 promoter is very efficient and is included in many expression vectors, together with gene for the T7 RNA polymerase (gene 1). Initially the SP6-containing vector made use of a single cloning site down stream of the promoter site. Later modifications inserted polylinkers very close to the initiation nucleotide. The T7 system uses the gene for the T7 polymerase and the T7 promoter and terminator signals flanking the DNA to be transcribed (fig).

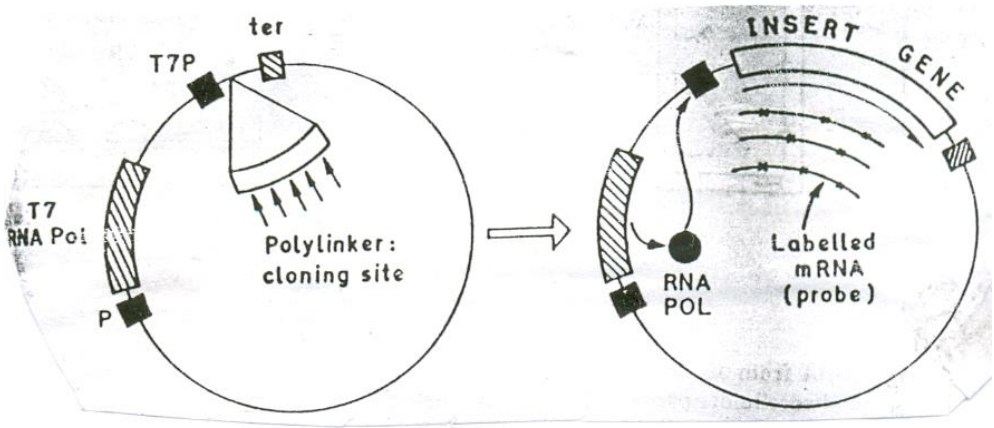



**Fig. synthesis of an RNA probe using the T7 promoter / RNA Pol system. a vector containing the promoter and gene for the phage T7 RNA Polymerase, and a cloning site flanked by the promoter and terminator sequences of phage T7 genes, also carries a DNA insert from which the probe is to synthesized. Phage promoters, in general, and the T7 promoter for late genes, in particular, are more powerful than those of bacterial genes. Cells transformed with r DNA are supplied with RNA transcripts from the insert gene are, therefore, labeled, and may be used as probes. The required RNA is isolated after fractionation by size on an agarose gel.**

The SP6 and T7 systems can be and have been utilized to express whole RNAs. However, for making a probe, only a short labeled RNA is sufficient. To enable such probes to be transcribed

into uniform lengths, it is a practice to linearize the plasmid by cleaving it with a restriction enzyme. The vector thus carries the input in the following order: phage promoter – Enz. 1-Enz.2. The template DNA is inserted at the number 1 site, and the composite treated with restriction enzyme 2. We now have a linear DNA with a promoter, the template DNA and an automatic termination site at the end cleaved with enzyme 2. The mRNAs fall of when they reach this end of the vector. Such templates, referred to as run – off linear templates (fig.) are of uniform size and are easier to isolate from the reaction mixture.

Transcription can also be controlled by placing the phage polymerase gene under the influence of an inducible promoter. A T7 expression system has been developed in which t7 polymerase gene is preceded by the E. coli lac UV5 promoter. The latter is repressed by the lac1 repressor and is induced by lactose or its analogue, IPTG (isopropylthioglactoside). The T7 polymerase is induced by IPTG, and the enzyme transcribes the cloned template from the T7 promoter. Krieg and Melton reported the synthesis of around 20mg of probe RNA from 1 mg of vector DNA in one hour at 40°C.

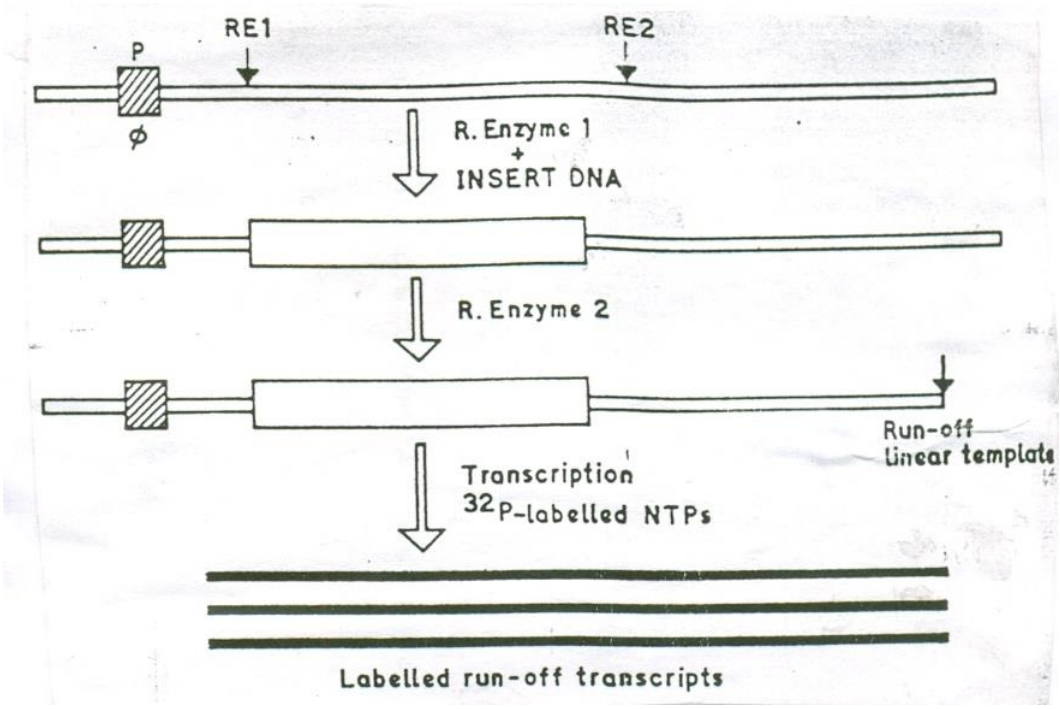



**Fig. Run off linear templates and labeled run-off transcripts.**

Probes to be utilized for Southern or Northern blotting (described later in this chapter) for in situ hybridization, or genomic sequencing (Church and Gilbert) need to be heavily labeled. Run-off transcripts can be made heavily radio active by including $^{32}$ P-labelled nucleotides in the reaction mixture. End – labeled probes are far less labeled than the transcribed ones with labels at several nucleotides in the strand.

**(2) cDNA Probes**

If a suitable mRNA is available, it can be copied into a cDNA (complementary) with the help of reverse trsncriptase (RNA – dependant – RNA polymerase). The enzyme used routinely is from the AMV (Avian mycloblastosis Virus), which does not popossess the exonuclase activity.

The polymerase requires a primer. In a eukaryotic mRNA, the polyadenylated tail may be utilized as one. A poly dT oligonucleotide primer (about 12TS) is synthesized chemically. It aligns with the poly dA region and transcribes the entire mRNA. Specific primers may also be synthesized chemically by deducing the base sequence of the probable primer from the amino acid sequences of the gene product (polypeptide). As the genetic code is degenerate, it is not possible to arrive at the exact sequence. In practice a mixture of the alternative sequences are made, one of which is likely to be the correct one. Methods of preparing oligonucleotides have improved and it is now possible to come very close to the actual sequence in the gene.

**(3) DNA Probes**

If the gene to be searched in situ, or from clones of a gene library, is already cloned the probe may be prepared from the vector carrying the gene. The vector DNA is dissociated and the cloned region cleaved out. The latter is then end – labeled with $^{32}$ P at the 5'- terminals. On dissociation of this fragment, a probe for the DNA and one for its mRNA will be available.

**(4) Nick – translated Probe**

One way to obtain short lengths of DNA probes is to nick a ds DNA probes is to nick a ds DNA with DNase I, and initiate strand synthesis at each 3'-end in the nicked single strands. The E. coli DNA Pol 1, that has the 5'-3'- exonuclease activity, is generally used as the enzyme. The latter has the ability to add a new nucleotide at the 3'-cut – end while removing the nucleotide ahead of its on the other side of the nick (Fig.). In this way one of the strands between two nicks becomes replaced by anew strand. If radioactively labeled nucleotides are provided for the synthesis, the new strand becomes labeled. This strand is said to be 'nick – translated'. This term should not be confused with the term 'translation' used for protein synthesis. To obtain a uniformly labeled probe it is better that all four NTPs be radioactively labeled. Otherwise, depending on the frequency of a particular nucleotide in a short stretch of DNA, a label in only one NTP will provide very non – uniformly labeled probes.

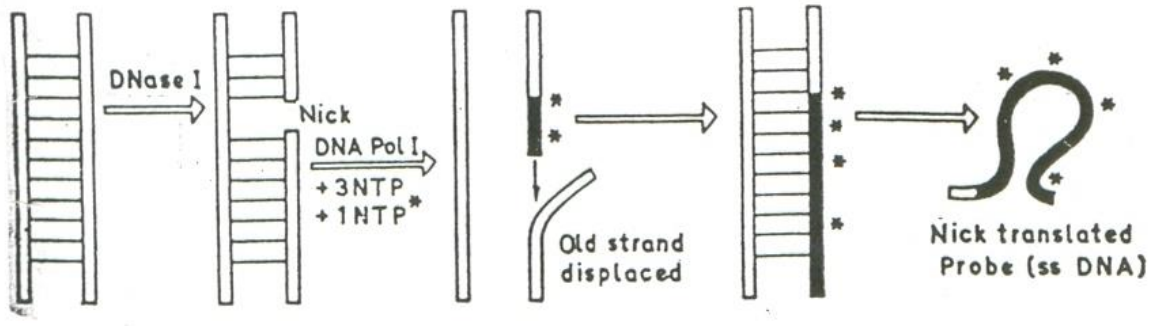


**Fig. Nick-translated probes. Short ss DNA probe molecules may be synthesized by exploiting the capability of the DNA polymerase I (essentially a DNA repair enzyme) to initiate DNA strand elongation from a free –OH end at a nicked region in a ds DNA. Pol 1, while removing the nucleotide with the free 5'- end. The DNA synthesis is carried out in the presence of at least one radioactively labeled deoxyribonucletide, so that the nick – translated DNA are labeled. The term ' translation' refers to the movements of the discarded and newly synthesized strands, and not to the usual term for decoding of the mRNA into a protein.**

## 4. Explain various hybridization techniques involve in analysis of DNA probes.

### Hybridization Techniques

The most accurate way of recognizing a specific piece of DNA or a clone is to search for it with an appropriate probe. The probe (mRNA, cDNA or nick – translated or other DNA probes) will be hybridized only with a complementary strand.

The technique for hybridization has been streamlined by E.M. Southern. The Southern blotting technique was originally developed for hybridizing DNA with probes. Later, it was modified to suit hybridization of RNA and proteins. These modifications acquired the names Northern and Western blotting techniques, respectively. These are extremely sensitive techniques for instituting searches for even very rare target molecules.

### (1) Southern Blotting

The Southern blotting technique consists of transferring DNA fragments from a gel slab to nitrocellulose (cellulose nitrate) filter paper (0.45 mm) hybridizing them with a radioactive probe, and identifying the hybridized DNA by autoradiography.

The technique is based on the strong affinity of DNA for nitrocellulose paper. The DNA to be searched is fractionated by agarose or polyacrylamide gel electrophoresis. The bands of DNA in the gel slab are eluted into a closely apposed nitrocellulose paper by a moving front of buffer solution. The adsorbed DNA becomes immobilized on the paper in the position in which they are transferred.
Hybridization is possible only if the DNA signal on the paper is single – stranded. The gel is, therefore, treated before the transfer step, with a mild alkali (5M Na Cl and 0.5M NaOH), and then neutralized using an NaCI solution in a tris – HCL buffer (pH 7.4).

The fel slab is then placed in an assembly having the following order of components (fig.), commencing form the bottom: buffer in a suitable tray – like container, a glass support a little larger than the size of the gel, a Whatman's 3 mm filter paper, the gel slab, a nitrocellulose paper of the same size as the gel surface, a second Whatman's 3 mm paper and a stack of absorbent paper.

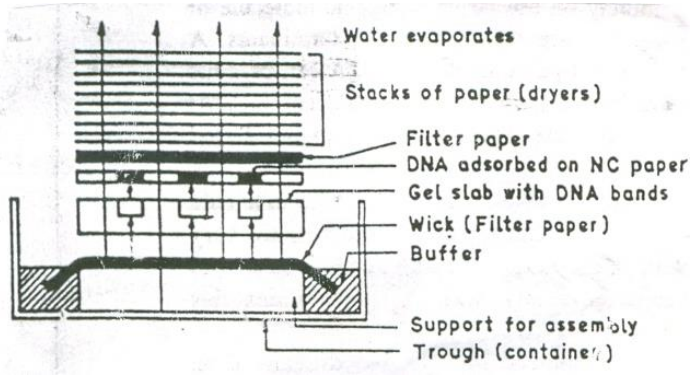



**Fig. Southern blotting assembly. The upward capillary movement of the buffer through the gel slab pushes the DNA of each band in the gel into the overlying nitrocellulose paper. The DNA binds itself to the paper, while the buffer solution moves upwards and evaporates. In this way, the DNA bands are blotted exactly on to the nitrocellulose paper.**

The buffer moves up through the gel towards the dry filter and absorbent papers. In its passage through the gel, the DNA fragments become eluted. The latter pass in to the nitrocellulose paper. The buffer solution moves upward but he DNA remains fixed to the blot paper.

In case the DNA fragments to be blotted are longer than 10 kb, it is customary to cleave them partially for more efficient transfer into the blot paper. The partial cleavage may be achieved by depurinating the DNA. This brought about by soaking the gel in HCI prior to the alkali treatment.

To fix the dissociated DNA fragments firmly, the blotted paper is baked at 65-80$^{o}$C in a vacuum oven, for about 2-3 hours. The Southern blot is now ready to screened with a probe.

Hybridization with the prove is performed by placing the blotted paper in a boilable plastic bag, containing a measured volume of the solution with the probe (pre-hybridizing and hybridizing solutions). The prehybridizing solution contains Denhardt's reagent and some denatured heterologous DNA. The hybridizing solution contains the probe in SSC (standard sodium citrate) or SSPE ( standard sodium phosphate – EDTA) buffer. Denhaardt's reagent, which contains ficoll poly vinyl pyrollidone and BSA (bovine serum albumin), prevents ss DNA from binding itself to the filter paper without hampering hybridization of the bound ss DNA with the probe.

The filter paper is washed and dried by appropriate methods and autoradiographed. As the patterns of bands on the nitrocellulose paper are identical to those which were on the agarose gel, the location of the DNA containing the wanted DNA sequences is identified easily by looking for the band that has exposed the film.

Hogness and grunestein's colonly hybridization technique is identical to the Southern blotting one, except that colonies are grown directly on nitrocellulose paper. Lysing of the cells in the colony is followed by an alkali treatment to dissociate the cellular DNA, and the paper is further treated as for Southern blots.

Plaques are also hybridized using a modification of the colony bybridization technique. The filter paper, when placed on a Petri dish with the plaques to be screened, picks up phages and naked DNA from the plaque regions. Several replicas may be made in filter paper discs from the same master plate.

At first only DNA could be Southern – blotted effectively. RNA was found not to adhere to the paper as well as DNA. Later modifications allow RNA also to be blotted efficiently on to nitrocellulose paper.

**(2) Northern Blotting**

RNA bands can also be transferred from electrophoresed gels to a solid substrate without displacement of the positions of the bands, initially, nitrocellulose paper was used, and the technique acquired the modified name of Northern blotting.

RNA molecules are mostly single- stranded, but duet o their secondary structure, they posses several regions of ds RNA.

This requires straightening out, and can be achieved by using one of the following strong denaturing agents: (i) glyoxal, alone, or in combination with DMSO (dimethyl sulphoxied); (ii) formaldehyde and formamide, (iii) methyl mercuric hydroxie; or (iv) urea.

RNA denatured by glyoxal can be directly blotted on to nitrocellulose paper. The use of the other agents requires a few additional steps before effective transfer can be made.

Of the denaturing agents mentioned above, methyl mercuric hydroxide reacts with free radicals of polyacrylamide gels; hence, only agarose gels may be used for use with this denaturing agent.
Nitrocellulose filter paper binds itself strongly to denatured RNA, but not to those with a secondary structure.

Another solid support for transfers of the nucleic acids from gels has been developed that binds equally well both denatured and non-denatured nucleic acids. It was initially employed for blotting RNA, but subsequently found to be equally useful for DNA transfer. The advantage of this technique is that the blotted paper may be reused several times for different probes, without appreciable loss of the nucleic acid signal, unlike nitrocellulose paper which is not reusable. The alternate filter paper is DBM or diazoienzyloxymethyl paper.

DBM can be converted from ABM (amino benzyloxy methyl) paper just before use. ABM is available commercially, or may be prepared in the laboratory by the following procedure. Whatman's paper is treated sequentially (with appropriate washings and dryings between steps) with n- nitrobenzyl-oxymethylpyridinum chloride, benzene and acetic acid. The paper is finally desiccated and stored at $4^{o}C$. DBM paper is prepared by treating ABM paper with $NaNO_2$ dissolved in HCI, and later washed with a chilled sodium borate buffer. The paper is usually referred to as diazotized paper.

Whatever the paper utilized for transfer of the RNA, the ethidium bromide stain for nucleic acids is added not to the nucleic acid, but to the latter. Acridine orange may be used to stain the glyoxal and formaide – treated gels. If methyl mercuir hydroxide treatment is employed, the reagent is removed before the blotting step; a treatment of the gel with a 2- mercaptoehanol and NaOH-containing regent commplishes this goal.

**(3) Western Blotting**

The technique of transferring protein fractional on a gel to a solid support inevitably became dubbed as 'Western Blotting'.

In the case of protein blotting the paper to be used is generally pretreated in a manner that is appropriate for identifying or probing for a particular protein. The analytical methods used for such identification include: (i) immunodetection, (ii) binding of proteins to ligands immobilized on the filter paper, and (iii) binding of tagged molecules to proteins immobilized on the filter paper.

Proteins are usually fractionated on polycrylamide gels, and transferred to nitrocellulose or DBM paper that has been appropriately pretrerated. One precaution has to be taken if DBM paper is used. As glycine reacts with the diazonium groups is DBM paper, the latter has to be washed free of glycine.

For immunodetection of a protein on the paper, the DBM is treated with the antibody for the protein (antiserum), which may be labeled or unlabelled. If unlabelled another labeled antibody or an antigen. The transferred protein has an affinity for its matching ligand, and so forms a complex

with it. The latter may be detected by autoradiogrphy if the protein in radioactively labeled. This immunodetection technique is a variant of the filter paper affinity one, where immunodiagonistic methods are employed to identify the wanter molecules.

The third technique reverse the positions of the protein and the binding molecule with respect to the blotting paper. In this case the protein is immobilized on the paper and treated with the labeled molecules ($^{32}$P-tagged nucleic acids or $^{125}$ I- tragged proteins). Protein – protein binding may be detected by labeled antisera, and protein nucleic acid associations by autoradiography.

**5. What are immunodiagnostic probes? Discuss on antigen and antibodies probes as molecular markers.**

**Immunodiagnostic Probes**

Antibodies or immunoglobulins constitute the main ammunition of the defence system of the animal body. Antibodies are also excellent biomolecular regents that may be used to identify and isolate even very small quantities of antigens form a heterogeneous mixture. The ability of the antibody to bind itself to a matching antigen is exploited in a variety of techniques employed inmmunochemistry and immunodiagnostic tests.

Immunodiagnoiss has become an essential tool in molecular biological investigations. It is most inseparable from most DNA cloning exercise. The methodologies and approaches of immunology are so different from those of other experimental biological systems that a little introduction to the basis of the immunological tools and familiarity with the paraphernalia of immunological exercises are necessary components of a course is genetic engineering. Let us take a quick look at some of the relevant terms and components utilized in immunodiagnositic probes and tests.

The probe may be the protein itself, or the antibody it provokes in a foreign body. The protein or the other molecular complex which provides and generation of antibody is usually referred to as the antigen. The serum containing the relevant antibody is usually called the antiserum (plural antisera).

**(1) The Antigen**

The immunoglobulin that matches an antigen does not necessarily cover the entire molecule or complex. It is usually a smaller moiety on the larger antigenic molecule or complex. The immune – provoking smaller moieties are the antigenic determinant. A cell or virus or an entire organism, such as protozoan and fungal parasites and pathogens possess several antigenic complexes on their surfaces. They are, therefore, sometimes referred to as ‘immunogens’.

Each antigenic determinant provokes the synthesis of an immunoglobulin molecule that binds itself only to this determinant, and not to others unless the others are very similar to the provoking antigenic determinant. In the latter case, the antibody is said to cross – react with two or more types of antigens; conversely, such as an antibody identifies antigens with close homologies in their make-up.

When a poly – determinant –studded antigen is introduced into the bloodstream of an animal, a heterogeneous mixture of antibodies is synthesized in the latter, each immunoglobulin matching one of the determinants in the immunogen. The proportions of the different antibodies varies from animal to animal, and from condition to condition, in the same animal. For general diagnostic purposes, antisera with such polyclonal antibodies are adequate as there is always at least one population that over helmingly represents a particular determinant and so reacts with the antigen in question.

If, however, a very specific probe is required, one has to make use of a monoclonal antibody (Mab), that has been raised specifically against a single determinant. Mabs are synthesized form tumours developed form a hybrid cell with special parentage; such a tumour has been named a hybridoma and the body of knowledge is synthesizing Mabs in vitro is known as hybridoma technology (Fig.).

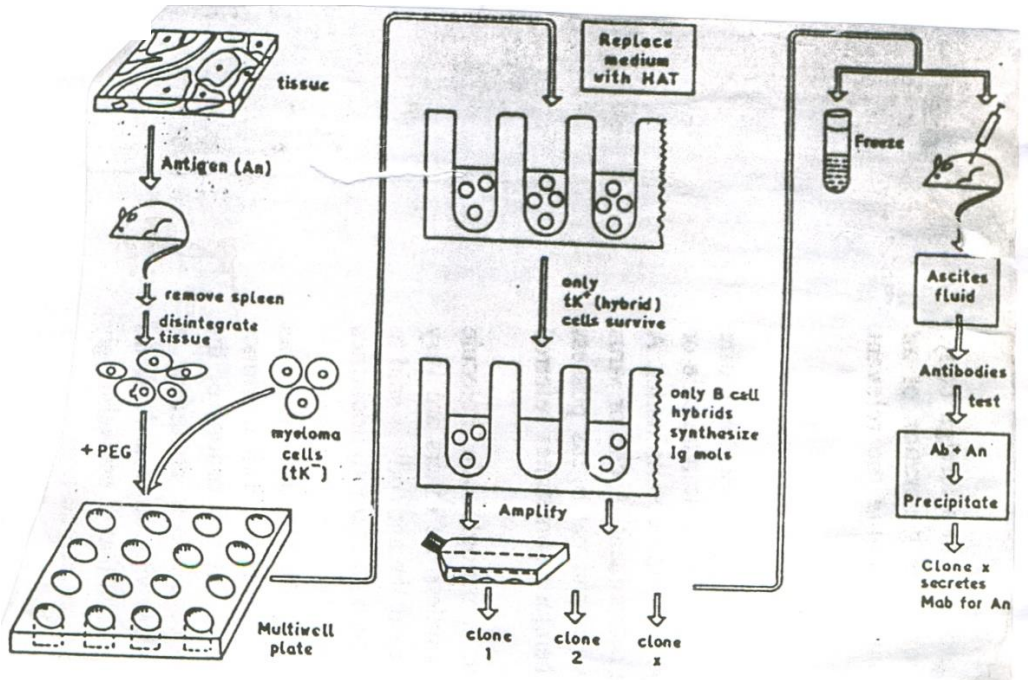



**Fig. hybridoma technology: the technique for the production of monoclonal antibodies (Mabs). Hybrid cells possessing the characteristics of continuous growth of cancerous cells and expression of a specific antibody of a B cell form tumours (hybridomas) that secrete antibodies made by a single clone of B cells. The parents for the somatic hybrid cells are a myeloma cell line and B cells stored in the spleen of a rabbit injected with a specific antigen. The fused cells are cultured in small volumes of media in the wells of a multivell plate, each well containing one fused cell. The medium when replaced with Hat selects for TK cells. These are amplified in T flasks (bottles with larger sides flat, on the inner surface or which normal animal cells get attached and divide**

**until the surface is covered with a single layer of cells). The cells are stored frozen or are introduced into periotional cavities of rabbits. The ascits fluid that develops contains clusters of cells or small tumours. The ascites fluid is withdrawn and tested with the antigen of interest and the ones secreting the matching Mab are identified.**

To be effective, the antigen used must be reasonably pure. Even biochemically pure proteins and other antigens ( complex carbohydrates, nucleic acids etc.) may contain traces of impurities that possess antigenic qualities.

There are several ways of purifying antigens. One of the most efficient ways of selecting a particular antigen from a mixture (such as membrane proteins or subunits of an enzyme) is that of fractionating the proteins in SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). The band corresponding to the expected molecular weight of the desired protein is cut out form the gel, the protein eluted form it, and used as the antigen. If the band contains more than one protein in the same range of molecular weight, two-dimensional gel electrophoresis may be employed to disperse the proteins in a band by a fractionation by electrophoresis in a second dimension (at right angles to the first).

The antigen is injected into the animal for raising antibodies. In practice, the antigen is introducd concurrently with a material that enhances that production of antibody and /or prolongs the life of the antigen in the body. This additional matial is called an 'adjuvant'. It may be a non-antigenic material such as mineral (paraffin) oil or one that itself elicites antibody formation, such as the tubercle bacilli (Mycobacteria). The adjuvant may be prepared in the laboratory although it is more usual to employ ready made ones. The most frequently utilized adjuvants are Freund's complete and incomplete aduvans (FCA,FIA). Both contain an oil and an emulsifier. The FCA contains, in addition, killed mycobacteria. The first of the weekly series of injections with antigen usually uses FCA while subsequent immunizations employ FIA.

Very little antigens is required to raise appreciable amounts of antibodies. A rabbit may be injected with 0.1 mg of a protein in each of four weekly doses, to obtain enough antibodies for use. Even as little as 1 mg of a protein attached to a solid support can induce the synthesis of sufficient antibodies. Very small molecular groups that do not elicit antibody response, can be made antigenic by conjugating them to a protein base. There is sufficient evidence (Medawar) that it is the small group, called the hapten, and not the supporting protein that provokes antibody synthesis.

**(2) The Antibody**

Antibodies are immunoglobulins, which are proteins with a very unique structure and function. In human beings, there are five types of immunoglobulin ($I_g$) molecules: $I_gM$, $I_gG$, $I_gA$, $I_gE$ and $I_gD$.

The bulk of the $I_g$ molecules in the body is due to the first two.
The different $I_g$ molecules differ in the number of components and other variations but all of them possess a common prototype unit. $I_gG$ has only one of these units, and will be used here to describe an $I_g$ molecule.

The molecule of $I_gG$(Fig.) consist of a pair of identical subunits, each consisting of a long (heavy or H) and a shorter (light or L) poly peptide chain. The H and L chains are held together by S-S bonds; so are the two H chains. The L and H chains of each subunit are aligned from the amino ends of the polypeptides. Each L and H chain can be divided into two distinct regions. The amino – terminal region is known as the 'variable or V region', and the remaining portion as the ' constant or C region', The V regions of the H and L chains of one unit bind themselves to the antigen. The amino acids in them possess a special affinity for the antigenic determinant that provoked antibody synthesis. The C regions have functions other than engaging the antigen; one of them is to get attached to surfaces of different cells or to certain other molecules of the immune system (e.g., complement Clq).

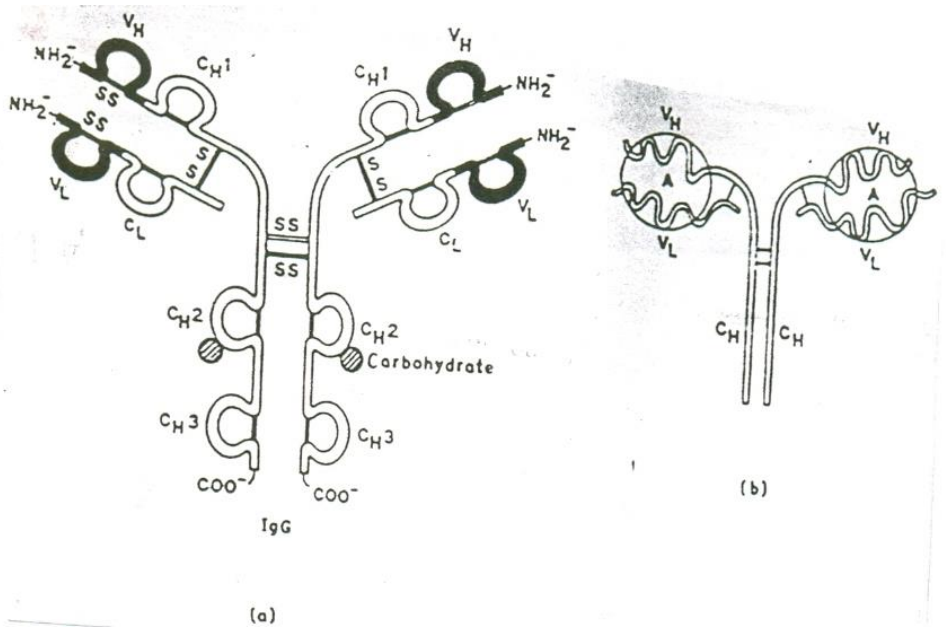



**Fig. Diagrammatic representations of a molecule of immunoglobulin G ($I_gG$), and of antibody antigen binding. $I_gG$ is one of the five human immunoglobulins, It possesses the prototype unit consisting of two identical copies of a pair of light (L) and heavy (H) chains (polypeptides). The amino – terminals of the H and L chains are the antigen – bindings sites.**

The genetics of antibody response and diversity is fascinating and extremely complex. Much of the knowledge in current immunology has been acquired by the use of recombinant DNA techniques. This, however, is not the forum for delving into these techniques.

It is sufficient to know that the antibody elicited by an antigen is highly specific for the latter, and either the antibody or the antigen may be recruited as probe to search for the other. The probe (antigen or antibody) may be labeled suitably if the test requires such tagging.

**6. What are the various immunological tests used for study of antigen and antibody probes?**

**Immunological Tests**

Immunochemical methods for detecting antigen – antibody complexes, and for estimating the amount of the antigen (or antibody), in the system make use of either the biological or the physical properties of the immune system components. Biochemical techniques are used for quantizing antibody – antigen complexes: the complexes are precipitated in a 2 percent solution of PBS. A second technique uses serum protein (complement) C1q immobilized on a solid substrate (plastic tube), to bind to $I_g$ molecules that have complexed with the antigen. A radioactively tagged anti – $I_g$ molecule is then used to identify and estimate the $I_gG$ of the complexes.

The antigen (protein products of cloned genes) to be detected in recombinant DNA essays are very little in quantity, and require rather sensitive tests that can be performed on minute amount of a substrate. The principles underlying some of them are described below.

**(1) The Precipitin Test**

When a polyvalent antigen solution is mixed with its matching antiserum (antibodies in serum), the two proteins combine to form a net-like spongy aggregate that precipitates. When there is more antigen than antibody in the mixture, the antigens usually form complexes that remain in solution. When the antibody is in excess, the complexes formed are insoluble. Some human sera form soluble complexes even in the presence of excess antibody. When the amounts of antibody and antigen are equivalent, there is a rapid aggregation and precipitation of the complexes.

Thus, by adding an equivalent volume of an antigen to that of its antisera, one obtains a preliminary idea about the ratios of the matching proteins in the mixture. If one solution is layered carefully over the other, within one day, a thin cloudy line will be seen at the junction of the two, if the antiserum matches the antigen. A quantitative estimate of the amount of antibody in the serum is obtained by adding different dilutions of the antigen to the latter at 37°C and then at 4°C. The precipitated complexes may be centrifuged out, washed in saline and tested for the quantity of antibody in the complexes. The above is known as the 'principitin test' (fig.).

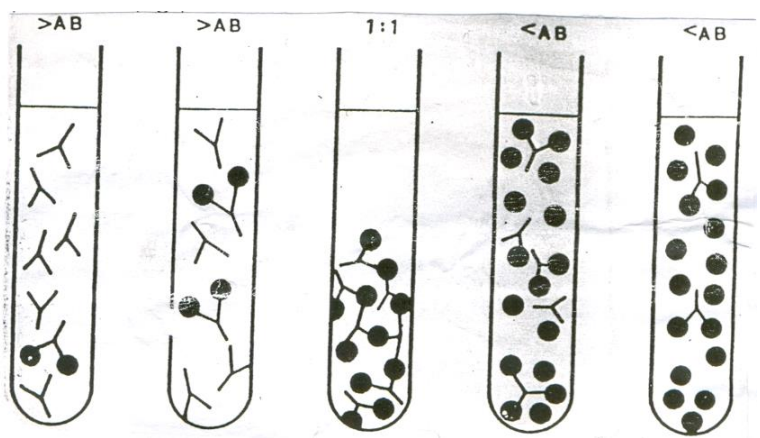


**Fig. The precipitin test. This is the simplest immunological test to determine the presence and proportion of an antibody in a mixture of the antibody and its matching antigen. Precipitates of An- Ab complexes are formed when the two components are in equal proportions. Excess or paucity of antibodies in relation to antigen concentration results in soluble complexes. If a series of antigen solutions of graded dilution are used for the test, an estimate may be made of the antibody concentration in the test sample.**

### (2) Immunodiffusion Technique

Titres of antisera may be obtained by immunodiffusion techniques. As in the case of the precipitin test the antigen and antibody are allowed to interact and form complexes at the interface of the two reactants. However, in immunodiffusion tests, the interfacing occurs between antigen and antibody that diffuse through a solid medium, such as an agar or agarose gel.

In the earliest of such as tests, the antibody was dispersed in the gel and the antigen placed in wells cut out form the gel. The antigen diffuses out form the well and interacts with the antibody to form complexes. The complexes appear as a ring of precipitates around the well. The width of the ring may be used to calculate the amount of antibody relative to the antigen present in the gel. The ring appears as an opaque band in a gel illuminated against a dark background. The ring may also be visualized by staining the precipitates with a protein stain, such as Coomassie brilliant blue.

The above mentioned single diffusion technique has been superseded by a double diffusion one developed by ouchterlony (fig.). In this case, the antigen is palced in a well with a antisera in wells surrounding it or vice versa. The radially placed wells may contain different antisera, or different dilustions of the same antibody.

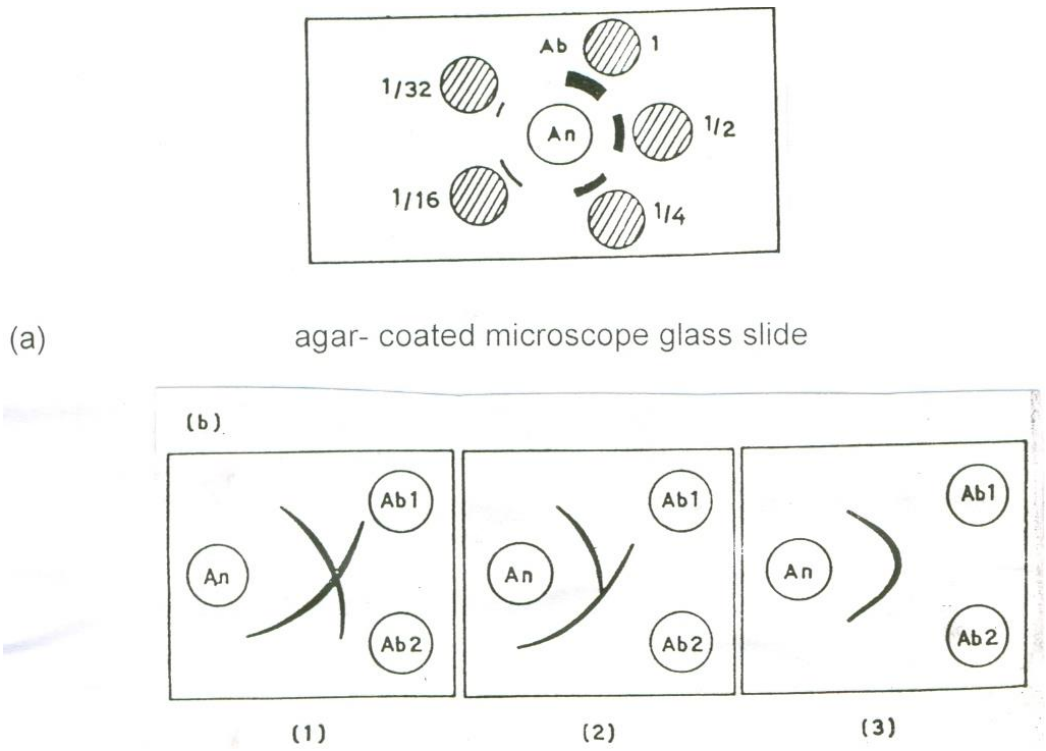



**Fig. Ouchterlony's double diffusion technique. This is a precipitin test carried out on an agarose (solid) medium. A glass slide is coated thinly with find quality agarose and wells made in various patterns as desired. (a) The antigen solution is placed in the centre well and dilutions of the antibody are placed in the wells surrounding it. Antigen and antibodies diffuse radially from their respective wells into the agarose gel. A line of precipitates is formed where they meet in equal concentration. The precipitates can be visualized as 'spus' after staining the proteins with Coomassie blue. (b) Two antibodies may be compared with respect to their affinities for a particular antigen by placing them in wells equidistant from the antigen – containing well. (1) If the spurs cross each other, the antibodies are unrelated. (2) If one spur merges with the other, and the latter extends beyond the first spur; the first antibody has some homology with the second one. (3) If both spurs combine to form one common curved spur, the two antibodies are identical.**

The advancing wave fonts of the diffusing antigen and antisera form precipitated complexes at the zones where they meet. The precipitate is soluble if there is more antigen than antibody. Hence, a very sharp precipitin line is made at the zone where amount of antigen and antibody are equivalent. The relative distance of the precipitin line from the two reactants indicates their relative strengths.

### (3) The Immunoelectrophoretic Technique

The Immunodiffusion and electrophoresing techniques may be combined to assess antigen antibody complex formation. The poly clonal antibodies in a serum may be fractionated into the components antibodies with different specificities by immunoelectrophoresis.

The technique consist of placing the serum in a well at one end of a thin agarose slab prepared on a microscope slide. When the slide is placed in an electrophoresis apparatus and the current turned on, the heterogeneous antibodies migrate in the gel at rats proportional to their size. A well may be cut along the longitudinal edge of the agarose slab and the antigen to be matched placed in this. The antigen will diffuse in the surroundings gel and precipitate the matching antibody. The precipitates are seen after staining with Coomassie blue. Other immunoelectrophoretic techniques have also been developed one of them is the rocket electrophoresis technique (fig.). In this case the antibody is mixed uniformly in the gel. It is kept immobile by choosing a pH that prevents its diffusion. The antigens are placed in small wells. Near one edge of the slide or on a piece of square glass coated uniformly with agarose gel. When the current is on, the antigens move out and the reaction with the antibody is visualized after staining the gel. Rocket – shaped precipitates are seen, the heights of the one – or rocket shaped stained region being proportional to antigen concentration. With the help of standard markers, the concentration of the unknown antigen may be determined.

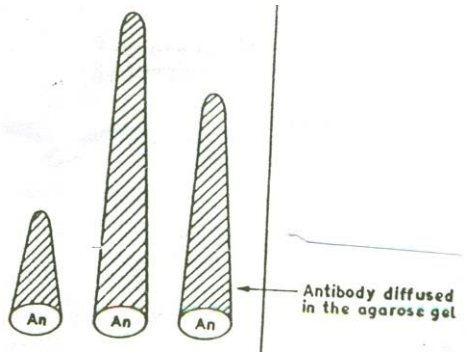



**Fig. The rocket elecrophoresis technique. Antigens are placed in a straight line at one edge of a rectangular gel, and the antibody is diffused in the latter. On electronphoresis, the antigens move out and complex with the antigen wherever they are in equal proportions. This results in cone – or rocket – shaped regions of An-Ab precipitates. The affinities of different antigen samples are indicated by the height and intensity of staining of the ' rockets' that they generate.**

**(4) Radioimmunoassay (RIA)**

Radioimmunoassay (RIA) is another technique that is used for precise and sensitive estimation of immune complexes. This technique involves the use of a known quantity of $^{125}$I-labelled antigen that is allowed to compete with the antigen to be estimated for a measured amount of the matching antibody; the degree of competition can be computed. A non-radioactive biotinylated label may be used instead of the radioactive iodine tag.

The RIA procedure is generally as follows (Fig.). The antigen in a saline solution is allowed to become adsorbed on the inner surface of a plastic tube (or microwell in a multiwelled plate). Non adsorbed antigen is removed by washing. To avoid non – specific protein solution is poured

into the tube (well). This is gain washed out. The antisera to be tested are poured in. the matching antibody alone will complex with the immobilized antigen. Again, excess antisera and unattached antibodies are washed out. A readioactively labelled ligand is included with the antiserum; the former binds itself to the antibody. After the reaction is over, and excess unused antibodies and ligands removed, the test tube (well) walls are tested for radioactivity. Sophisticated variation of this basic procedure are employed to even distinguish the type and subtype of Ig molecules that complex with a given antigen.

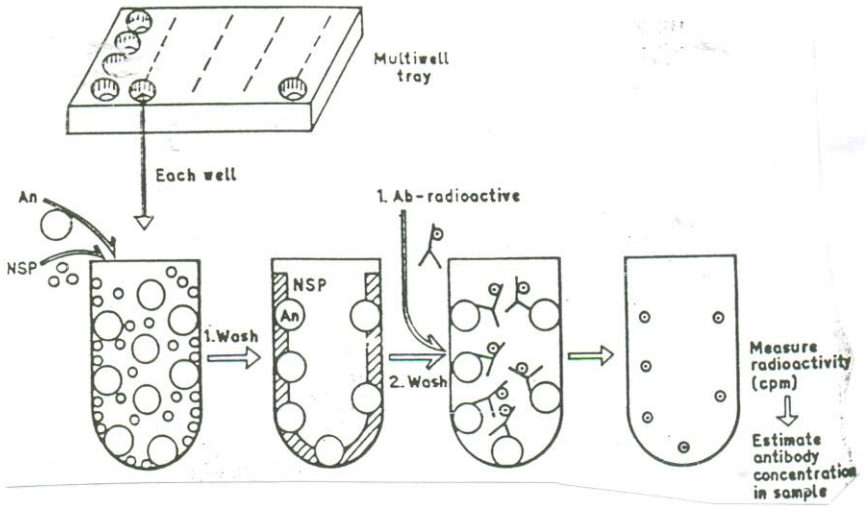



**Fig. Radioimmunioassay (RIA). The antigen to be tested is mixed with an excess of a non – specific protein (NSP) and pourd into wells in a multiwell culture plate. The antigen is adsorbed on the plastic surface of the well. Surface areas not covered by antigen are lined with NSP. The excess mixture is pipetted out, the wells washed free of proteins and solution of radioactively labeled antibody introduced into each well. The excess antibody solution is removed and the wells washed to remove free labeled antibody. Each well is cut out from the plate and monitored for radioactivity (cpm or counts per minute) in a scintillation counter. The amount of antibody bound to antigen is estimated from the cpm in each well. The amount of antigen is calculated from this value. The technique may be utilized to estimate the amount of antibody by reversing the roles of An and Ab in the above procedure.**

**(5) Enzyme – linked Immunoabsorbent Assay (ELISA)**

The ELISA technique is (fig.). similar to RIA up to the addition of the test antiserum or antigen. The antibody or antigen is immobilized (conjugated) on a solid base (test – tube or multi – well microtitre plate surfaces, or plastic beads). The matching protein, labeled with an enzyme (e.g., horseradish peroxidase) is added to the bound protein and the excess enzyme – labeled protein removed. The amount of complexes formed (immobilized can be estimated by adding the substrate which is a chromogen for the enzyme and measuring the products (one of which is coloured) of the reaction with a spectrophotometer.

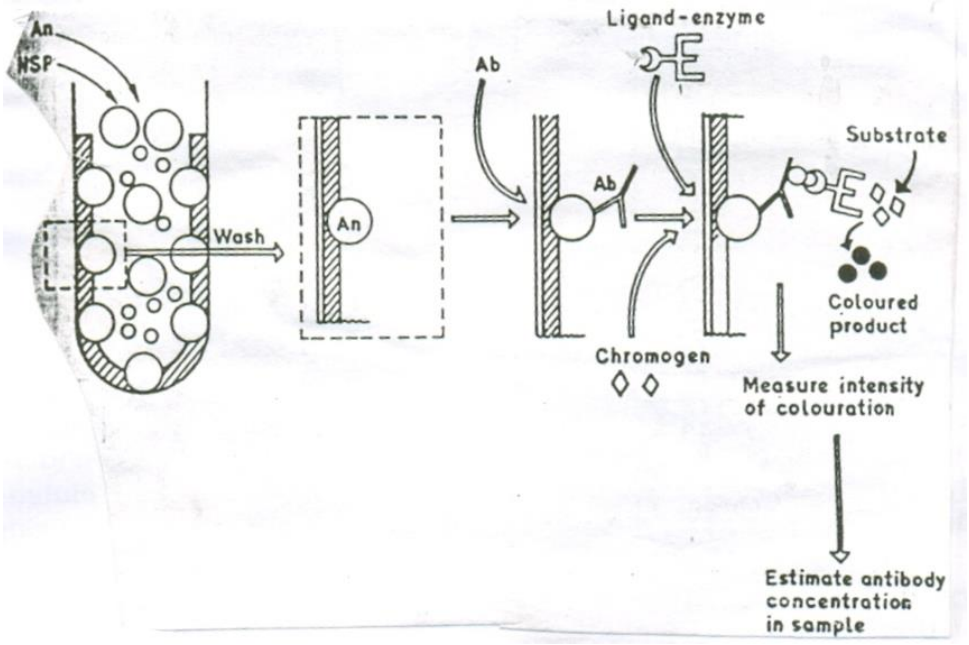



**Fig. Enzyme – linked immunoabsorbent assay. (ELISA). This technique estimates the amount of antigen (or antibody) using a non –radiactive probe. As in RIA (fig.) antigen and NSP are first allowed o line the inner surface of the wells of the culture plate. Now, a non – radioactive antibody (Ab) is added in the following sequence: the Ab is added to each well and free Ab removed. The Ab molecules bound to the An are conjugated to an enzyme (such as horseradish peroxidase) which catalyses a reaction that results in a coloured product. A substrate for the enzyme is added. The enzyme – catalysed reaction colurs the solution in the well. The intensity of colouration (measured by a colorimeter) is utilized to estimate the concentration of the antibody, and eventually that of the antigen in the test sample.**

### (6) Immunoaffinity Chromatography

Once a clone that is expressing the protein from the recombinant DNA is identified, it may be necessary to collect sufficient amounts of the antigen in a pure form. One of the best techniques for doing so is to use the bio chemical method of affinity chromatography (Fig.), with the matching antibody in the column gel. The unpurified mixture of antigen and impurities is passed through this column; the required antigen complexes with the immobilized antibody; everything else is eluted. The antigen can then be released from the complexes. The reverse may be followed to obtain an antibody in a pure form: the antigen is immobilized in this case.

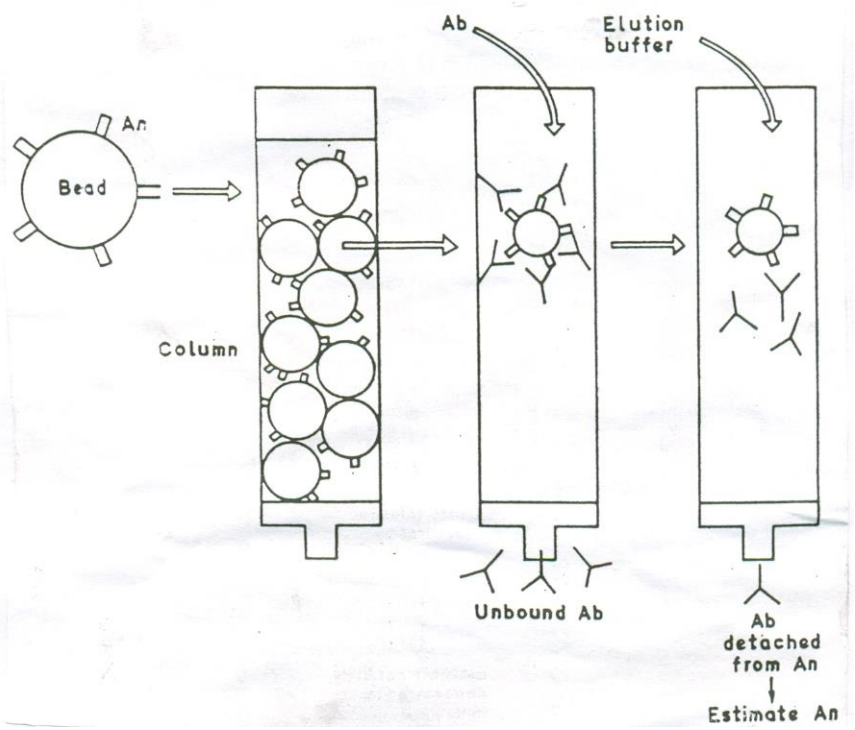



**Fig. Immunoaffinity chromatography. Polystyrene beads coated with the antigen (AN) are used to pack a column through which the solution of a matching antibody (Ab) in passed. Unbound Ab is removed by repeated elutions. Finally, the An-Ab complexes are released from the beads and eluted. Pure antigen or antibody (by reversing the roles of An and Ab in the above procedure) may be collected using this technique.**

**7. Write short notes on the following;**

**i) Plasmid and marker rescue techniques**

**ii) Transposan Tagging**

**I. Plasmid and marker rescue techniques**

Plasmid and marker rescue techniques are essentially similar. In both, the DNA is cloned as a library into a plasmid, usually pBR322 (or a cosmid). The clones are used to select primary transformants. The DNA from these is used to obtain secondary transformants which may possess the entire pBR322 (if that were the vector) or only parts of it. In the plasmid rescue method, the clones that are positive for pB322 are isolated and their DNA extracted and cleaved with an enzyme that does not cleave within the gene or between the gene and the plasmid DNA (ascertained before the protocol is followed). The cleaved fragments are circularized and used to transform E. coli cells. Those that are amplicillin – resistant (due to $Amp^R$ on pBR322) are allowed to grow into large colonies, and DNA extracted form them to transform once more the mammalian cell. Perucho utilized this method to isolate the TK gene from chicken DNA. The cells

were selected on a HAT medium for the tk $^{+}$ transformants. On sequencing the DNA of transformed cells, the entire TK gene was found with the pBR322 sequences.

In the marker rescue scheme, the DNA of the secondary transformants is cloned into a phage library, and each clone searched with a pBR322 probe. The ones that are positive for the probe are used to transform or complement a host defect (mutation) is the gene being searched. Pellicer et al. (1980) employed this strategy is isolate the APRT gene form hamster DNA. The aprt cells that became aprt$^{+}$ were selected on a special medium. The DNA of these cells contained the APRT gene together with a small region only of the pBR322.

A some what similar strategy was used to isolate oncogenes. In this case the tag for the chromosomal DNA was a plasmid carrying the SupF (suppressor tRNA that suppresses amber mutations) gene. Transformation of hosts. The DNA of the secondary transformants was made into a phage library, with the amber mutation included in one arm of the phage. When the phages were palted on su E. coli, only Sup F-carrying phages cold grow. The DNA from the latter was used to transform redent cells is culture. Those that acquired the characteristics of oncogenically transformed cells were selected. The DNA of the latter contained the onognes c-Ha-ras (Goldfarb et al 1982) and N-ras (Shimizu et al., 1983).

**Transposon Tagging**

Isolating a eukaryotic gene by trasposon tagging involves inserting transposons in the target DNA, fractionating fragments of the latter on Southern blots and hybridizing the transposon with a probe. Cleaving out the transposon, together with flanking DNA, has revealed adjoining sequences of a gene. Probes made from the latter are then utilized to look for the fragments having the gene.

The transposon may make its presence felt by altering a normal phenotype. The method of transposon tagging was first employed to isolate the white locus in Drosophila. The mutants with white – apricot eyes harboured a copia retrotransposon in the white locus. A probe made form copia identified the transposon as well as the accompanying white locus. Many eukaryotic genes have been purified by this method.

**8. How will you construct the genomic library used in recombinant DNA technology?**

**GENE LIBRARIES**

The collection of cloned DNA fragments that represents the entire genome of a species is referred to as a ‘gene library’. A gene bank or a clone bank may be a complete or an incomplete gene library.

The gene library may be a genomic or a cDNA one. A genomic library ideally contain every region of the genome represented in the collection of cloned fragments. A cDNA library consist of the genic regions of the genome. As cDNA is copied from RNAs, non-genic regions and genic stretches of sequences not represented in the transcripts are also not present in a cDNA library. A cDNA library is further liable not to be representative of all the genes, as the ratios of RNA species form different genes vary is proportion and stability and may not, therefore, be included in the RNAs that are copied into cDNAs.

**Genomic Library**

For a genomic library the total DNA is cleaved into smaller fragments(mechanically or enzymatically), each one of which is cloned in a suitable vector. The rDNA carrying such fragment is amplified in E. coli cells. One can calculate the minimum number of clones that must be present to represent every region of the genome. For this calculation, one needs to know the average length of the fragments and the approximate total length of the genomic DNA. Clarke and Carbon (1976) proposed a formula that is generally utilized, keeping in mind that the values obtained are good approximations and not exact ones. One may find the value of N, the number of clones needed to represent every bit of the total DNA from the relationship

$$N = \frac{\ln\ (1\text{-}P)}{\ln\ (1\text{-}F)}$$

Where, F is the ratio of the length of the fragment to the entire genomic length and P is the probability of finding a specific sequence.

Fragments for a genomic library should be long enough to include an entire gene sequence. It is customary to have average fragment lengths of 15-20 kb. This can be obtained by mechanical shearing of the DNA or by the use of restriction enzymes. What enzymes would one use? The criterion for the use of the particular restriction enzyme is partly how often it is likely to come across its cleaving site in the DNA. A recognition motif with four nucleotides is likely to be present every $4^4$ or 4096 bases. The latter will produce fragments too long for cloning and characterization by restriction mapping. The preferred fragment length is 15-20 kb as mentioned earlier.

The next decision to be taken is how long should the DNA be treated with the chosen enzyme? It complete digestion is allowed, every DNA will be cut up into identical sets of fragments. Which will be very large in number. In practice, partial digestion is preferred as it produces long fragments; these are also random ones, as the enzyme does not cleave at every site, nor at the same sites in every DNA strand. Such random fragments, therefore, over lap at places, a feature that is useful for lining up the fragments in the correct original order during the melding operations in

DNA sequencing. The procedure of connecting sequences of consecutive fragments by aligning their overlapping regions (ends) is popularly known as chromosome walking.

There is one other reason for the fragments to be about 15-20 kb in length. The fragments usually need to be mapped by restriction enzymes other than the one used for the initial fragment production. Restriction mapping is done best if the fragment is not too long (i.e., > 20 kb). Such a long fragment is likely to posses more than one cutting site for an enzyme. This complicates the mapping operation.

The geomic library will be screened for desired regions of the DNA. Since genes and their flanking regions are of primary interest, it would be ideal to have fragments with unmultilated genes – that is, genes without a portion cleaved out with the enzyme. Often, it is desirable to know the nature of the sequences on either side of a gene. A good collection of random fragments usually satisfies the above mentioned needs.

The cloing vector can theoretically by any one of the several available vectors. However, for a variety of reasons, λ -replacement vectors and cosmids are used for making gene libraries. The merits of these two will be discussed later.

The first step in making a genomic library is to isolate genomic DNA with as little shear as possible. The high molecular weight DNA is then digested partially with a restriction enzyme. The fragments are fractioned by gel electrophoresis or sucrose (or sodium) gradient velocity centrifugation. The sodium gradient centrifugation is preferred as it does not require an additional step of removing the salt (as is done for the sucrose). A size standard is run in the same gel, and lengths of 18-22 kb are identified among the fragmented genomic DNA. Fragments larger than these are not packaged in to phage particles. While ones much smaller may join tandemly in a random order to make up the optimum packaging size. The latter are not useful for obvious reason.

Of course, by confining onself to a size range, one may miss larger fragments that have no restriction sites, as well as very small ones that are lost during processing. By creating a library of a sufficient number of clones, one may hope to ensure the inclusion of every region of the genome.

The enzyme used for fragmenting the DNA are usually Mbo I and Sam 3, both of which recognize a tetranucleotide motif, GATC, which is the core of the Bam HI motif, GGATCC. Sticky ends caused by these three enzymes are compatible. With the calculation that a 4- base motif occurs every 256 bases, a 15kb DNA would be expected to posses about 50 such sites; to recover fragments of this size only 1/60 of the total cutting sites may be engaged by the enzyme. Controlled partial digestion would ensure this result. In the same vein, a 20kb fragment can be recovered by using only 1/80 of the possible cleavage sites.

To prevent donor DNA fragments from joining with each other, or from circulating the 5'-ends of the stands are dephosphorylated with alkaline phosphatase. This DNA when purified, is ready for ligation to the phage λ - vector. The unligated nicks at each juncture of vector and donor DNA are joined by the ligase in the E. coli cell in which the hybrid molecule is introduced. The donor DNA is spliced to the arms of the vector and recombinant DNAs are packaged with packaging mixes.

The hybrid DNA from each phase particle is amplified in E. coli either by phage infection or transfection with the naked DNA. The method of choice will depend partially on the resources of the user; packaging mixes are not inexpensive.

The library can be stored appropriately, and used to fish out a desired gene or other regions of the genome or for chromosome walking. Probes are required for both operations the hybridization is usually carried out on nylon filters or on nitro cellulose paper to which replicas of plaques have been transferred by blotting ( a modification of the Hogness – Grunestein technique). The blots are treated with a mild alkali (NaOH), which release the DNA from intact cells and denatures the DNA into single strands. Free, unpackaged DNA present in the lysate in each plaque also becomes adsorbed on the blotting paper. The filter is then flooded with the probe, treated as usual to fix the hybridized DNA and autoradiographed for detecting the locations of the latter.

In practice, the term 'genomic library' is used for a library of clones made form the native DNA, as opposed to that from cDNAs.

**9. How the cDNA library is constructed and screened for recombinant DNA technology?**

**Construction of cDNA Library**

cDNA (complementary DNA) is synthesized against an RNA template using the enzyme reverse transcriptase. A cDNA library or clone bank contains cDNA rather than genomic DNA fragments spliced to the vector.

Although any RNA may be copied into a cDNA, in practice, mRNAs have been mostly used as templates. A cDNA clone bank, therefore, usually represent the heterogeneous population of mRNAs present in a cell.

A complete clone bank should represent every mRNA in a species. This rarely happens as all mRNAs are not synthesized in every cell, nor are the same sets found in cells of different tissues and / or at different developmental stages. A cDNA cloen bank would thus reflect the genes expressed in the cell from which the mRNA templates are taken.

Again, a cDNA clone bank even from the same cell need not contain representatives of every transcripts that this cell synthesizes. Transcripts that are not very stable or long lasting, or those which are produced at negligibly low levels, are likely to be missed.

Despite these shortcomings, and inspite of the fact that it is more time consuming and expensive to prepare cDNA and not the genomic DNA banks or libraries. Let us list some of these requirements.

First of all, there are far less numbers of clones of cDNA in a bank than in a genomic library may require 100-1000 k fragments, whereas a cDNA bank may contain only 10-30 k clones to choose from.

Screening a cDNA bank also provides fairly unambiguous results. The probes utilized for screening genomic fragments are made by labeling in vitro mRNAs or cDNAs. In practice, there is no guarantee that the isolated mRNA is not contaminated with the rRNAs that occur in profusion in every cell. Some of these rRNAs also become labeled, and result in 'false positives' when used to hybridize the genomic clones. No such mistake can occur with a cDNA clone bank. There is always a unique mRNA that will bind itself with the correct cDNA.

A cDNA bank is useful when one is particularly interested in recovering a specific gene. If the cDNA is synthesized against the mRNA from cells expressing the latter at a high level, a large percentage of the clones will represent this mRNA, and hence its gene.

There are at least four other justifications for preparing cDNA clone banks. One of them is for cloning genomes of RNA viruses; cDNA copies are the only possible inserts that can be spliced to vector DNA. Secondly, one can get an idea about the base level house- keeping genes by comparing cDNA. Secondly, one can get an idea about the base level house-keeping genes by comparing cDNA clone banks from cells at difference stages of development and from different tissues. The luxury of special mRNAs in them may be different, but there will always be one set of genes that is expressed in all of them. Again, if it were necessary to express a eukaryotic split gene in a bacterial cell, a cDNA prepared form the spliced mRNA would be translated in the prokaryots the latter will not be able to handle splicing of the mRNA, since the in fracture for such operations is not present in them. Further, a cDNA is useful for delineating the exon and intron regions of a split gene. When a cDNA is hybridized with a genomic counterpart, only the exon regions pair; the introns are thrown out as loops.

As in the case of preparing a genomic library, Clarke and Carbon (1976) have provided a formula for estimating the number of clones (N) that will guarantee the presence of the minimum number (n) of clones required to represent all classes of abundance of mRNAs. There are three classes of abundance: high, medium and low. The low abundance class may have several thousand different

species, but only a very few copies per species. In contrast, the other two classes may represent fewer number of genes, but the latter produce several thousands or hundreds of copies of mRNA per gene.

If one was interested in screening a cDNA clone bank for one of the high or medium abundance class genes, only a few (7000-8000) clones would be enough as many of them would be duplicates from the same mRNA species Low abundance clones may be recovered only from a much large collection. If a specific probe was available for the low abundance species of interest, it would not be very cumbersome to screen even a large collection.

The cDNA bank is prepared by first extracting the total RNA from a cell and them purifying it from any contaminating DNA by caesium chloride density centrifugatice. The already purified mRNAs are further purified by running them through a colures containing poly-dT oligonucleotides. The poly dA tails of the mRNAs become hybridized to these oligonucleotides; the mRNAs are eluted form the column.

The mRNAs are next copied into ss cDNA using a poly dT – oligonucleotide as prmer and the AMV reverse transcriptase as the DNA polymerase. There are at least two procedures for synthesizing the second – strand of the cDNA.

To enable the ds cDNA to be joined to the vector DNA, it is customary to provide suitable joining ends to the cDNA. If the ends of the latter are not 'flush', they are made so using S1 nuclease. Flush-ended cDNA may be joined to flush-ended vector ends using t4 ligase. Or else, a staggered end, specific for a particular restriction enzyme, may be created at the cDNA terminally by first joining the restriction enzyme linker (synthetic short length of DNA containing the cleavage site of an enzyme which is subsequently cut with the restriction enzyme. The sticky tails of vector and cDNA may be extended by complementary homopolymer tails and TNT.

The vector and cDNA are ligated and E., coli cells transformed with them. When the transformant colonies are grown, representatives from each are stored in a storage medium (usually containing DMSO), at -20$^{o}$C or -70$^{o}$C. Hanahan and Meselson have developed a neat way of storing the transfomants in a very small space. They grew the bacteria on Millipore filters placed on the nutrient agar. The filters with the colonies were frozen and stored at -70$^{o}$C. These can be utilized for screening for the desired done. Usually, the screening is performed in two steps: the primary screening identifies the clones likely to possess the sequence in question, and the secondary screening main points the ones with the sequence. The latter are then characterized.

**Phage Lambda versus Cosmids for Gene Libraries**

Cosmid can carry a maximum of 45kb of insert DNA, whereas phage λ vectors can bear a maximum of 25kb of the donor DNA. The larger insert DNA can encompass long genes, particularly eukaryotic split genes. This advantage of cosmids, as well as that of requiring fewer

clones to include all fragments in the genome, is offset by the fact that they are less easy to handle, the longer DNA does not always become packaged, and several recombination events may reduce the size of the cloned fragment.

Phage λ-based libraries, on the other hand, do not present the above problems, in spite of the larger number of clones that have to be screened, the techniques for screening are so simple that the larger number of clones is not much of a problem. The smaller insert DNAs may cut through a gene, or very large or very small fragments may be left out during packaging. By ensuring a sufficient number of clones of random fragments, the sequences in adjacent fragments may be determined, and the intact gene characterized.

**10. How cDNA libraries are generated by using reverse transcription of mRNA?**

**cDNA is representative of the mRNA population, and therefore reflects mRNA levels and the diversity of splice isoforms in particular tissues**

Complementary DNA (cDNA) is prepared by reverse transcribing cellular mRNA. Cloned eukaryotic cDNAs have their lack introns and other non-coding sequences present in the corresponding genomic DNA. Introns are rate in bacteria but occur in most genes of higher eukaryotes. They can be situated within the coding sequence itself, where they then interrupt the colinear relationship of the gene and its encoded polypeptide, or they may occur in the 5′ or 3′ untraslated regions. In any event, they are copied by RNA polymerase when the gene is transcribed. The primary transcript goes through a series of processing events in the nucleus before appearing in the cytoplasm as mature mRNA. These events include the removal of intron sequences by a process called splicing. In mammals, some genes contain numerous large introns that represent the vast majority of the sequence. For example, the human dystrophin gene contains 78 introns, representing over 99% of the sequence. The gene is nearly 2.5 Mb in length yet the corresponding cDNA is only just over 11 kb. Thus one advantage of cDNA cloning is that in many cases the size of the cDNA clone is significantly lower than that of the corresponding genomic clone. Since removal of eukaryotic intron transcripts by splicing does not occur in bacteria, eukaryotic cDNA clones find application where bacterial expression of the foreign DNA is necessary, either as a prerequisite for detecting the clone or because expression of the polypeptide product is the primary objective. Also where the sequence of the genomic DNA is available, the position of intron/exon boundaries can be assigned by comparison with the cDNA sequence.

Under some circumstances, it may be possible to prepare cDNA directly from a purified mRNA species. Much more commonly a cDNA library is prepared by reverse transcribing a population of mRNAs and the screening for particular clones. An important concept is that the cDNA library is representative of the RNA population from which it was derived. Thus, whereas

genomic libraries are essentially the same regardless of the cell type or developmental stage from which the DNA was isolated, the contents of cDNA libraries will vary widely according to these parameters. A given cDNA library will also be enriched for abundant mRNAs but may contain only a few clones representing alternative splice variants.

Table shows the abundances of different classes of mRNAs in two representative tissues. Generally, mRNAs can be described as abundant, moderately abundant, or rate. Notice that in the chicken oviduct, one mRNA type is superabundant. This encodes ovalbumin, the major egg-white protein. Therefore, the starting population is naturally so enriched in ovalbumin mRNA that isolating the ovalbumin cDNA can be achieved with out the use of a library. An appropriate strategy for obtaining such abundant cDNAs is to clone them directly in an M13 vector such as M13 mp8. A set of clone can then be sequenced immediately and identified on the basis of the polypeptide that each encodes. A successful demonstration of this strategy was reported by Puteney et al. (1983), who determined DNA sequences of 178 randomly chosen muscle cDNA ciones. Based on the amino acid sequences available for 19 abundant muscle-specific proteins, they were able to identify clones corresponding to 13 of these 19 proteins, including several protein variants.

For the isolation of cDNA clones in the moderate and low abundance classes it is usually necessary to construct a cDNA library. Once again the high efficiency obtained by packaging in vitro makes phage λ vectors attractive for obtaining large number of cDNA clones. λ insertion vectors are particularly well suited for cDNA cloning and some of the most widely used vectors are discussed in box.

| Source | Number of different mRNAs | Abundance (molecules/cell) |
|---|---|---|
| Mouse liver cytoplasmic poly(A)$^+$ | 9 | 12,000 |
| | 700 | 300 |
| | 11,500 | 15 |
| Chick oviduct polysomal poly(A)$^+$ | 1 | 100,000 |
| | 7 | 4,000 |
| | 12,500 | 5 |

**λgt 10 and λgt 11**

Most early cDNA libraries were constructed using plasmid vectors, and were difficult to store and maintain for long periods. They were largely replaced by phage-λ libraries, which can be stored indefinitely and can also be prepared to much higher titers. λ gt 10 and λgt 11 were

largely replaced by phage- λ libraries, which can be stored indefinitely and can also be prepared to much higher titers. λgt 10 and λgt 11 were the standard vectors for cDNA cloning until about 1990. both λgt 10 and λgt11 are insertion vectors, and they can accept approximately 7.6 kb and 7.2 kb of foreign DNA, respective. In each case, the foreign DNA is introduced at a unique EcoRI cloing site. λgt 10 is used to make libraries that are screened by hybridization. The EcoRI site interrupts the phage cl gene, allowing selection on the basis of plaque morphology. λgt 11 contains an E. coli lacZ gene driven by the lac promoter. If inserted in the correct orientation and reading frame, cDNA sequences cloned in this vector can be expressed as β-galactosidase fusion proteins, and can be detected by immunological screening or screening with other ligands λgt 11 libraries can also be s creened by hybridization, although λgt 10 is more appropriate for this screening strategy because higher titers are possible.

**λZAP series:**

While phage-λ vectors generate better libraries, they cannot be manipulated in vitro with the convenience of plasmid vectors. Therefore, phage clones have to be laboriously sub cloned back into plasmids for further analysis. This limitation of conventional phage-λ vectors has been addressed by the development of hybrids, sometimes called

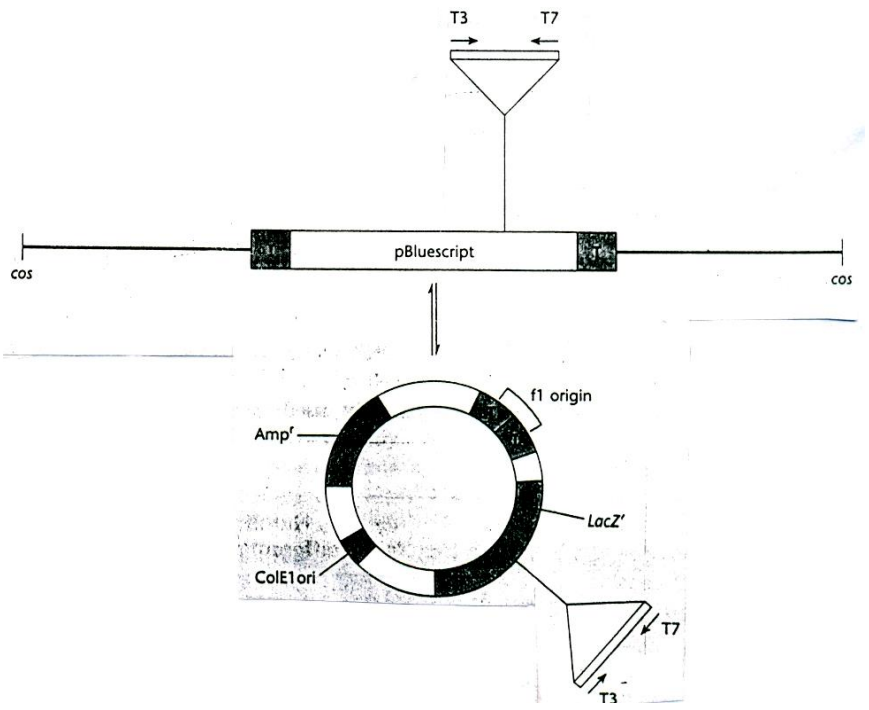



**Figure: Linear phage map of the prototype λZAP vector with the circular map of the excised pBluescript plasmid shown below it.**

Phagmids, which possess the most attractive features of both bacteriophage λ and plasmids. The most popular current vectors for cDNS cloning are undoubtedly those of the λZAP

vector is shown opposite. The advantageous features of this vector are:

(i) The high capacity – up to 10 kb of foreign DNA can be cloned, which is large enough to encompass most cDNAs,
(ii) The presence of a polylinker with six unique restriction sites, which increases cloning versatility and also allows directional cloning; and
(iii) The availability of T3 and T7 RNA polymerase sites flanking the polylinker, allowing sense and antisense RNA to be prepared from the insert.

Most importantly, all these features are included within a plasmid vector called pBluescript, which is itself inserted into the phage genome. Thus the cDNA clone can be recovered from the phage and propagated as a high-copy-number plasmid without any subcloning, simply by coinfecting the bacteria with a helper f1 phage that nicks the λZAP vector at the flanks of the plasmid and facilitates excision. Another member of this series, λZAP Express, also includes the human cytomegalovirus promoter and SV40 terminator, so that fusion proteins can be expressed in mammalian cells as well as bacteria. Thus, cDNA libraries can be cloned in the phage vector in E. coli, rescued as plasmids and then transfected into mammalian cells for expression cloning.

Typically clones is sufficient for the isolation of low-abundance mRNAs from most cell types, i.e. those present at 15 molecules per cell or above. However, some mRNAs are even less abundant than this and may be further diluted if they are expressed in only a few specific cells in a particular tissue. under these circumstances it may be worth enriching the mRNA preparation prior to library construction, e.g. by size fractionation, and testing the fractions for the presence of the desired molecule. One way in which this can be achieved is to inject mRNA fractions into Xenopus oocytes and test them for production of the corresponding protein (Melton 1987).

**The first stage of cDNA library construction is the synthesis of double-stranded DNA using mRNA as the template:**

The synthesis of double-stranded cDNA suitable for rinsertion into a cloning vector involves three major steps as;

1. first –strand DNA synthesis on the mRNA template, carried out with a reverse transcriptase
2. removal of the RNA temple and
3. Second – strand DNA synthesis using the first DNA strand as a template, carried out with a DNA – dependent DNA polymerase such as E.coli DNA polymerase 1.

All DNA polymerase, whether they use RNA or DNA as the template, require a primer to initiate strand synthesis.

The first reports of cDNA cloning were published in the mid-1970s, and were all based on the homo-polymer tailing technique, of several alternative methods, the one that became the most popular was that of Maniatis et al. (1976). This involved the use of an oligo-dT primer annealing at the polyadenylate tail of the mRNA to prime first-strand cDNA synthesis, and took advantage of the fact that the first cDNA strand has the tendency to transiently food back on itself, forming a hairpin loop, resulting in self-priming of the second strand (Efstratiadis et al. 1976). After the synthesis of the second DNA strand, this loop must be cleaved with a single-strand –specific nuclease, e.g. S1 nuclease, to allow insertion into the cloning vector.

A serious disadvantage of the hairpin method is that cleavage with S1 nuclease results in the loss of a certain amount of sequence at the 5′ end of the clone. This strategy has therefore been superseded by other methods in which the second strand is primed in a separate reaction. One of the simplest strategies is shown in figure. After fist –strand synthesis, which is primed with an oligo-dT primer as usual, the cDNA is tailed with a string of cytidine residues using the enzyme terminal transferase. This artificial oligo-dC tail is then used as an annealing site for a synthetic olig-dG primer, allowing synthesis of the second strand. Using this method, Land et al. (1981) were able to isolate a full-length cDNA

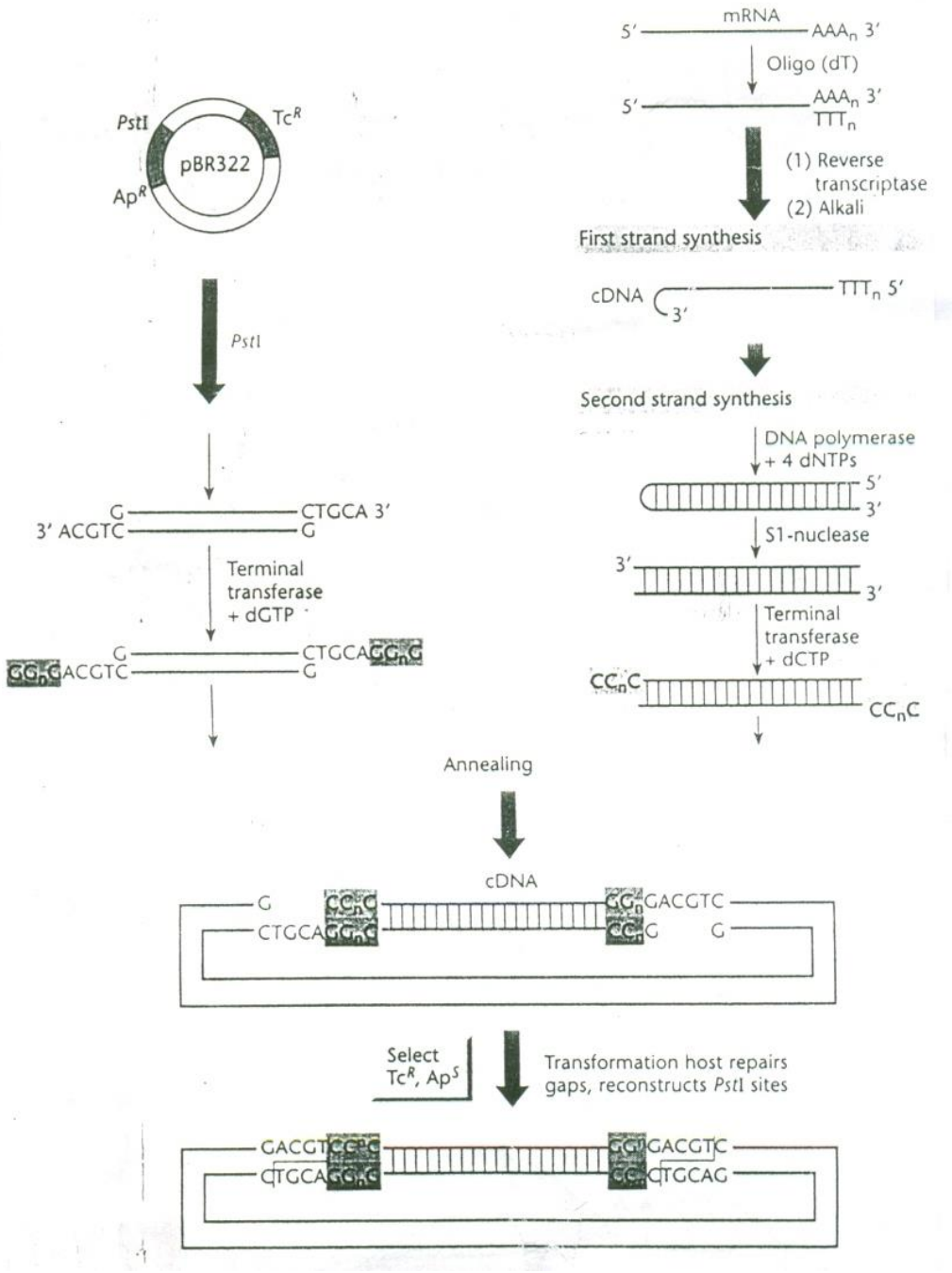



**Figure: An early cDNA cloning strategy, involving hairpin-primed second-strand DNA synthesis and homo-polymer tailing to insert the cDNA into the vector.**

Corresponding to the chicken lysozyme gene. However the efficiency can be lower for other cDNAs (e.g. Cooke et al. 1980).

For cDNA expression libraries, it is advantageous if the cDNA can be inserted into the vector in the correct orientation. With the self-priming method, this can be achieved by adding a synthetic linker to the double-stranded cDNA molecule before the hairpin loop is cleaved (e.g.

Kurtz & Nicodenmus 1981) where the second strand is primed separately, direction cloning can be achieved using a oligo-dT primer containing a linker sequence (e.g. Coleclough & Erlitz) An alternative is to use primers for cDNA synthesis that are already linked to a plasmid. This strategy was devised by Okayama & Berg (1982) and has two further notable characteristics. First full-length cDNAs are preferentially obtain because an RNA-DNA hybrid molecule, the result of first –strand synthesis, is the substrate for a terminal transferaser reaction. A cDNA that does not extend to the end of the mRNA will present a shielded 3-dydroxyl group, which is a poor substrate for tailing. Second – strand synthesis therefore occurs by a nick-translation types of reaction, which is highly efficient. Simpler cDNA cloning strategies incorporating replacement synthesis of the second strand is widely used (e.g. Gubbler & Hoflman 1983, Lapeyre & Amalric 1985). The Gubbler-Hoffman reaction as it is commonly known is shown in figure.

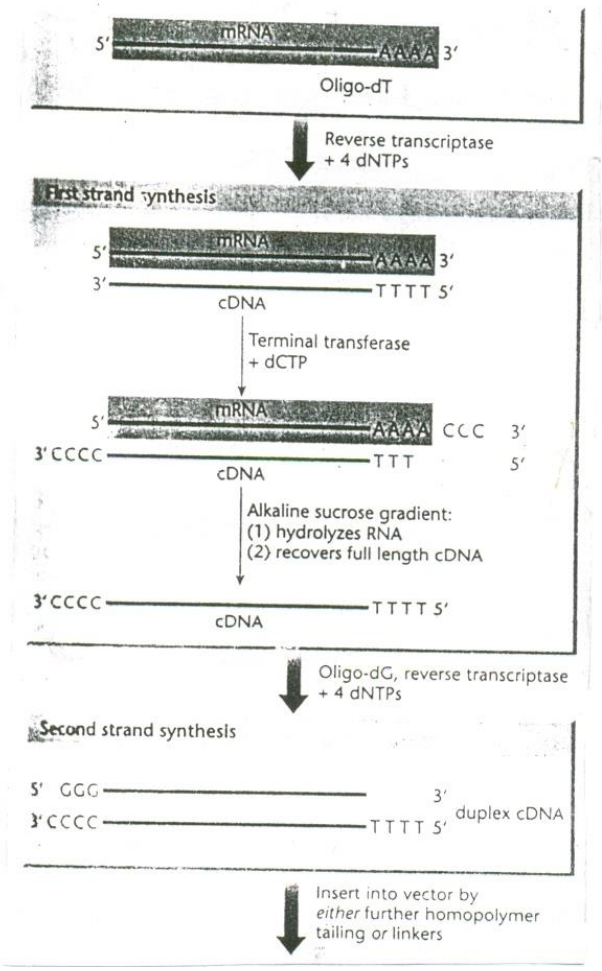



**Figure: Improved method for cDNA cloning. The first strand is tailed with oligo(dC) allowing the second strand to beinitated using an oligo(dG) primer.**

**11. Explain in detail about the preparation of full length cDNA for cloning.**

**Obtaining full-length cDNA for cloning can be a challenge:**

Conventional approaches to the production of cDNA libraries have two major drawbacks. First, where oligo-dT primers are used to initiate first-strand synthesis, there is generally a 3′ end bias (preferential recovery of clones representing the 3′ end of Cdna sequences) in the resulting library. This can be addressed through the use of random oligonucleotide primers, usually hexamers, for both first-and second strand cDNA synthesis. However, while this eliminates 3′ end bias in library construction, the resulting clones are much smaller, such that full-lenth cDNAs must be assembled from several shorter fragements. Second as the size of a cDNA increases, it becomes progressively more difficult to isolate full-length clones. This is partly due to deficiencies in the reverse transcriptase enzymes used for first-strand cDNA synthesis. The enzymes are usually purified from avian myelobastosis virus (AMV) or produced from a cloned Moloney murine leukemia virus (MuLV) gene in E. coli. Native enzymes have poor processivity and intrinsic RNase activity, which leads to degradation of the RNA template (Champoux 1995). Several companies produce engineered murine reverse transcriptases that lack RNase H activity, and these are more efficient in the production of full-lenth cDNAs (Gerard & D′ Allesio 1993). An example can also carry out reverse transcription at temperatures of up to 50C. the native enzymes function optimally at $37^0$ C, and therefore tend to stall at sequences that are rich in secondary structure, as often found in 5′ and 3′ untranslated regions.

Despite improvements in reverse transcriptases, the generation of full-length clones corresponding to large mRNAs remains a problem. This has been addressed by the development of cDNA cloning strategies involving the selection of mRNAs with intact 5′ ends. Nearly all eukaryotic mRNAs have a 5′ end cap, a specialized, methylated guanidine residue that is inverted with respect to the rest of the strand and is recognized by the ribosome prior to the initiation of protein synthesis. Using a combination of cap selection and nuclease treatment, it is possible to select for full-length first-strand cDNAs, and thus generate libraries highly enriched in full-length clones.

An example of the above is the method described, BY Edery et al (1995). In this strategy, first-strand cDNA synthesis is initiated as usual, using an oligo-dT primer. Following the synthesis reaction, the hybrid molecules are treated with RNase A, which only digests single – stranded RNA. DNA-RNA hybrids therefore remain intact. If the first – strand cDNA is full length, it reaches all the way to the 5′ cap of the mRNA, which is therefore protected from cleavage by RNase A. however, part-length cDNAs will leave a stretch of unprotected single-stranded RNA between the end of the double-stranded region and the cap, which is digested away with the enzyme.

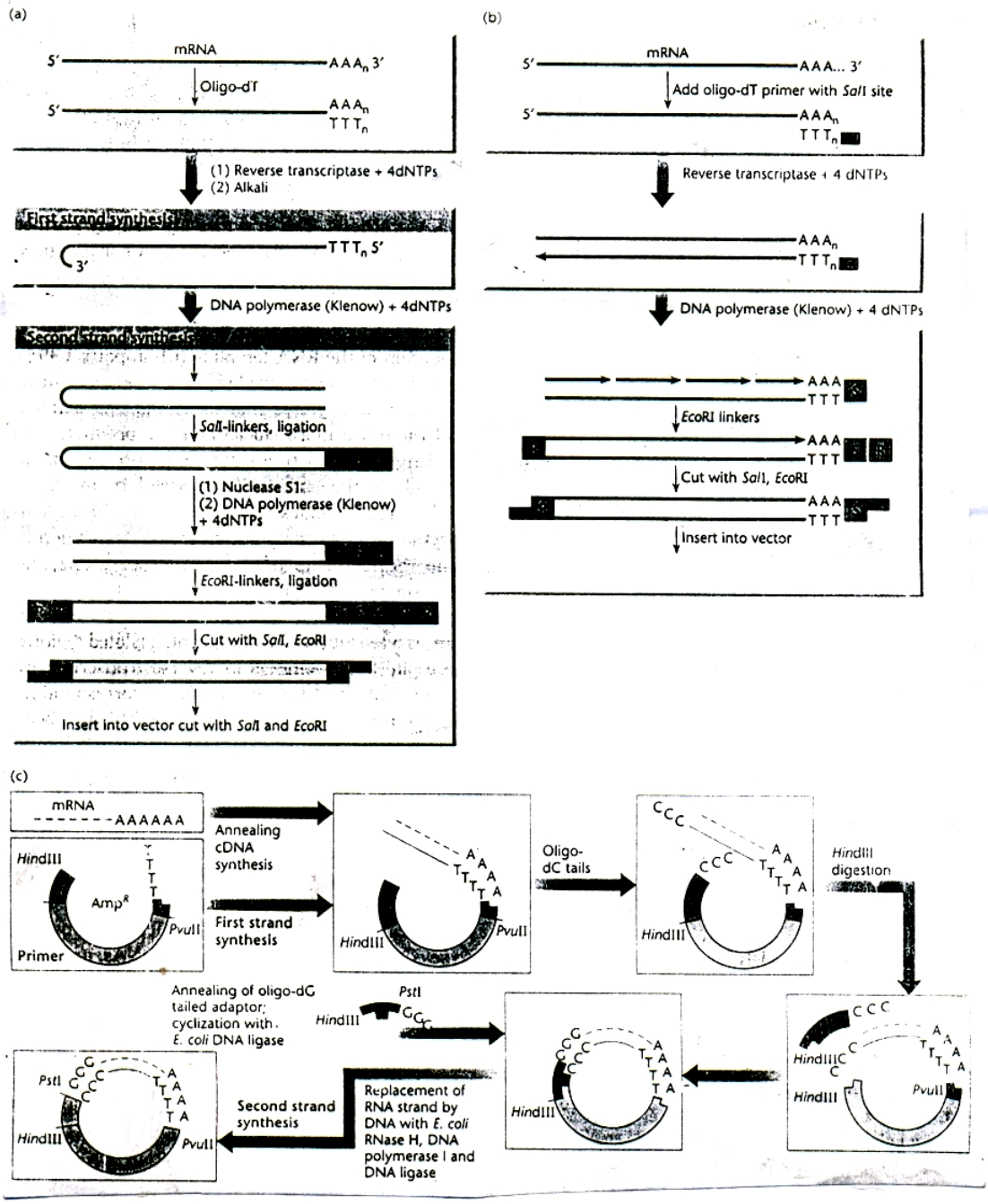



**Figure: Methods for directional cDNA cloning. (a) An early strategy in which the formation of a loop is exploited to place a specific linker (in this example, for Sall) at the open end of the duplex cDNA. Following this ligation, the loop is cleaved and trimmed with S1 nuclease and EcoR1 liakers are added to both ends. Cleavage with EcoR1 and Sall generates a restriction fragment that can be unidirectionally inserted into a vector cleaved with the same enzymes.**

**(b) A similar strategy, but second-strand cDNA synthesis is random – primed. The oligo (dT) primer carries an extension forming a SalI site. During second-strand synthesis, this forms a double-stranded SalI linker. The addition of further EcoRI linkers to both ends allows the cDNA to be unidirectional cloned, as above.**

**(c) The strategy of Okayama & Berg (1982), where the mRNA is linked unidirectionally to the plasmid cloning vector prior to cDNA synthesis, by virtue to a cDNA tail.**

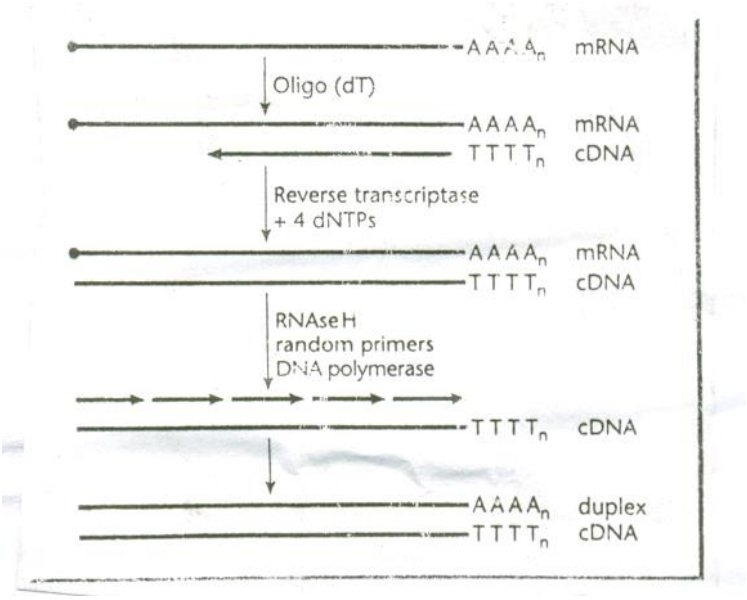



**Figure: The Gubbler – Hoffman method, a simple and general method for non-directional cDNA cloning. First strand synthesis is primed using an oligo (dT) primer. When the first strand is complete, the RNA is removed with RN ase H and the second strand is random-primed and synthesized with DNA polymerase I. T4 DNA polymerase is used to ensure that the molecule is blunt-ended prior to insertion into the vector.**

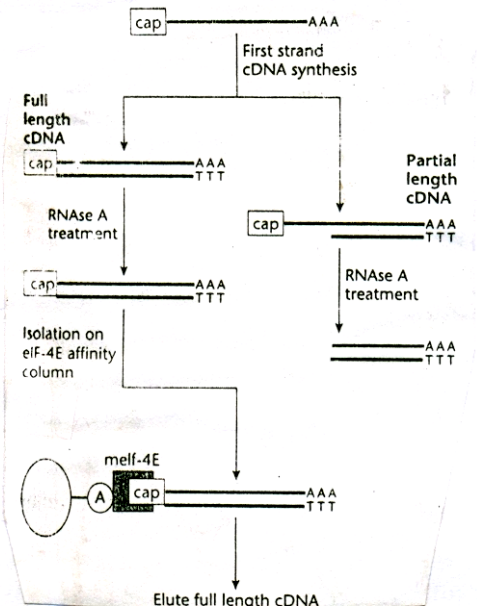



**Figure: The CAP ture method of full length cDNA cloning, using the eukaryotic initiation factor eIF -4E to select mRNAs with caps protected from RN ase digestion by a complementary DNA strand.**

In the next stage of procedure, the eukaryotic translational initiation factor eIF -4E is used to isolate full-length molecules by affinity capture. Incomplete cDNAs and cDNAs synthesized on broken templates will lack the cap, and will not be retained. A similar method based on the biotinylation of mRNA has also been reported (caminci et al.1996). Both methods, however, also co-purify cDNAs resulting from the mispriming of first-strand synthesis, which can account for

up to 10% of the clones in a library. An alternative method, oligo-capping, addresses this problem by performing selection at the RNA stage (Maruyama & Sugano 1994, Suzuki et al. 1997, Suzuki et al. 2000) the basis of the method is that RNA is sequentially treated with the enzymes alkaline phosphatase and

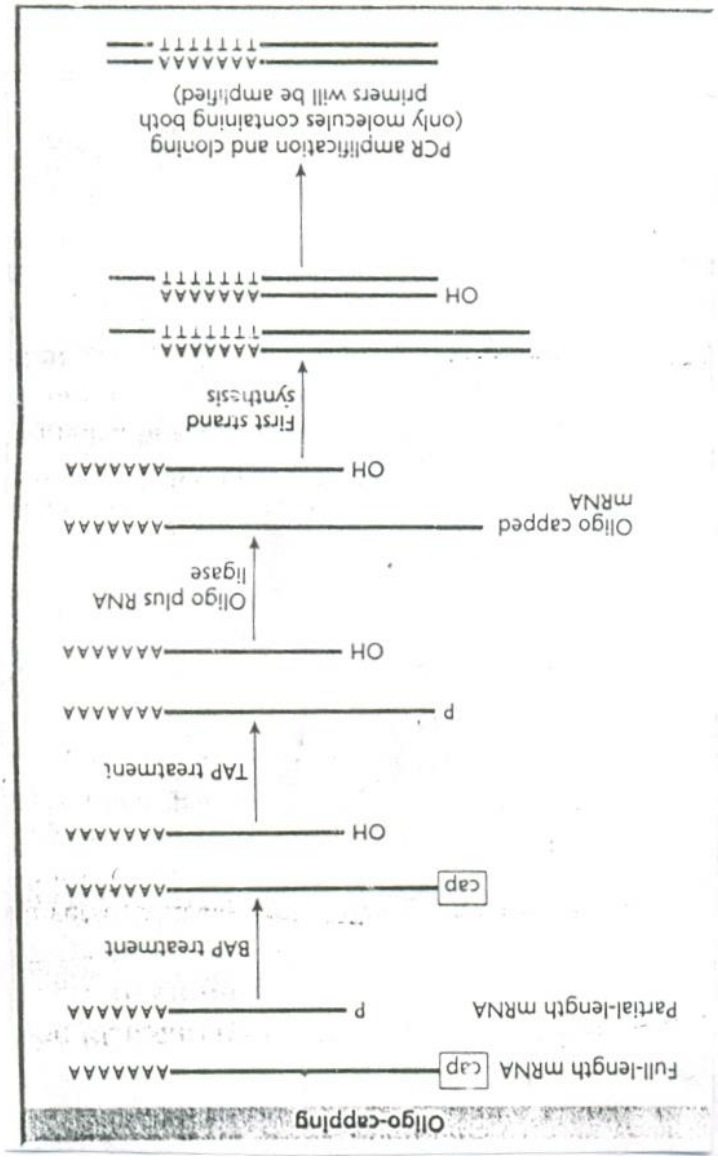



**Figure: Oligo-capping, the addition of specific oligonucleotide primers to full-length RNAs by sequential treatment with alkaline phosphatase and acid pyrophosphatase. Once the oligo cap has annealed to the 5′ end of the mRNA, It can serve as a primer binding site for PCR amplification.**

Acid pyrophosphatase. The first enzyme removes phosphate groups from the 5′ ends of

uncapped RNA molecules, but does not affect full-length molecules with a 5′ cap. The second treatment removes the cap from full-length RNAs leaving a 5′ terminal residue with a phosphate group. Full-length molecules can beligated to a specific oligo-nucleotide, while broken and degraded molecules cannot. The result is an oligo-capped population of full-length mRNAs. this selected population is then reverse trascribed using an oligo-dT primer. Second-strand synthesis and cloning is then carried out by the PCR using the oligo-dT primer and a primer annealing to the oligo nucleotide cap. Only full-length cDNAs annealing to both primers will be amplified, thus eliminating broken or degraded RNAs, incomplete first cDNA strands (which lack a 5′ primer annealing site), and misprimed cDNAs (which lack a 3′ primer annealing site).

## UNIT – IV

## PART – A

**1. What is polymerase chain reaction (PCR)?**

The polymerase chain reaction (PCR) is an in vitro technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequences. PCR amplify any short length of DNA several million folds in a test-tube, by using only the DNA to be amplified, two primers each matching the 5 extremity of the single strands of the said DNA and a DNA polymerase that works at a high temperature. The mixture is heated to dissociate the strands. After a while the mixture is cooled. The primer attached to the ss DNA; the DNA pools begin and synthesis more DNA molecules.

**2. What are the different PCR methods involved for analysis of DNA?**

There are different PCR methods involve which developed based on the modification of basic PCR.

1. Inverse PCR
2. RACE PCR
3. Reverse Transcriptase PCR
4. Nested PCR
5. Quantitative PCR
6. Anchored PCR
7. Real-time PCR
8. In-situ PCR

**3. What is PCR assisted DNA sequencing?**

Polymerase chain reaction (PCR) is used for sequencing the amplified DNA product. This method also called as ligation mediated PCR (LMPCR). This method of sequencing is farter and more reliable and can utilize either the whole genomic DNA or closed fragments for sequencing a particular DNA segment. The sequencing using PCR involves two steps as

1. generation of sequencing templates (ds DNA to ss DNA) using PCR and

2. sequencing of PCR products either with the thermo labile DNA polymerase or with the thermos table Tag DNA polymerase

**4. What is PCR walking (or) Primar walking?**

When a fragment of DNA with unknown sequences need to be amplified, this DNA fragment is cloned in a vector, so that the known sequences at the insertion site of the vector can be used as a primers. More recently PCR methods have been designed, which allow amplification of DNA with unknown sequences, which are present adjoining known sequence with in a DNA fragment. The technique is described as PCR walking or primer is walking.

**5. How the PCR is involved in site directed mutagenesis?**

This technique is used for introducing mutations at the desired place in a DNA sequence by alerting the sequences of primers. Since mutation are introduced only through primers, mutations are limited to the ends of the gene sequence. A variation of this technique allows mutations to be introduced at any place of interest in the gene. This method is described as ova lop extension.

**6. What is inverse PCR (Chromosome crawling)?**

In this method, the amplification of those DNA sequences, which are away from the primers and not those which are flanked by the primers are done. For instance, if the border sequences of a DNA are not known and those of a vector are known, then the sequence to be amplified may be cloned in the vector and the border sequence of the vector may be used with primers in such a way that the polymerization proceeds in an inverse direction, i.e., a way from the vector sequence flanked by the primers and towards the DNA sequence of inserted segment. Similarly if the give sequence is known, one can use its border sequences as primers, for an inverse PCR, to amplify the sequences flanking this gene, e.g., the regulatory sequences.

**7. Write the application of Nested PCR.**

The sensitivity and specificity of PCR can be increased by using nested PCR (in PCR). On nested PCR, two separate amplifications are used. The first uses a set of primers that yields a large product, which is then used as a template for the second amplification. The second set of primers anneal to sequences with in the initial product producing a second smaller product. Nested PCR increases the specificity of the reaction because the formation of the feral product depends up on the binding of two separate sets of primers.

**8. Write short notes on Tag man's assay.**

This is used in PCR amplification technique. In this assay method an oligonuclcotide probe is labeled with a fluorescent reporter molecule (e.g., FAM or TAT) at 5′ end and a quencher dye (e.g., TAMRA) at the 3′ end. This probe is called Tag man probe. The Tag man probe after hybridization to the template DNA is degraded at its 5′ end during extension phase of PCR due to exonucleass activity of Tag polymerase enzyme, so that the reporter is released leading to a rise in

fluorescence signal. Additional reporter –dye molecules are cleaved from their respective probes with each cycle, effecting an increase in fluorescence intensity proportional to the amount of amplicor produced.

**9. What are molecular beacons?**

It is used in polymerase chain reaction technique molecular beacons are hairpin-shaped oligomucleotides with a fluorophore at one end and a fluorescence quencher at the other end. While in the hairpin structure the fluorescence is quenched but when the probes bind to the target it undergoes a confirmational reorganization that restores fluorescence. The oligonucleotide probe (molecular beacons) consist of the target SNP sequence, with the two ends binding complimentary each other. The fluorescence signals in molecular beacons can be detected by appropriate sensing devices.

**10. Write short notes on scorpion probes.**

A scorpion probes also has a hairpin structure configuration but differs from a molecular beacons in that it has a 3′ complimentary sequence that acts as primer and is extended in the PCR reaction. After denaturation, the probe sequence binds to the target sequence and this restores fluorescence. Scorpions perform better under rapid cycling conditions because binding obeys first – order kinetics whereas molecular beacons obey second – order kinetics.

**11. Write short notes on RAPD? Give the importance.**

RAPD is abbreviated as Randomly Amplified Polymorphic DNA. RAPDs are widely used by plant molecular biologists to construct maps because they provide very lage numbers of markers and are very easy to detect by agarose gel electrophoresis. RAPDs are generated by producing the random sequences in the polymorphic DNA are used as primers for amplification by PCR. RAPDs are smaller is size and generated large number from DNA. Lays number of RAPD marker are communally available which increased the number of amplified products.

**12. Mention any 4 methods involved in detecting SNP, (single Nucleotide phosphates).**

1. Enzymatic method – RFLP, AFLP, CFLP, RAPD
2. Electrophoretive method –

(i) Single confirmation polymorph sin
(ii) Heterodepler analysis
(iii) DNA sequencing

3. Solid phase method
4. Chromatographic method

(i) Denaturing high performance liquid chromatography (DHPLC)
5. Examining EST data
6. Physical – Fluorescence exchange – based methods

**13. What is site directed mutagenesis? Give its importance in molecular probe analysis.**

It is a technique to use the mutational effects in nucleotide substation is DNA fragment. By using site directed-mutagenesis it is possible to change two or three adjacent nucleotides, so that every possible amino acids substitution is made at a site of interest. This generates a requirement for 19 different mutagenic oligonucleotides assuming only one codon will be used for each substitution.

**14. Write short notes on cassette mutagenesis.**

Cassette mutagenesis is a alternative way of changing one amino aid to all the alternatives. This involves replacing a fragment of the gene with different fragments containing the desired codon changes. It is a simple method for which the efficiency of mutagenesis is close to 100%.

**15. What is mean by DNA sequencing or gene sequencing? Write the significance.**

Sequencing of DNA or gives means mapping of the DNA in form of its nucleotide sequences. By give sequencing it is possible for generating DNA fragments with a defined nucleotide terminus. By sequencing methods, the DNA fragmented into four aliquots, with fragments in each aliquots ending at one end with one of the four nucleotides. The other ends of fragments are identical. These results in each aliquote are separated on a DNA that forms a nested collection. By using sequence method the cohesive ends of DNA wave obtained. To facilitate the isolation of single stands, the DNA molecule is the to be sequenced and closed in cloning sites.

**16. What are the methods covertly used for gene sequencing?**

The following methods are commonly used for used sequencing

1. Enzymatic method
2. Chemical method

Enzyme method:
- Sanger and coulson's enzymatic method
- Messing's shot – Gun method

Chemical method:
- Maxam and Gobnert's chemical method

**17. How computers are applied for analysis of DNA sequence datas?**

The larger DNA to be sequenced is converted to these sequence able lengths. In essence each fragment is compared with sequences of all other fragments in order to look for regions that overlap or are common to each other. By placing these fragments together so that the common regions overlap, one can produce larger sequences.

Computers have been recruited to carry out these comparison and melding operation. Several computer programs have been developed to do this job quickly and efficiently. This method consist of recording the sequence of each fragments in a memory bank. The sequence of each fragments in a memory bank. The sequence from one set of fragments is then compared with those from every fragment of second test.

**18. Write short notes on Sanger's method of sequencing.**

It is an enzymatic method of sequencing DNA, which results in an array of fragments, each of which differs from the ones larger and smaller than it by one nucleotide each. In this case the fragments are synthesized in four reaction mixtures, up to a particular nucleotide.

The method is based on synthesizing a lapelled stand of DNA standing from a primer annealed to a template and terminating the synthesis at one of the form nucleotides. The terminator is a dide enucleated that has no 3.04 to which another nucleotide may be added.

**19. What are the requirements in gene sequencing by Sanger's method?**

The following materials are required for DNA sequencing by sayesis enzymatic method.

1. a short primer
2. a pure ss DNA template
3. the four terminators (dd ATP, ddGTP, ddCTPR, ddGTP)
4. the four dNTPs of which some or one or five are 32p labelled in each reaction mixture
5. a DNA polymerase without 5′→ 3′ exonuclease activity.

**20. How the replacement of radioactive labels with fluorescent labels is possible in automated DNA sequencing?**

To automate the sequencing process it is desirable to acquire sequence data in real-time by detecting the DNA bands with in the gel during the elatrophotration. On practice, the fluorescent tags are attached to the chain-terminating nucleotides. Each of the dideoxy nucleotides carries a spectrally different fhuorophose. The tag is in corporate into a DNA molecule by the DNA polymerase and auomplishes two operation in one step; it terminates the synthesis and it attaches the flourophore at the end of the molecule. By using form different fluorescent dyes it is possible to electrophones all form chain terminating reaction together is one lave of a sequencing gel.

**21. Write short notes on RACE – PCR.**

This is a method by which the 3′ and 5′ ends of CDNA are amplified using small street of known sequence with in a gene. This small stretch can be derived by back translation from the amino arid sequence of the protein or can be from DNA sequence homology with other members of the gene family. For 3′ RACE , a reverse transcriptase primer that contain oblige dT is used. For 5′ RACE, CDNA is prepared with either specific primer or random hexamers and them the newly synthesized CDNA stand is has homopolymeric tail added to its 3′ end using terminal transferase.

**22. What are the applications of PCR?**

1. Detecting pathogens by PCR
2. Study of DNA polymorphism
3. Molecular mapping
4. Gene tagging
5. Use in human genetics
6. Prenatal die gnosis
7. Recombination data analysis
8. Sexing of Embrges using PCR
9. Confirmation of the presence of transferred gene
10. DNA finger printing.

**23. What are the major steps involve in PCR?**

There are 3 major steps involve in PCR technology.

(i) **Melting of target DNA**: The target DNA containg sequence to be amplified in heat natured (around $94^0$C for 15 second) to separate complementary stands.

(ii) **Ann eating of primers**: The second step is the Ann eating of two oligonuclotide primers to the denatured lowered to about $68^0$C for 60 secs consequently the primers form hydngen bonds i.e. anneal to the DNA on both sides of the DNA sequence.

(iii) **Primer extension**: Finally, nucleoside triphosplate (dATP, dGTP,dCTP and dTTP)and a there mutable DNA polymerase and added to reaction mixture and extortion of primers (at $68^0$C) resulting synthesis of target sequence DNA.

**24. How will you calculate theoretically the copies of DNA sequence amplified by PCR technology?**

PCR amplification of DNA is achieved by using oligonucleotide primers, also known as amplifiers. Primen are short sequenced SS DNA template. The enzyme DNA polymerase (Tag polymerase) involved in the presence of deoxynucleoside triphosphates (dNTPs)

The final number of copic of the tayet sequence, is expressed by the formula as

$(2^n - 2n)$ x, when

n = number of cycles
2n = first product obtained after cycle 1 and second product obtained after cycle 2 with in defined length
x = number of copies of the original template.

Potentially after 20 cycles of PCR, there will be $2^{20}$ – fold amplification, assuming 100% efficiency during each cycle. However, in practice, only 20 – 30 % efficiency is achieved in PCR methods.

**25. What are primers in PCR?**

Primers are short sequenced and designed to be exactly complementary to the template DNA. The primers used in PCR are between 20 – 30 nucleotides in length. The primer should if possible to have equal number of each of four bases, avoiding regions of unusual sequences such as stretches of polyp urines or polypyrimidines. Primer should not be complementary to each Other; otherwise, they will form primer-dimer'. The primer concentration should not be greater than 1ul. High primer concentration promotes the mispriming, the formation of primers or the generation of non-specific products and reducing the yield of product.

## PART – B

**1. Describe at least four methods of PCR for analysis of DNA.**

**1. Inverse PCR (Chromosome Crawling):**

In this method, the amplification of those DNA sequences, which are away from the primers and not those which are flanked by the primers are done. For instance, if the border sequences of a DNA are not known and those of a vector are known, then the sequence to be amplified may be cloned in the vector and the border sequence of the vector may be used with primers in such a way that the polymerization proceeds in an inverse direction, i.e., away from the vector sequence flanked by the primers and towards the DNA sequence of inserted segment. Similarly, if the gene sequence is known, one can use its border sequences as primers, for an inverse PCR, to amplify the sequences flanking this gene, eg., the regulatory sequences.

In this technique, the primers are designed so that their 3′ end face away from each other. Carrying out PCR will not be expected to amplify the target sequence, since the extension products of either primer will not contain sequences complementary to each other. However, if the linear DNA molecule contain the target and primer sequences is first circularized by restriction enzyme digestion followed by ligation, the target can be amplified by PCR.

This clever modification of the basic PCR technique allows amplification of unknown DNA sequences to one other or both sides of a known DNA segment (depending upon which restriction enzyme sites are chosen) with result, it resembles chromosome walking by traditional cloning methods.

**2. Anchored PCR:**

In the basic PCR technique and in inverse PCR, one has to use two primers representing the sequences lying at the ends of sequences to be amplified. But sometimes, we may have knowledge about the sequence at only one of the two ends of the DNA sequence to be amplified. In such cases, anchored PCR may be used, which will utilize only one primer instead of two primers. In this technique, due to the use of one primer, only one strand will be copied first, after which a poly G tail will be attached at the end of the newly synthesized stand. This newly synthesized stand with 'poly G tail' at its 3′ end will then become template for the daughter strand synthesis utilizing an anchor primer with which a poly C sequence is linked to complement with poly G of the template. In the next cycle, both the original primer and anchored primer will be used for gene amplification. There are two anchored PCR methods for the DNA isolation: rapid amplification of CNA ends (RACE-PCR) and ligation anchored PCR.

**3. RACE – PCR:**

This is a method by which the 3′ and 5′ ends of a cDNA are amplified using a small stretch of known sequence within a gene. This small stretch can be derived by back translation from the amino acid sequence of the protein or can be from DNA sequence homology with other members of the gene family. The procedure was made more powerful by the use of nested primers, which reduce non-specific amplification and ensures the production of relatively pure specific product. For 3′ RACE, a reverse transcriptase primer that contains an oligo dT sequence linked to an adapter sequence (which may be long enough to permit binding of two nested primers or shorter for single primer binding) is used to prime the first stand cDNA synthesis. Primary amplification is then performed with a gene-specific primer (GSP – 1) and the outer primer R1. A small fraction of the first amplification is then used for secondary amplification that is nested gene-specific primer (GSP-2) and inner primer R2.

For 5′ RACE, cDNA is prepared with either a specific primer or random hexamers and then the newly synthesized cDNA strand has homo polymeric tail (eg. A residues) added to its 3′ end using terminal transferase. The complementary reverse transcription anchor primer is then used to generate second stand cDNA. This ds DNA can then serve as the template for PCR reaction as described for 3′ RACE.

**4. Ligation-anchored PCR:**

This is a simple, efficient and sensitive technique for the amplification of cDNAs where the 5′ end has unknown sequence. In this method, T4 RNA ligase is used to covalently link the 'anchor' oligonucleotide to first-strand of cDNAs. Amplification is then carried out with one primer specific for a sequence within the gene of interest and one primer specific for the anchor. The archor oligonucleotide must be 5′ phosphorylated, this is necessary for ligation of the cDNA. The 3′ end of the anchor oligonucleotide is blocked, for example, by the addition of a dideoxynucleotide is blocked, for example, by the addition of a dideoxynucleotide using terminal deoxynucleotidy1 transferase or by the incorporation of amino acid group during oligonucleotide synthesis. This 3′ modification is required to prevent the ligation of more than one anchor to the cDNA.

**5. Reverse transcriptase PCR:**

The RT-PCR protocol requires two separate major reactions, a reverse transcriptase step, followed by PCR amplification. Recently, a DNA polymerase known as Tth DNA polymerase, isolated from a Thermus thermophilus is found to have both the properties of reverse transcription and DNA amplification. This enzyme, in the presence of managanese can reverse transcribe RNA. Because the Tth DNA polymerase can utilize both DNA and RNA templates, the whole procedure can be carried out in a single tube.

For mRNA that contains a poly (A) tract at the 3'end, oligo dT, random hexamers or a gene-specific primer can be used to prime cDNA synthesis. RT-PCR is a highly sensitive tool in the study of gene expression at the RNA level and in particular, in the quantitation of mRNA or viral RNA levels. This technique, also known as message amplification phenotyping (MAPPing), permits simultaneous analysis of a large number of mRNAs from small numbers of cells. RT-PCR can also be used as a first step in preparing a cDNA library by PCR of all of the mRNAs in a sample of cellular DNA.

Reverse transcriptase is usually used to synthesize first – stand cDNA from RNA. Reverse transcriptase can be purified from several sources, eg. Avian myeloblastosis vitud (AMV) and Moloney Murine Leukemia Virus (MMLV). AMVRT is an RNA-dependent DNA polymerase that uses single-stranded RNA as a template and can synthesize a complementary DNA (cDNA) in the 5' to 3' dhection if the primer is present. In addition, it has DNA polymerase activity and this enzyme also exhibit ribonuclease H activity, which is specific for RNA:DNA heteroduplex molecules.

MMLV RT acts in the same way as AMV RT; however, it lacks DNA endonuclease activity and has lower RN ase H activity. Thus, it has a greater chance of producing full-length copies of large mRNA species.

**6. Nested PCR:**

The sensitivity and specificity of PCR can be increased by using nested PCR (nPCR). In nPCR, two separate amplifications are used. The first uses a set of primers that yields a large product, which is then used as a template for the second amplification. The second set of primers anneal to sequences within the initial product producing a second smaller product. The primers for the second round of amplification are either both different from the first set and both located within the amplified DNA region. If only one of the second round primers is located within the amplified region and is used together with one of the first round primers, it is termed as 'semi-nested PCR'. Nested PCR increases the specificity of the reaction because formation of the final product the need for verification of the PCR product by blotting, restriction digestion or sequencing. The second set of primers also serves to verify the specificity of the first product.

Nested PCR primers are ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. The sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using the nested PCR method. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products. This is because, after the first round of PCR the non-specific products are unlikely to be sufficiently complementary to the nested

primers to be able to serve as template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity.

**2. Describe the basic PCR and the variants of basic PCR. Discuss their utility with specific examples.**

**Basic PCR:**

In order to understand PCR, the readers should be familiar with the mechanism of DNA replication within the cell. DNA replication involves polymerization of nucleotides using a template DNA strand with the help of an enzyme DNA polymerase, but this reaction invariably requires a primer strand to which further nucleotides can be added using the DNA polymease enzyme. If the primer strand is not available, the reaction can not proceed. In the living cells this primer strand is not a DNA

**Figure: A thermal cycler for PCR amplification (the instrument has a microprocessor- controlled temperature cycling needed for PCR).**

Strand but is a small single stranded RNA molecule synthesized with the help of RNA polymerase enzyme, which does not need a primer. In PCR, a similar reaction takes place in an 'eppendorf tube', where the primer strand is added from outside in the form of a deoxyribo – oligonucleotide, and DNA polymerase enzyme is added to help in polymerization. Unlimited supply of amplified DNA is obtained by repeating the reaction, which is made possible by regular denaturation of freshly synthesized double stranded DNA molecules by heating it to 90- $98^0$ C. At this high temperature the two strands separate. Once the double stranded DNA is made single stranded by heating upto 90-$98^0$C, the mixture of these two strands with two primers that recognize the sequences bordering the sequence to be amplified is cooled to 40-$60^0$C. This allows the primers (which are in excess) to bind to their complementary strands through renaturation. The presence of Taq DNA polymerase enzyme and all the four essential nucleoside triphosphates

allow synthesis of complementary strands in the usual manner. In a thermal cycler, this process is automatically repeated 20-30 times (as predetermined by a computer device), so that in a single afternoon, a billion copies of the sequence flanked by the left and right primers can be produced.

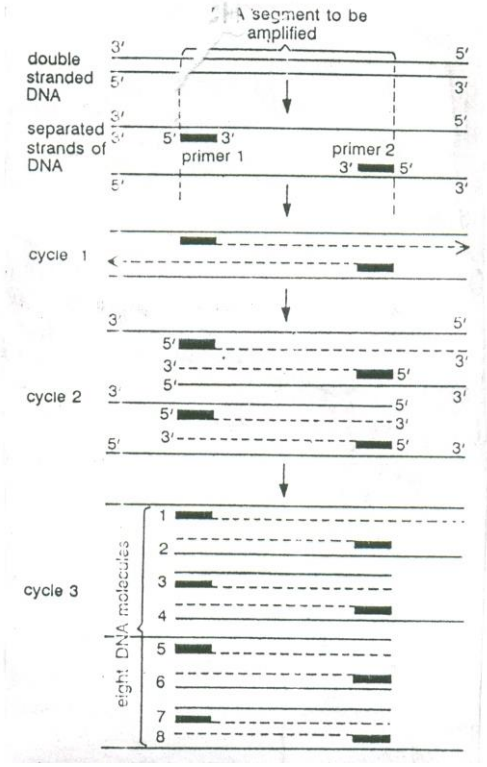



**Figure: Basic reaction of the PCR (only three cycles of PCR are shown; in each cycle primers are shown by solid boxes, template strands are shown by continuous lines and newly synthesized strands are shown by broken lines). (Modified and redrawn from Dharmalingam. K. –Biospectra, Nov-Dec., 1990**

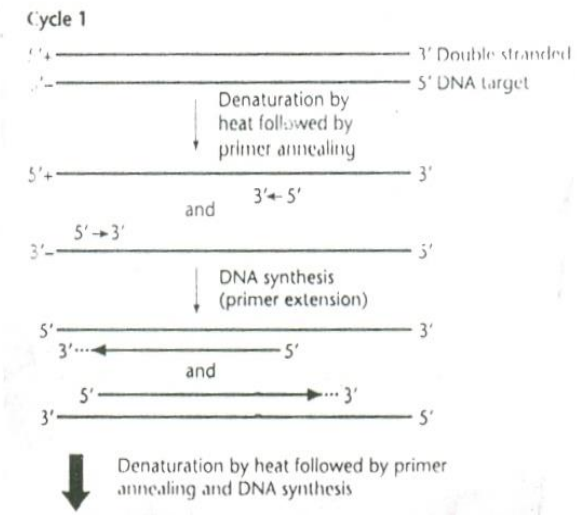
In order to continue the synthesis, the temperature of the mixture is alternately increased (for denaturation) and decreased (for renaturation) once every 1-3 minutes (as fixed by the computer device). This requires that during temperature rise, the enzymatic activity of DNA polymerase should not be destroyed; otherwise one may have to add a fresh aliquot of enzyme in each cycle of amplification. This became possible only by the discovery of thermostable enzyme **Taq DNA polymerase**, isolated from Thermus aquaticus growing in hot springs. This enzyme acts best at $72^0$C and the denaturation temperature of $90^0$C does not destroy its enzymatic activity. Later, other thermostable enzymes like **Pflu DNA polymerase** isolated from pyrococcus furiosus and **Vent polymerase** isolated from Thermococcus litoralis, were discovered and were discovered and were found more efficient. These enzymes allowed automation of the entire process and automatic PCR thermal cyclers are now available (each for a price of around Rs 2-4 lakhs), which can amplify DNA sequences at a fast speed unattended.

**Comparison of PCR and gene cloning (modified from Dharmalingan, 1990)**

| Parameter | PCR | Gene cloning |
|---|---|---|
| 1. Final result | Selective amplification of specific sequence | Selective amplification of specific sequence |
| 2. Manipulation | In vitro | In vitro & in vivo |
| 3. Selectivity of the specific segment from complex DNA | First step | Last step |
| 4. Quantity of starting material | Nanogram (ng) | Microgram (μg) |
| 5. Biological reagents required | DNA polymerase (Taq polymerase) | Restriction enzymes, ligase, vector, bacteria |
| 6. Automation | Yes | No |
| 7. Labour intensive | No | Yes |
| 8. Error probability | Less | More |
| 9. Applications | More | Less |
| 10. Cost | Less | More |
| 11. User's skill | Not required | Required |
| 12. Time for a typical experiment | Four hours | Two to four days |

As pointed out earlier, PCR may eventually replace gene cloning technique discussed in chapter 2 and 3. This technique is much easier and requires much smaller quantity (nanogram or ng = $10^9$ g) of DNA. For cloning, DNA is needed, as the starting material, in microgram (μg) quantities. A comparison between PCR and gene cloning is given in table.

**3. Describe the PCR technique and its application. Explain the different steps involved in the amplification of a DNA fragment by PCR.**



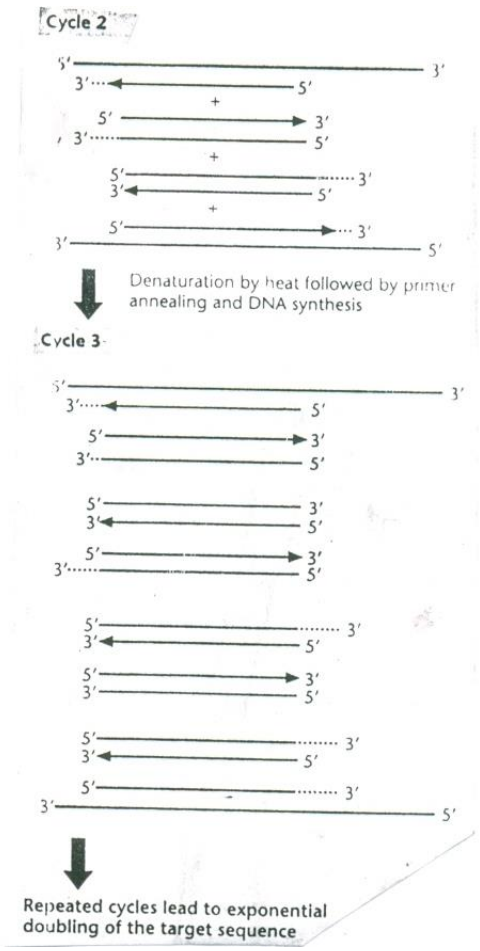



**Figure: The polymerase chain reaction. In cycle 1 two primers anneal to denatured DNA at opposite sides of the target region, and are extended by DNA polymerase to give new strands of variable length. In cycle 2, the original strands and the new strands from cycle 1 are separated, yielding a total of four primer sites with which primers anneal. The primers that are hybridized to the new strands from cycle 1 are extended by polymerase as far as the end of the template, leading to a precise copy of the target region. In cycle 3, double-stranded DNA molecules are produced (highlighted in color) that are precisely identical to the target region. Further cycles lead to exponential doubling of the target region. The original DNA strands and the variably extended strands become negligible after the exponential increase of target fragments**.

**The polymerase chain reaction (PCR) has revolutionized the way that biologists manipulate and analyze DNA:**

The impact of the PCR upon molecular biology has been profound. The reaction is easily performed, and leads to the amplification of specific DNA sequences by an enormous factor. From a simple basic principle, many variations have been developed with applications throughout gene technology (Erlich 1989, Innis et al. 1990). Very importantly, the PCR has revolutionized prenatal diagnosis by allowing tests to be performed using small samples of fetal tissue. in forensic science, the enormous sensitivity of PCR- based procedures is exploited in DNA profiling; following the publicity surrounding Jurassic Park, virtually every one is aware of potential

applications in paleontology and archeology. Many other processes have been described which should produce equivalent results to a PCR (for review, see Landegran 1996) but as yet none has found widespread use.

In many applications of the PCR to gene manipulation, the enormous amplification is secondary to the aim of altering the amplified sequence. This often involves incorporating extra sequences at the ends of the amplified DNA. In this section we shall consider only the amplification process. The applications of the PCR will be described in appropriate places later in the book.

**The principle of the PCR is exceedingly simple:**

First we need to consider the basic PCR. The principle is illustrated in the figure. the PCR involves two oligonucleotide primers. 17-30 nucleotides in length, Which flank the DNA sequence that is to be amplified. The primers hybridize to opposite strands of the DNA after it has been denatured, and are orientated so that DNA synthesis by the polymerase proceeds through the region between the two primers. The extension reactions create two double stranded target regions, each of which can again be denatured ready for a second cycle of hybridization and extension. The third cycle produces two double stranded molecules that comprise precisely the target region, in double-stranded form. By repeated cycles of heat denaturation, primer hybridization and extension, there follows a rapid exponential accumulation of the specific target fragment of DNA. After 22 cycles, an amplification of about $10^6$ – fold is expected and amplifications of this order are actually attained in practice.

In the original description of the PCR method (Mullis & Faloona 1987 Saiki et al., 1988, Mullis 1990). Klenow DNA polymerase was used and, because of the heat-denaturation step, fresh enzyme had to be added during each cycle. A breakthrough came with the introduction of Taq 1) NA polymerase (lawyer et al. 1989) from the thermophilic bacterium Thermus aquaticus. The Taq DNA polymerase is resistant to high temperatures and so does not need to be replenished during the PCR (Erlic et al. 1988. Sakai et al 1988). Furthermore, by enabling the extension reaction to be performed at higher temperatures, the specificity of the primer annealing is not compromised. As a consequence of employing the heat-resistant enzyme, the PCR could be automated very simply by placing the assembled reaction in a heating block with a suitable thermal cycling program.

| Cycle number | Number of double-stranded target molecules |
|---|---|
| 1 | 0 |
| 2 | 0 |
| 3 | 2 |
| 4 | 4 |
| 5 | 8 |
| 6 | 16 |
| 7 | 32 |
| 8 | 64 |
| 9 | 128 |
| 10 | 256 |
| 11 | 512 |
| 12 | 1024 |
| 13 | 2048 |
| 14 | 4096 |
| 15 | 8192 |
| 16 | 16,384 |
| 17 | 32,768 |
| 18 | 65,536 |
| 19 | 131,072 |
| 20 | 262,144 |
| 21 | 524,288 |
| 22 | 1,048,576 |
| 23 | 2,097,152 |
| 24 | 4,194,304 |
| 25 | 8,388,608 |
| 26 | 16,777,216 |
| 27 | 33,554,432 |
| 28 | 67,108,864 |
| 29 | 134,217,728 |
| 30 | 268,435,456 |

**Figure: Theoretical PCR amplification of a target fragment with increasing number of cycles.**

Recent developments have sought to minimize amplification times. Such systems have used small reaction volumes in glass capillaries to give large surface area-to-volume ratios. This results in almost instantaneous and denaturation times. This accompanied by temperature ramp rates of 10-$20^0$ C/s, made possible by the use of turbulent forced hot-air systems to heat the sample, results in an amplification reaction completed in tens of minutes.

While the PCR is simple in concept, practically there are a large number of variables which can influence the outcome of the reaction. This is especially important when the method is being used with rare samples of starting material or if the end result has diagnostic or forensic implications.

| **The PCR achieves enormous amplifications of specific target sequence, very simply** | |
|---|---|
| The reaction is assembled in a single tube, and then placed in a thermal cycler (a programmable heating / cooling block), as described below<br><br>A typical PCR for amplifying a human genomic DNA sequence has the following composition. The reaction volume is 100µl<br><br>Input genomic DNA, 0.1 – 1 µg<br>Primer 1, 20 pmol<br>Primer 2, 20 pmol<br>20 mmol/l Tris-HCI, Ph 8.3 (at $20^0$ C)<br>1.5 mmol/l magnesium chloride<br>25 mmol/l potassium chloride<br>50 mmol/l each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)<br>2 units Taq DNA polymerase<br><br>A layer of mineral oil is placed over the reaction mix to prevent evaporation.<br><br>The reaction is cycled 25-35 times, with the following temperature program: | Denaturation $94^0$ C, 0.5 min<br>Primer annealing $55^0$ C, 1.5 min<br>Extension $72^0$ C, 1 min<br><br>Typically, the reaction takes some 2 -3 h overall<br>Notes:<br><br>The optimal temperature for the annealing step will depend upon the primers used. The pH of the Tris-HCI buffer decreases markedly with increasing temperature. The actual pH varies between about 6.8 and 7.8 during the thermal cycle.<br>The time taken for each cycle is considerably longer than 3 min (0.5 + 1.5 + 1 min), depending upon the rates of heating and cooling between steps, but can be reduced considerably by using turbo systems.<br><br>The standed PCR does not efficiently amplify sequences much longer than about 3 kb. |

For a detailed analysis of the factors affecting the PCR, the reader should consult Pavlov et al. (2004). There are many substances present in natural samples (e.g. blood, feces, environmental materials) which can interfere with the PCR, and ways of eliminating them have been reviewed by Bickley and Hopkins (1999).

**4. Explain in detail the two methods involved in DNA sequencing.**

**Sequencing genomes:**

**High-throughput sequencing is an essential prerequisite for genome sequencing:**

As noted in the previous chapter genomes range in size from millions of base pairs to thousands of millions. Given that a single Sanger sequencing reaction allows 500-600 bases to be sequenced. It is clear that automation is essential. The theoretical sequencing capacity of an automated DNA sequencer is easy to calculate. For a four-dye slab system, the capacity is the number of sequencing reactions that can be loaded on each sample, times the number of gels that can be run at once, times the number of days this can be carried out per year. For a 24-channel sequencer the capacity calculated in this way is 2.7 million bases per year. To use just one such

instrument to sequence the human genome would require over 1000 years for single pass coverage and this clearly is not a practicable proposition.

To meet the demands of large-scale sequencing 96-channel instruments have become common place and at least one 384-channel instrument has been developed (shibata et al. 2000). By switching from slab gels to capillary systems, the electrophoresis run time is greatly reduced and nine runs can be achieved per 24 h period. various other improvements to the biochemistry of the sequencing reactions and the chemistry of the gel matriz mean that the read length can be extended from the usual 500-600 bases to 800 bases. As a consequence it now is possible to generate 1-6 million bases of sequence per machine per month (Meldrum 2000 b, Flkin et al. 2001) and Amersham Biosciences claim over 1 million bases in 8 hours or 2.8 million bases in 24 hours for their MegaBACE 4000 instrument. By way of comparison, 10 years ago the best that could be achieved was only (!) 40,000 bases per month (Fleischmann et al. 1995). Those laboratories engaged in sequencing large genomes have large numbers of sequencing machines and can generate millions of base sequences per day, e.g. in excess of 18 million (Elkin et al. 2001).

To achieve the levels of sequence data quoted above it is not sufficient to have sequencing instruments with a high capacity. There are many manipulative steps required before samples are loaded on gels in preparation for electrophoresis. For example DNA has to be isolated, fragmented, and then cloned or amplified. The DNA then has to be re-isolated and subjected to the various sequencing reactions described earlier. Each of these procedures is labour intensive. Not surprisingly, there now are machines that can automate every one of them and details of these machines have been provided by Meldrum (2000a).

**There are two different strategies for sequencing genomes:**

Two different strategies have been developed for the sequencing of whole genomes: the "clone-by-clone" approach and "whole – genome shotgun sequencing". The relative merits of the two strategies have generated much debate, particularly in relation to the sequencing of the human genome. An understanding of this debate, and the terminology used is necessary to appreciate some of the landmark genome sequencing publications. More recently, the debate has cooled with the adoption of hybrid strategies for the sequencing of genomes.

As noted earlier, Sanger sequencing has an accurate read length of 500-800 bases. One approach to sequencing a genome would be a shotgun approach in which a random selection of sequencing reads would be collected from a larger target DNA sequence. With sufficient over sampling (coverage) it should be possible to infer the complete genome sequence by piecing together the individual sequence reads. In practice, two problems will arise. First, the presence of dispersed repeated sequences will confound the sequence assembly. Second, assembly of sequences into contigs will be possible but there will be gaps between contigs that will need

closing by other means. The numbers of these problems should increase linearly with the size of the genome to be analyzed. Consequently, it was assumed that targets the size of cosmids represented the limit of the shotgun approach. Thus, whole genomes would be sequenced by first developing a set of overlapping cosmids that had been ordered by physical mapping. This is the "clone-by-clone" or "map-based" approach and it was used successfully to generate the complete genome sequence of saccharomyces cerevisiae (Goffeau et al. 1996) and the nematode Caenorhabditis elegans (C.elegans Sequencing Consortium 1998).

The view that cosmids represented the size limit for shotgun sequencing was destroyed when Fleischmann et al. (1995) determined the 1,830,137 bp sequence of the bacterium Hemophilus influenzae without prior mapping. The starting point was the preparation of genomic DNA, which then was mechanically sheared and size fractionated. Fragments between 1.6 and 2.0 kb in size were selected.

| **Some definitions used in genome sequencing projects** | |
|---|---|
| **Contig** An overlapping of clones or sequence reads that corresponds to a contiguous segment of the source genome | **Finished sequence** The complete sequence of a clone or genome with a defined level of accuracy and contiguity |
| **Coverage** The average number of times a genomic segment is represented in a collection of clones or sequence reads. Coverage is synonymous with redundancy | **Full – shotgun sequence** A type of pre-finished sequence with sufficient coverage to make it ready for sequence finishing |
| **Minimal tiling path** A minimal set of overlapping clones that together provides complete coverage across a genomic region | **Prefinished sequence** Sequence derived from a preliminary assembly during a shotgun-sequencing project |
| **Sequence-ready map** An overlapping bacterial clone map with sufficiently redundant clone coverage to allow for the rational selection of clones for sequencing | **Working draft sequence** A type of prefinished sequence with sufficient coverage (8 – to 10 – fold) to make it ready for sequence finishing |

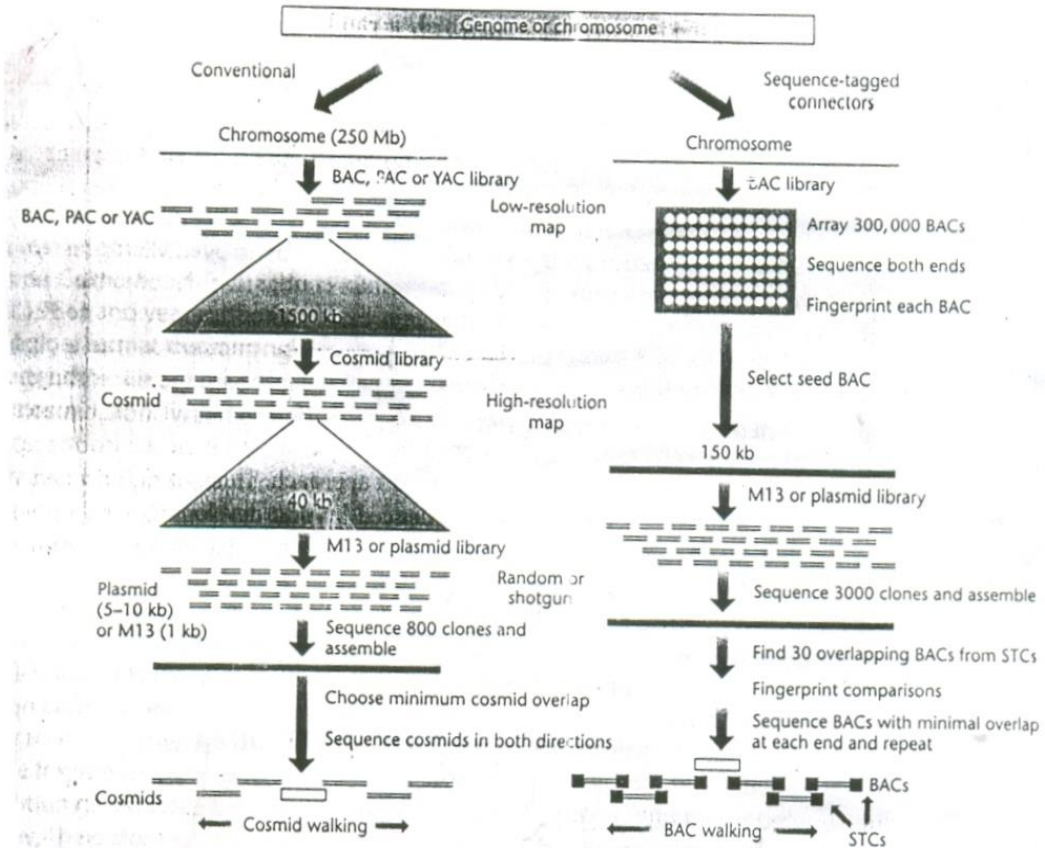



**Figure: Comparison of the conventional sequence approach with that proposed by Venter et al. (1996) (see text for full details). BAC, bacterial artificial chromosone: STC sequence-tagged connector. (Redrawn from Venter et al, 1996 with permission from Nature, © Macmillan Magazines Ltd.)**

This narrow range being chosen to minimize variation in the growth of clones. In addition, with a maximum size of only 2.0 kb the number of complete genes on a DNA fragment is minimized thereby reducing the chance of their loss through expression of deleterious gene products. The selected fragments were ligated to a sequencing vector and again size fractionated to minimize contamination from double-insert chimeras or free vector. Finally, all cloning was undertaken in host cells deficient in all recombination and restriction functions to prevent deletion and rearrangement of inserts.

Many other bacterial genomes have been sequenced using the same basic method as was used for the H. influenzae genome . however, it should be realized that much of the success of the method stems from the fact that bacterial genomes contain virtually no repeated DNA to complicate conting assembly. Nevertheless, the success of the method led Venter et al. (1996) to propose a method for sequencing the entire human genome without first constructing a physical map. Rather than using yeast artificial chromosomes (YACs) and cosmids it would use bacterial artificial chromosomes (BACs) which can accept inserts up to 350 kb in length. A BAC library is prepared which has an average insert size of 150 kb and a 15 fold coverage of the genome in

question. The individual clones making up the library are arrayed in microtiter wells for ease of manipulation. Starting at the vector-insert points, both ends of each BAC clone are then sequenced to generate 500 bases from each end. These BAC end sequences will be scattered approximately every 5 kb across the genome and make up 10% of the sequence. These "sequence-tagged connectors" (STCs) will allow any one BAC clone to be connected to about 30 others. In effect. STCs are simply very long STSs.

Each BAC clone would be fingerprinted using one restriction enzyme to provide the insert size and detect artificial clones by comparing the fingerprints with those of overlapping clones. A seed BAC of interest is sequenced and checked against the database of STCs to identify the 30 or so overlapping BAC clones. The two BAC clones showing internal consistency among the fingerprints and minimal overlap at either human genome could be sequenced with just over 20,000 BAC clones. This proposal was not universally accepted but subsequently a modification of it was used to sequence chromosome 2 of Arabidopsis thahana (Lin et al. 1999b) and the entire human genome (International Human Genome Sequencing Consortium 2001).

Whereas Venter et al. (1996) proposed a hierarchical shotgun approach in which end sequences (STCs) are used to provide long-range continuity across the genome, Weber & Myers (1997) proposed a total shotgun approach to the sequencing of the human genome. In this approach DNA would be sheared and size-selected before being cloned in E. coli. Cloned inserts would fall into two classes: long inserts of size 5-20 kb and short inserts of size 0.4 – 1.2 kb. Sequencing read lengths would be of sufficient magnitude so that the two sequence reads from the ends of the short inserts overlap. Both ends of the long inserts also would be sequenced and, because their spacing and orientation are known, they can be used to create a scaffold on which the short sequences can be assembled. This approach was not well received (Green 1997, Marshall & Pennisi 1998) but Myers et al. (2000) were able to show the validity of the approach by applying it to the 120 Mb euchromatic protion of the Drosophila melanogaster genome. Venter et al, (2001) then applied the method to the sequencing of the human genome. However, in both these cases, end sequences also were determined for 50 kb inserts in BACs to provide additional scaffolding information. Also in the case of the human sequence, any sequence – tagged sites (STSs) that were detected helpedd to locate the fragment in the context of the overall genome STS map. An indication of the workload associated with sequencing the human genome in this way can be obtained by consideration of the data in table.

Sequencing statistics for the shotgun sequencing of the human genome as undertaken by Venter et al. (2001). The DNA sequenced was derived from five different individuals (A, B, C, D, and F)

| Number of reads for different insert libraries | | | | | | Total number of base pairs |
|---|---|---|---|---|---|---|
| | **Individual** | **2 kbp** | **10 kbp** | **50 kbp** | **Total** | |
| No, of sequencing reads | A | 0 | 0 | 2,767,357 | 2,767,357 | 1,502,674,851 |
| | B | 11,736,757 | 7,467,755 | 66,930 | 19,271,357 | 10,464,393,006 |
| | C | 853,819 | 881,290 | 0 | 1,735,109 | 942,164,187 |
| | D | 952,523 | 1,046,815 | 0 | 1,999,338 | 1,085,640,534 |
| | F | 0 | 1,498,607 | 0 | 1,498,607 | 813,743,601 |
| | Total | 13,543,099 | 10,894,467 | 2,834,287 | 27,271,853 | 14,808,616,179 |
| Fold sequence coverage (2.9 Gb genome) | A | 0 | 0 | 0.52 | 0.52 | |
| | B | 2.20 | 1.40 | 0.01 | 3.61 | |
| | C | 0.16 | 1.17 | 0 | 0.32 | |
| | D | 0.18 | 0.20 | 0 | 0.37 | |
| | F | 0 | 0.28 | 0 | 0.28 | |
| | Total | 2.54 | 2.04 | 0.53 | 5.11 | |
| Fold clone coverage | A | 0 | 0 | 18.39 | 18.39 | |
| | B | 2.96 | 11.26 | 0.44 | 14.67 | |
| | C | 0.22 | 1.33 | 0 | 1.54 | |
| | D | 0.24 | 1.58 | 0 | 1.82 | |
| | F | 0 | 2.26 | 0 | 2.26 | |
| | Total | 3.42 | 16.43 | 18.84 | 38.68 | |
| Insert size (mean) | Average | 1951bp | 10,800bp | 50,715bp | | |
| Insert (SD) | Average | 6.10% | 8.10% | 14.90% | | |

**5. Describe the gene sequencing method by Sanger and Coulson's enzymatic chain terminator method.**

**Sanger and Coulson's Enzymatic/Chain Terminator Method:**

The enzymatic method of sequencing DNA also results in an array of fragments each of which differs from the ones larger and smaller than it by one nucleotide each. In this case the fragments are synthesized in four reaction mixtures, up to a particular nucleotide.

The method is based on synthesizing a labelled strand of DNA starting from a primer annealed to a template and terminating the synthesis at one of the four nucleotides. The terminator is a dideoxynucleotide that has no 3-OH to which another nucleotide may be added. The terminator is mixed with the corresponding deoxynucleotide in a proportion that allows the

terminator to be incorporated randomly in at least one of each of the sites for the particular nucleotide. The reaction mixture thus contains, at completion time, fragments that represent lengths of DNA from one identical end to each of the sites for a particular base. The gels are run, autoradiographed and interpreted as already described for the chemical sequencing method.

The enzymatic synthesis (or dideoxynucleotide chain terminator) method requires the following material:

(i) a short primer
(ii) a pure as DNA template
(iii) the four terminators (ddATP, ddGTP, ddCTP and ddGTP)
(iv) the four dNTPs of which some or one or two are P labelled in each reaction mixture; and
(v) a DNA polymerase without 5′ → 3′ exonuclease activity.

The reactions are carried out in four aliquots in small volumes (0.5 – 10.0ml) either in micropipettes or microfuge tubes. The polymerase is usually the Klenow fragment. Avian reverse trtanscriptase may be utilized with appropriate alterations in the buffer and proportions of dNTPs.

**The Primer:**

The length of the primer should be between 30 and 80 kb. The primer may be a nick-translated oligonucleotide or a short restriction fragment. The primer DNA is usually purified by fractionati ng on a passing the eluted DNA through a DEAE cellulose column. The primer fragments may be concentrated by cold ethanol precipitation and stored a $-20^0$ C in an appropriate solution.

**The Template:**

The DNA to be sequenced is usually cloned in a λ vector. The λ DNA has regions rich in cytosine. By adding uracil and guanidine, the strand with more Cs is made heavier than the other. The two strands are pooled in separate tubes and dialysed.

Messing's M13 phage method simplifies the procedure for obtaining ss DNA templates for enzymatic synthesis. This will be described later.

**The Dideoxynucleotide Terminators and Deoxynucleotides:**

In each reaction four deoxynucleotides and one dideoxynucleotide are mixed in a given proportion. One of the deoxynucleotides is α P-labelled. The dideoxynucleotide that is to

compete with its deoxynucleotide version is kept at a higher concentration with respect to the cognate deoxynucleotide. The chains to be stopped at As have dideoxyadenine as the terminator. Similarly, ddGTP, and ddTTP terminate chains in nucleotides with the corresponding bases.

**The Polymerase:**

Klenow fragment or avian reverse transcriptase are used, as both lack the 5′ – 6o 3′ – activity. Its absence prevents degradation of the primer from the 5′ end.

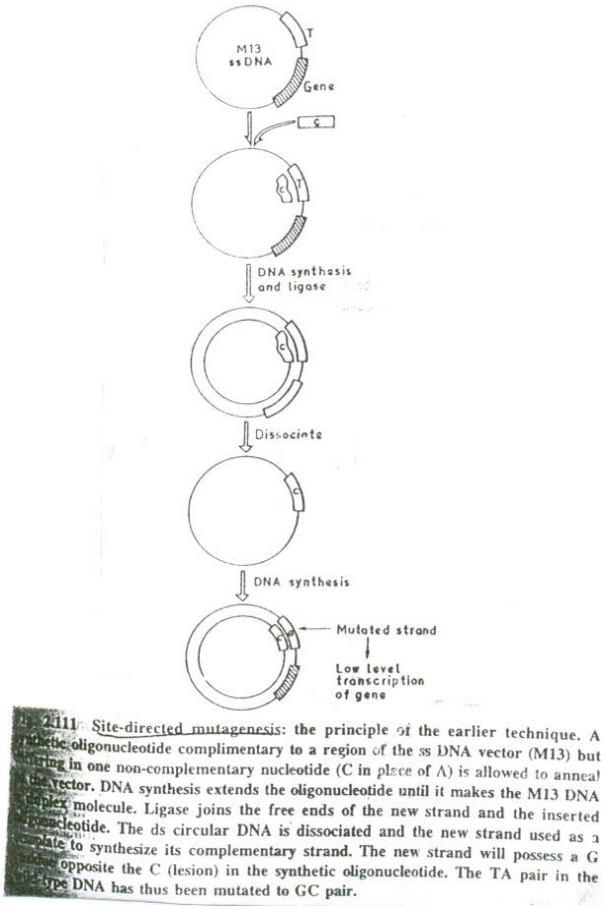
The primer is annealed to the template before the synthetic reaction is begun. The primer (ds DNA) and the ss DNA template are boiled in a mixture, and the latter transferred to an oven at $60^0$ C. (Boiling dissociates the primer strands, one of which hybridizes with the template at $60^0$ C). The operation is performed in small capillary tubes that are sealed after filling with the primer and template mixtures.

Four reaction mixtures are prepared with a unique terminator in each. The primer-template mixture is added to this together with the DNA polymerase and incubated at 18-$24^0$ C for 15 minutes. The reaction is stopped and the mixture loaded into a sequencing gel. The primer may be removed before placing the mixture in the gel by cleaving with a restriction enzyme (the site for which is restored at the 3′-end of the primer as strand synthesis begins).

**6. What is site directed mutagenesis How it is analyzed by PCR and explain with example?**

**(1) Oligonucleotide-directed Muragenesis:**

Oligonucleotide directed mutagenesis can install a desired alteration in any site in a stretch of DNA. The technique is based on the use of a synthetic oligonucleotide that is identical to (complementary) the site to be mutated, except in one or more bases. This synthetic short length of DNA is hybridized with a ss DNA vector (usually from ϕ M13), and acts as the primer which elongates to make the M13 template duplex. The two strands of this circular DNA are separated and the new strand used as a template to synthesize its partner.




2.11 Site-directed mutagenesis: the principle of the earlier technique. A synthetic oligonucleotide complimentary to a region of the ss DNA vector (M13) but differing in one non-complementary nucleotide (C in place of A) is allowed to anneal to the vector. DNA synthesis extends the oligonucleotide until it makes the M13 DNA a duplex molecule. Ligase joins the free ends of the new strand and the inserted oligonucleotide. The ds circular DNA is dissociated and the new strand used as a template to synthesize its complementary strand. The new strand will possess a G residue opposite the C (lesion) in the synthetic oligonucleotide. The TA pair in the wild-type DNA has thus been mutated to GC pair.

**Figure:**

The mismatched bases in the original principle of the earlier technique. A synthetic oligonucleotide complimentary to a region of the ss DNA vector (M13) but differing in one non-complementary nucleotide (C in place of A) is allowed to anneal to the vector. DNA synthesis extends the oligonucleotide until it makes the M13 DNA a duplex molecule. Ligase joins the free ends of the new strand and the inserted oligonucleotide. The ds circular DNA is dissociated and the new strand used as a template to synthesize its complementary strand. The new strand will possess a G residue opposite the C (lesion) in the synthetic oligonucleotide. The TA pair in the wild-type DNA has thus been mutated to GC pair.

Oligonucleotides are now matched with the correct complementary base(s). a mutation becomes established in this manner at the site of the bases that were different in the oligonucleotide. Almost all techniques based on this pioneering one (Hutchinson and associates. 1978) utilize a ss DNA as the template. Vectors from phage M13 and fd as well as plasmids (ss) of the pEMBL series are suitable for the purpose. All of these may be packaged as ss DNA – containing particles.

While the technique appeared to be quite simple in principle, the strategy was found to encounter certain snags during practice. The chief among these obstacles was the propensity of cells to repair mismatched regions of a DNA. The second difficulty was the pushing out of the oligonucleotide by the advancing 3′-end of the new DNA strand which had reached the 5′end of the oligonucleotide. Thirdly, the primer often hybridized to unintended regions of the template with which it had partial homology.

The first difficulty (of removal by repair or proofreading by DNA Pol I) is avoided by ensuring that the mutated strand (i.e., the one with the oligonucleotide) is methylated at appropriate bases. Endonucleases do not attack fully methylated or even hemimethylated (one strand) DNA, except for a very few known restriction enzymes (e.g., Dbn I). The mutated strand is maintained by ligating the end of the new strand with the oligonucleotide. Hybridization at incorrect locations is avoided by allowing only a limited region of the template for attaching the oligonucleotide primer.

These trouble-shooting jobs led to various improvements in the technique, so that it is now a reliable and routinely used one for site-specific mutagenesis. Kramer at al. (1984) developed the following elegantly simple protocol. The template was a ss M13 DNA containing a short insert at the region to be mutate. This rDNA is an RF molecule synthesized while in a methylation-deficient (dam) E. coli host. A non-recombinant M13 DNA (ss) is obtained by infection of a methylation – competent strain (dam). The non-methylated RF recombinant DNA is dissociated and mixed with the methylated M13 DNA, which has been linearized. Double-stranded hybrid. There is a gap in the latter opposite the insert in the former. The oligonucleotide is introduced to the left end of this gap allowed to synthesize a new strand to fill the gap. The nicks between the new strand and the existing M13 DNA are ligated. By these tactics,

1. The mutated strand is not vulnerable to attack by endonucleases (particularly the four GATC nucleosides in M13 DNA, which have been methylated in the N of the adenine),
2. The primer does not hybridize at unwanted regions, and
3. The length of new strand to be synthesized is quite short.

This last feature prevents possible non-completion of strand synthesis due to incorrect primer attachment etc., and saves on time and material.

The preceding ‘gapped duplex’ approach has been further refined and modified both by Kramer and associates and others. Instead of having an insert in the template DNA, a gap may be generated by removing the short Eco R1 – Hind fragment present in the M13 DNA. When this shortened DNA is hybridized with a full length DNA cleaved elsewhere, a gap occurs in one strand and in a single strand region opposite the gap in the other strand. Dalbadic –McFarland et al (1982) produced a similar gap in one strand by nicking the latter at a site and degrading the nicked strand using exonuclease III that had a 3′ $\rightarrow$ 5′ activity.

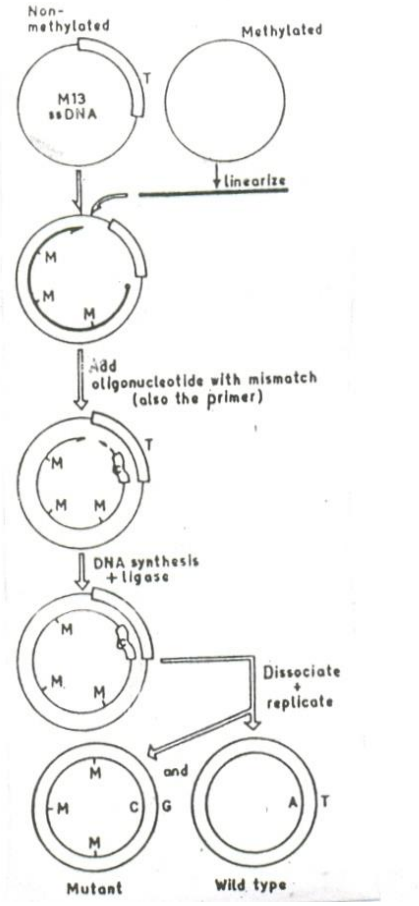



**Figure: The site – directed mutagenesis: An improved technique DNA to be mutagenized is cloned in an M13 vector. The ss DNA of the vector, without the insert, and which has been methylated at the C residues, is linearized and allowed to hybridize with the vector containing the insert. There will be a gap in the linear DNA opposite the insert DNA. An oligonucleotide with the desired mismatched base and a primer for DNA synthesis are added to the incomplete M13 duplex. DNA synthesis from the 3′ end of the methylated strand generates the complementary strand generates the complementary strand for the insert DNA (except for the mismatch, C, in place of A). Ligase circularizes the linear DNA. The ds DNA is dissociated and each strand allowed to replicate. Half the recovered circular DNAs will be of the wild-type, while the other half will be the mutants with the GC pair in place of the wild-type AT pair. This method limits DNA synthesis to a defined short region, feature which makes it less vulnerable to various defects including non-specific annealing of the primer.**

The approaches mentioned above also reduced the number of steps in producing the ds mutant DNA. After the filling up and ligating in the gap region, it may not be necessary to dissociate the two strands and use the mutated one to acquire a new complementary strand. As the template strand is unmethylated, the DNA repair enzymes favour correcting the mismatch in this strand rather than in the one with the introduced oligonucleotide. Such repair would result in a ds DNA that was mutant and without a heteroduplex region. However, all mismatches are not corrected with equal frequencey, in which event, it is better to rely on other means of selection.

One such strategy is to use selection by complementation. The strand with the oligonucleotide could carry a lacZ gene, while its partner carries the same but with an amber mutation. So, the template DNA will be able to grow only on E. coli with a suppressor for the amber mutation. The mutated DNA will grow on a wild-type (non-suppressor) E. coli and will transform a lac host strain (Kramer et al., 1984).

**7. Explain the method of sequencing of genes by chemical method of Maxam and Gilbert's method.**

**Maxam and Gilbert's Chemical Method:**

Maxam and Gilbert's chemical method depends on the production of four sets of fragments, each set with one identical end and the other ending in one particular nucleotide. That is in one aliquot, all fragments end in G, in another in A and so on. The identical end is labelled. These fragments are created by chemical reactions that cleave the DNA preferentially after a particular type of nucleotide.

The cleavage reaction consists of three steps: (1) modification of a base next to the base of interest (the one that is to terminate a fragment in one reaction mixture); (2) removal of the modified base, thus creating a gap in the base sequence; and (3) cleavage of the backbone at the site of the gap.

The DNA fragment to be sequenced is usually a restriction fragment, one end of which is labelled at only the 5′ or 3′ nucleotide or at both. The labelled DNA is dissociated and the single strands fractionated on a denaturing gel. If the DNA had lables on ends of each strand, the ds DNA is first cleaved into asymmetric sized fragments and each of the latter sequenced separately. The two strands of each fragment are sequenced individually. This is a check for the validity of the derived sequence; the results from the two strands should be complementary to each other. The ds strands are dissociated by mild alkali or heat.

The cleave is carried out in four reaction mixtures, with conditions in each aliquot for cleaving after one particular kind of nucleotide. The reaction conditions (i.e., reaction temperature, reaction time and concentrations of reagents) are chosen to allow only one base to be modified per DNA strand, or a very few in the entire strand. Since modification occurs at random in the various likely sistes, the reaction mixture contains the end fragments that possess the labelled end the terminate in one of the required positions. Say, the reaction is set for cleaving the DNA only after a G. The final collection of fragments will have every length of the DNA that ends in a G, and begins with the labelled nucleotide.

The fragments from each reaction mixture are fractionated and read as described earlier.

The four reactions that cut the DNA at G.G+A.T+C and C (and also if needed at A>C) are based on the following principles. In each case the reaction weakens the N-glycosidic bond between the ribose and the base moieties:

1. To cleave after A G, the DNA is methylated with dimethy1 sulphate and treated with alkaline piperidine that removes the modified G (N –methyldoxyguanidine) and cleaves the backbone at the site of the gap.
2. To cleave after an A, the DNA is methylated as done earlier, but treated subsequently with acid and piperidine. Only the backbone at the site of the N-3 methylated adenine is cleaved. Thus in one reaction mixture, fragments end in G. and in the other in A, depending on the treatment after methylation.

3. Both T and C are modified by hydrazine and removed by piperidine; the latter also breaks the DNA backbone at the site of the gap as in (1 ) and (2)

4. If hydrazine treatment is carried out in a background of IM NaCl, only C is affected, and the DNA cleaved at the gap left by removal of a C.

A fifth reaction, involving NaOH treatment followed by piperidine, is sometimes set up; the data from this merely confirming the positions of bases derived from the use of the first four reactions.

You will notice that there is no reaction specifically terminates a DNA at a T site. The positions of T's are determined by comparing the lanes in the gels for fragments cleaved at T and C and only at C. The bands that are not common to both lames are due to cuts made at Ts.

A minimum of 1 proomole of DNA is sufficient for sequencing by this method. Although factors such as the incubation time, the concentration reagents and the temperature of reaction are the parameters that control how many bases are to be modified by a reaction, in practice, incubation time is used as the controlling variable.

**8. Describe in detail on gene sequencing by automated methods as shot-Gun method and compute analysis.**

**Messing's Shot – Gun Method:**

Messing developed a rapid sequencing variation of the enzymatic method that bypasses the error-prone and laborious steps of isolating pure ss template DNA and primers specific for one of its ends. The method is said to be a 'shot-gun' one as the DNA to be sequenced is broken or cleaved into random fragments. Each fragment is cloned into an M13 RF vector and the latter

hybrid DNA used to transform JM101 strains of E. coli Each clone of cells will represent one of the fragments. The vectors used are of the M13p series, of which M13p series, of which M13p2 is the most widely used for sequencing.

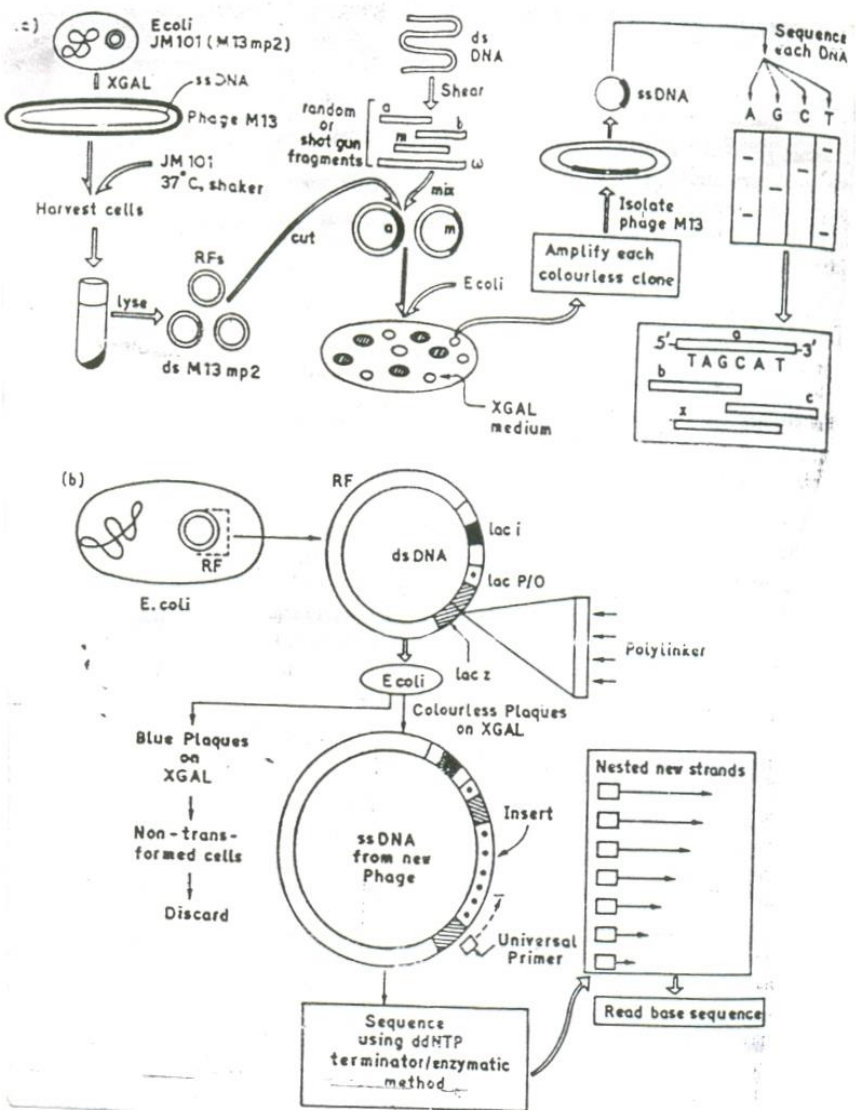



**Figure: Algorithm for Messing's shot-gum method of DNA sequencing. RF (replicative form) molecules of vector M13mp2 are extracted from the E. coli strain JM101 which harbours the vector, and are used to ligate randomly (shot-gun) sheared DNA molecules of the DNA to be sequenced. Cells transformed with the mixture of hybrid and unhybridized vectors are identified on XGAL media. The recombinant DNA – containing cells produce colourless plaques. Each colourless plaque is amplified, its hybrid ss DNA extracted from the phage M13 particles and each hybrid DNA sequenced by the enzymatic dideoxynucleotide termination method. Use is made of computer programs for melding the sequenced fragments into the integrated sequence.**

The M13p2 contains a part of the lac operon of E. coli, including the lacI gene (repressor), the promoter and the first section of the lacZ gene that encodes the α-region of β-galactosidase. A polylinker at an Econ R1 site between the 5th and 6th codons of lacZ is present in M13p2. the insert

DNA is cloned is cloned by cutting the polylinker with the appropriate restriction enzyme.

The RF molecule of the M13 phase is cleaved and the insert DNA ligated at this site. The hybrid RFs are used to transform the E. coli cells. After a few rounds of replication of the ds DNA, ss circular DNA (positive strand) are synthesized. These the growth medium. The DNA is released from the phage particles and used for sequencing. The template is already in an ss DNA stae, and all phages from the same plaque have template DNA in the same orientation. Other plaques may possess the same DNA fragment, but in the opposite orientation.

The defined nature of the M13p2 makes it easy to have a single primer for every sequencing project that uses this vector. The primer is complementary to a region on the vector, having its 3-OH end at the start of the cloning site. The common primer, available commercially, is known as the 'universal primer'. The latest one is a 15-base oligonucleotide that hybridizes just to the right of the polylinker (Norrander et al. 1983 Messing et al., 1981). A vector with the insert DNA disrupts the lacZ gene, so that recombinant cells show up as colourless colonies in an XGAL supplemented medium. The colourless colonies in an XGAL supplemented medium. The colourless colonies are picked up, grown to amplify the number of cells, and the DNA from them extracted. This DNA (circular, single-stranded) is utilized for sequencing, which is performed using the Sanger's chain terminator method.

The following is an outline of the steps followed in Messing's method of sequencing DNA:

1. Pure colonies of E. coli, JM101 and blue plaques of M13p2 phage streaked on JM101 in an XGAL plate, are isolated.
2. The phage is mixed with the lost cells in suspension and incubated with shaking (at $37^0$ C). Mature phages will be extruded in the surrounding fluid.
3. This mixture is added to a suspension of JM101 cells grown from a single colony innoculum. The mixture thus obtained is incubated with shaking at $37^0$ C for 4 to 6 hours.
4. The cells are harvested, lysed and the ds RF DNA extracted from them. This DNA is purified by CsCl gradient centrifugation at $15^0$ C (in three steps at 136 k, 90 k and 90 k x g respectively) for 24, 36 hours, respectively.
5. The DNA to be cloned and sequenced is cleaved or sheared, and the ends modified suitably.
6. Each fragment is ligated to an RF molecule and used to transform E. coli. The treated cells are plated with XGAL. Colourless plaques are picked out and transferred to individual plates to amplify the clones of each fragment. DNA isolated from each clone (phage) and used for sequencing. The ss DNA within each phage contains the vector DNA, the lacZ sequences, the insert DNA and the appropriate sequence at the beginning of the insert for annealing to the universal primer.
7. The DNA from each clone is sequenced using the chain terminator method.

**9. Write short notes on the following.**

i) Molecular Beacons

ii) RACE

iii) RFLP

iv) Taqman Assay

**Use of molecular beacons:**

In this assay an oligonucleotide probe (molecular beacon) is used, which consists of the target SNP sequence, with its two ends being complementary to each other. The two ends of the oligonucleotide are labelled just like the oligonucleotide probe used in TaqMan assay. The probe, when fails to form a duplex with the template DNA, generates a hairpin structure due to self-annealing of its two ends, thus quenching the reporter. But when the probe anneals with the template, it gets linearized, thus separating the reporter from quencher and permitting fluorescence signal. The fluorescence signals, both in TaqMan and molecular beacon can be detected by appropriate sensing devices.

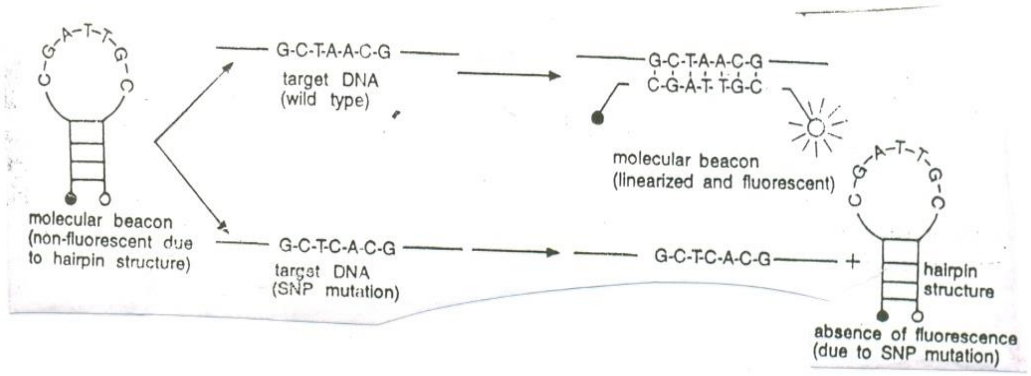



**Figure: Use of 'molecular beacon' as a probe for detection of SNPs.**

**Full – length cDNA cloning is facilitated by the Rapid Amplification of cDNA Ends (RACE):**

Another way to address the problem of incomplete cDNA sequences in libraries is to use a PCR-based technique for the rapid amplification of cDNA ends (RACE) (Frohman et al. 1988). Both 5′ RACE and 3′ RACE protocols are available, although 3′ RACE is usually only required if cDNAs have been generated using random primers. In each case only limited knowledge of the mRNA sequence is required. A single stretch of sequence within the mRNA is sufficient, so an incomplete clone from a cDNA library is a good starting point. From this sequence, specific primers are chosen which face outwards, and which produce overlapping cDNA fragments. In the two RACE protocols, extension of the cDNAs from the ends of the transcript to the specific primers is accomplished by using primers that hybridize either at the natural 3′ poly(A) tail of the mRNA, or at a synthetic poly (dA) tract added to the 5′ end of the first –strand cDNA. Finally,

after amplification, the overlapping RACE products can be combined if desired, to produce an intact full-length cDNA.

Although simple in principle, RACE suffers from the same limitations that affect conventional cDNA cloning procedures. In 5′ RACE, for example, the reverse transcriptase may not, in many cases, reach the authentic 5′ end of the mRNA, but all first-strand cDNAs whether full length or truncated, are tailed in the subsequent reaction, leading to the amplification of a popultion of variable-length products. Furthermore, as might be anticipated, since only a single specific primer is used in each of the RACE protocols, the specificity of amplification may not be very high. This is especially problematical where the specific primer is degenerate. In order to overcome this problem, a modification of the RACE method has been devised which is based on using nested primers to increase specificity (Frohman & Martin 1989). Strategies for improving the specificity of RACE have been reviewed (Schaefer 1995, Chen 1996).

**AFLPs resemble RFLPs and can be detected in the absence of sequence information:**

Amplified fragment length polymorphism (AFLP) is a diagnostic fingerprinting technique that detects genomic restriction fragments and in that respect resembles the RFLP technique (Voss et al. 1995). The major difference is that PCR amplification rather than southern blotting is used for the detection of restriction fragments. The resemblance to the RFLP technique was the basis for choosing the name AFLP. However, the name AFLP should not be used as an acronym because the technique detects presence or absence of restriction fragments and not length differences. The AFLP approach is particularly powerful because it requires no previous sequence characterization of the target genome. For this reason it has been widely adopted by plant geneticists. It also has been used with bacterial and viral genomes (voss et al. 1995). It has not proved useful in mapping animal genomes because it is dependent on the presence of high rates of substitutional variation in the DNA; RFLPs are much more common in plant genomes compared to animal genomes.

The AFLP technique is based on the amplification of subsets of genomic restriction fragments using the PCR. To prepare an AFLP template, genomic DNA is isolated and digested simultaneously with two restriction endonucleases, EcoRI and MseI. The former has a 6-bp recognition site and the latter a 4-bp recognition site. When used together these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1 kb) for separation on denaturing polyacrylamide gels. Following heat inactivation of the restriction enzymes the genomic DNA fragments are ligated to EcoRI and MseI adaptors to generate template DNA for amplification. These common adapter sequences flanking variable genomic DNA sequences serve as primer binding sites on the restriction fragments. Using this strategy it is possible to amplify many DNA fragments without having prior sequence knowledge.

The PCR is performed in two consecutive reactions. In the first pre-amplification reaction, genomic fragments are amplified with AFLP primers each having one selective nucleotide. The

PCR products of the pre-amplification reaction are diluted and used as a template for the selective amplification using two new AFLP primers which have two or three selective nucleotides.

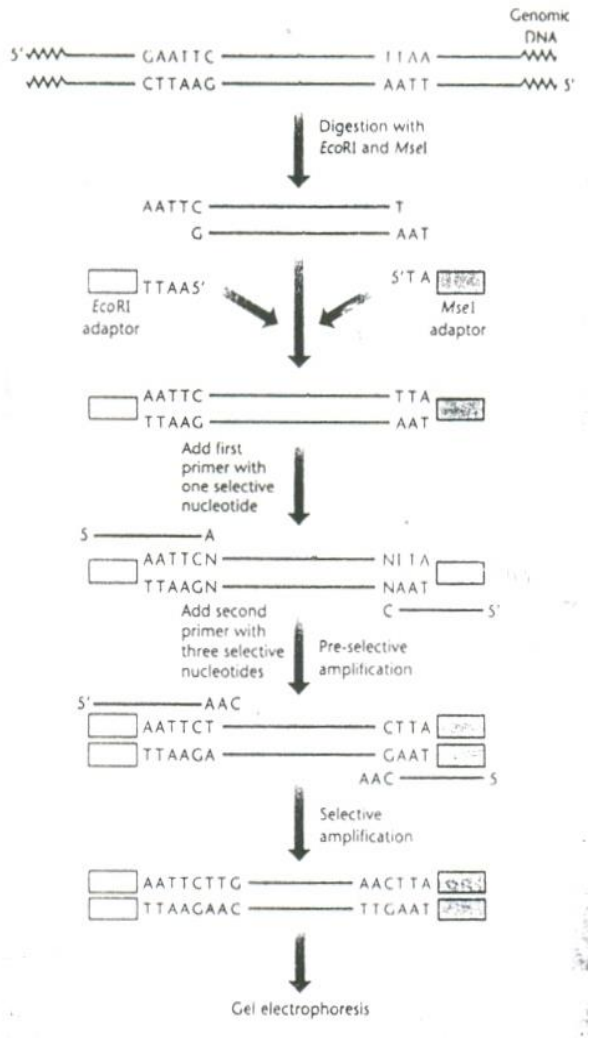



**Figure: Principle of the amplified fragment length polymorphism (AFLP) method**

In addition, the EcoRI selective primer is radiolabeled. After the selective amplification the PCR products are separated on a gel and the resulting DNA fingerprint detected by autoradiography.

The AFLP technique will generate fingerprints of any DNA regardless of the origin or complexity. The number of amplified fragments is controlled by the cleavage frequency of the rare cutter enzyme and the number of selective bases. In addition, the number of amplified bands may be controlled by the nature of the selective bases. Selective extension with rare di-or trinucleotides will result in a reduction of the number of amplified fragments.

The AFLP technique is not simply a fingerprinting technique; rather, it is an enabling

technology that can bridge the gap between genetic and physical maps. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited.

**Tagman assay:**

In this assay an oligonucleotide probe is labelled with a fluorescent reporter molecule (e.g. FAM or TET) at 5′ end and a quencher (e.g. TAMRA) at the 3′ end. The TaqMan probe after hybridization to the template DNA is degraded at its 5′ end during extension phase of PCR due to exonuclease activity of Taq polymerase enzyme (TaqMan polymerase), so that the reporter is released leading to a rise in fluorescense signal. However, when due to the presence of an SNP, the probe mismatches with the template leading to a failure in duplex formation, no such degradation at 5′ end of the probe is possible and there is no rise in fluorescence signal. Different combinations of reporters and quenchers, will also permit multiplexing so that as many as six SNPs can be scored in a single PCR reaction.

**10. Write short notes on following.**

**i) RACE – PCR**

**ii) Nested PCR**

**RACE- PCR:**

This method by which the 3′ and 5′ends of a cDNA are amplified using a small stretch of known sequence within a gene. This small stretch can be derived by back translation from the amino acid sequence of the protein or can be from DNA sequence homology with other members of the gene family. The procedure was made more powerful by the use of nested primers, which reduce non-specific amplification and ensures the production of relatively pure specific product. For 3′ RACE, a reverse transcriptase primer that contains an oligo dT sequence linked to an adapter sequence (which may be long enough to permit binding of two nested primers or shorter for single primer binding) is used to prime the first strand cDNA synthesis. Primary amplification is then performed with gene-specific primer (GSP-1) and the outer primer R1. a small fraction of the first amplification is then used for secondary amplification that is nested gene-specific primer (GSP-2) and inner primer R2.

For 5′ RACE, cDNA is prepared with either a specific primer or random hexamers and then the newly synthesized cDNA strand has homo polymeric tail (rg. A residues) added to its 3′ end using terminal transferase. The complementary reverse transcription anchor primer is then used to generate second strand cDNA. This ds DNA can then serve as the template for PCR reaction as described for 3′ RACE.

**Nested PCR:**

The sensitivity and specificity of PCR can be increased by using nested PCR (nPCR). In nPCR, two separate amplifications are used. The first uses a set of primers that yields a large product, which is then used as a template for the second amplification. The second set of primers anneal to sequences within the initial product producing a second smaller product. The primers for the second round of amplification are either both different from the first set and both located within the amplified DNA region. If only one of the second round primers is located within the amplified region and is used together with one of the first round primers, it is termed as 'semi-nested PCR'. Nested PCR increases the specificity of the reaction because formation of the final product the need for verification of the PCR product by blotting, restriction digestion or sequencing. The second set of primers also serves to verify the specificity of the first product.

Nested PCR primers are ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. The sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using the nested PCR method. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products. This is because, after the first round of PCR the non-specific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity.

## UNIT – V

## PART – A

**1. Write the principles involved in gene-gun technique.**

Gene-gun is otherwise called as particle bombardment, which can be used to transfer of genes in plants. In this system, heavy microparticles (tungsten or gold) coated with the DNA of interest are accelerated to a very high initial velocity (1,400 ft. per sec). These microprojectiles, each normally 1-3 μm in diameter are carried by a microprojectile, or the 'bullet' and are accelerated into living plant cells, so that they can penetrate cell walls of intact tissue. The acceleration is achieved either by an explosive charge or by using shock waves initiated by a high voltage electric discharge.

**2. Give two examples of plant viruses that can be used as plant expression vectors.**

These two groups of plant viruses that infect plants, which are used as a vectors for plant gene transfer.

i) Caulimoviruses (CaMV) – Cauliflower mosaic viruses
- The 8 Kb double stranded DNA genome.

ii) Geminiviruses – Bipartite genome;
(geminate – twin virions)
- dsDNA virus

**3. What is T – DNA? Give the significance of selectable markers included in T-DNA.**

Agrobacterium tumefaciens contain Ti – plasmid which includes T-DNA region. T – DNA is called as Transfer DNA region contain 23 to 25 kb. T-DNA carries genes that confer both unregulated growth and the ability to synthesize opines up on the transformed plant tissue. The T-region in both octopine and nopaline plasmids is flanked by a direct repeat of 25 base pairs, which is known as border sequences. The border sequences are involve in transfer process. The Right and Left border sequences are involve in T-DNA transfer process. Among that, the right border sequences are having high efficiency in transfer of T-DNA.

**4. What is Embryo sexing? Give its application.**

The embryo sexing involves in the microinjection of DNA into male pronucleus and subsequent injection into fertilized female egg nucleus. This is a gene transfer method used in animal cloning method. Just after fertilization, the small egg nucleus was injected with the male pronucleus carrying targeted DNA with fine needle. Then the embryo is cultured in vitro to the

morula stage and then transferred to pseudo pregnant foster mothers. The application is to produce transgenic animals including transgenic mice.

**5. What are Edible vaccines? Give 2 examples.**

Edible vaccines are known to produced by plants, which are orally administered by eating plant parts. Plants have been explored as a cheap, safe and efficiency production systems for subunit vaccines, with the added advantage of oral consumption. Therefore, it is limiting the need for processing and purification. For example, expression of a surface antigen from streptococcus mutans in tobacco in the earliest stage.

e.g. 1. Tomato - for rabies glycoprotein
2. Potato - Cholera antigen
3. Lettuce - Cholera antigen

**6. Give four examples of DNA viruses as expression vectors.**

1. Cauliflower mosaic virus (CaMV) - DNA viruses
2. Gemini viruses - DNA viruses
3. African cassava mosaic viruses - DNA
4. Tomato golden mosaic virus - DNA
5. Maize streak virus - DNA

**7. What are the gene transfer methods available for plants?**

i. Agrobacterium – mediated gene transfer method through Ti – plasmid
ii. Direct DNA transfer through transformed protoplasts.
iii. Direct DNA transfer through particle bombardment or by gene – gun method.
iv. Other Direct method is by Electrophoration.
v. Direct DNA transfer through chloroplast transformation.

**8. What are knockout mice? How they are established?**

Knockout mice are produced by gene manipulation technology in which the particular gene is removed in embryonic cell and the mice produced are either lost the character or dies after sometime. Cells are selected that have had the gene removed (knock out), and are then implanted into blastocysts (early embryos). The embryonic cell matures, where they form part of the new animal. This animal (mice) is chimera.

**9. What is good about chloroplast transformation?**

The chloroplast is used as a target for genetic manipulation because thousands of chloroplasts are present in photosynthetic cells and this can result in levels of transgene expression up to 50 times higher than possible using nuclear transformation. Transgenes integrated into chloroplast DNA do not appear to undergo silencing or suffer from position effects that can influence the expression levels of transgenes in the nuclear DNA chloroplast transformation also provide natural containment method for transgenic plants. Since the transgenic cannot be transmitted through the pollen.

**10. What is plant promoter? How could you increase the activity of plant promotor?**

The promoter region of the plant gene of target to be transferred for cloning is involve in expression is called plant promoter. We could increase the activity of plant promotor as;

1. by using naturally occurring inducible promotors
2. by using promoter inducible proteins (activator)
3. by use of hormones (steroids)
4. by using certain inducer chemicals

**11. What is nuclear cloning?**

The nuclei of the animal species can be isolated and reprogrammed at appropriate circumstances in cytoplasm of the egg to recapitulate the development. Nuclear transplantation can be used to generate clones of animals with the same genotype by transplanting many somatic nuclei from the same individual into a series of enucleated eggs. This allows animals with specific and desirable traits to be propagated. Application is mainly in farming.

**12. What are Plantibodies?**

Plantibodies are the antibodies produced from plants against certain pathogenic microorganism which are used as a health care product. For example plants producing recombinant sIgA against the oral pathogen streptococcus mutans have been generated and these plant derived antibodies are called as 'Plantibodies'. This being commercially developed as the drug CaroR$_x$™ , marketed by planet Biotechnology Inc, US.

**13. What are vaccine candidates? Give 4 example.**

The edible vaccines against certain diseases caused by microorganisms are generated (or) produced by plants known as candidate vaccines.

E.g. 1. Bovine herpes virus (type I) glycoprotein produced in Tobacco leaves.
2. Bacillus anthracis protective antigen in Tobacco leaves.

3. V. Cholera toxin, B subunit in potato tubers.
4. Simian / Human immunodeficiency virus (SHIV) 89.6 $p^{Tat}$ from potato tubers.

**14. Give any 4 example of therapeutic proteins expressed in GM plants.**

1. Epidermal growth factors, human in tobacco transgenic for wound repair and cell proliferation
2. Erythropoitin, human in Tobacco suspension cells for Anemia.
3. Interlukin-2 in potato for Antiviral and Anticancer.
4. α-Interferon, human in Rice, Turnip for Hepatitis.

**15. What are the strategies involve in gene transfer to animal cells?**

i. Biological mechanism involve through using viral vector. The delivery mechanism is called as 'transduction'.
ii. Delivery of gene in animals through bacterial vectors termed as bactofection – i.e. through plasmid vectors.
iii. Chemical transfection of gene transfer through synthetic complex make electric charges and promote uptake of DNA.
iv. Physical transfection method involve by exploiting physical force to introduce DNA directly. E.g. microinjection, particle bombardment.

**16. Mention the physical transfection mechanism involved in gene transfer in animals.**

i. Microinjection through fine needle to embryos.
ii. Particle bombardment through gene – gun by applying high pressure force.
iii. Electrophoration through applying high voltage.
iv. Ultra sound by using ultrasonic waves make entry of cell membrane.

**17. Give any 4 commonly used dominant selectable marker genes in animals.**

i. as – Asparagine synthetase (E-Coli) toxic glutamine analog albizzin.
ii. his D - Hitidinol dehydrogenase (salmonella typimurium) confers resistance to histidinol.
iii. hpt - Hygromycin phosphotransferase (E-Coli) confers resistance to hygromycin-B.
iv. trpB - Tryptophan synthesis (E. Coli) confers resistant to in dol.

**18. Give any 4 viral vectors system involve in gene transfer in animals.**

Virus particles have a natural ability to adsorb to the surface of cells and gain entry, and this can be exploited to deliver recombinant DNA into animal cells. Main examples are:

i. Adenoviruses vectors – DNA viruses, 36 kb size – short term transgene expression.
ii. Bacilovirus vectors – ds DNA viruses. Has High level transgene expression.
iii. Herpes viruses – large ds DNA, promote long term transgene expression.
iv. Retrovirus vectors, RNA viruses, integrate efficiently into the host cell genome.

**19. Write short notes on vaccinia and other pox virus vectors.**

Vaccinia and other pox viruses are widely used for vaccine delivery. Vaccinia virus is closely related to variola virus, the agent responsible for small pox. A world-wide vaccination program using vaccinia virus resulted in the elimination of small pox as an infectious disease. The success of the program raised hopes that recombinant vaccinia viruses, carrying genes from other pathogens, could be used as live vaccines for other infectious diseases.

The pox viruses have a complex structure and a large double-stranded linear DNA genome (up to 300 kb).

**20. What is mean by transgenic organisms?**

The desired gene of interest from Host organisms are isolated and purified for transformation into the test organism (or) experimental organisms, in which the genetic pattern is modified and expressed. Those genes transferred or genetically modified organisms are called either transgenic animals (or) transgenic plants. Transgenics are having advantages in characteristic features over the normal one.

**21. What is mean by Gain-of-function diseases? Give suitable examples.**

The gain-of-function disease defines as it caused by a dominantly acting allele and can be modeled simply by adding that allele to the normal genome. e.g. by microinjection into eggs. Some of the earliest transgenic disease models were mice predisposed to particular forms of cancer because the germ line contained exogenously derived oneogenes.

e.g. Gerstmann – Straussler – Scheinker (GSS)

i. Syndrome a neurodegenerative disease,
ii. Alzheimer's disease which is over expressed by amyloid precursor protein.

**22. Write short notes on gene therapy.**

Gene therapy can be used to treat diseases caused by mutations in the patients' own DNA (inherited disorders, cancers) as well as infection diseases, and is particularly valuable in cases where no conventional treatment exists or that treatment is inherently risky. The strategies are;

i. gene augmentation therapy (GAT), where DNA is added to the genome with the aim of replacing a missing gene product.
ii. gene targeting to correct mutant alleles.
iii. gene inhibition therapy such as antisense RNA expression or the expression of expression of intracellular antibodies to treat dominantly acting diseases.
iv. the targeted ablation of specific cells. In these, only somatic cells can be used as a targets.

**23. What is cystic fibrosis (CF)? How will you treat the disease?**

Cystic fibrosis (CF) is a disorder which predominantly affects the lungs, liver, and pancreas. This is caused by the loss of a cAMP regulated membrane - spanning chlorine channel. This results in in electrolyte imbalance and the ammulation of mucus, often leading to respiratory failure.

The CF can be corrected by introducing a functional copy of the gene. Indeed, epithelial cells isolated from CF patients can be restored to normal by transfecting them with the cloned cystic fibrosis transmembrane regulator (CFTR) cDNA.

**24. Give any 4 applications of transgenic technology of plants.**

i. To produce Drought resistance in plants.
ii. To produce insert resistant in plants.
iii. Resistance of plants against Bacterial diseases.
iv. To enhance the plants to produce mere vitamin E.
v. To produce herbicide resistant transgenic plants.
vi. To production of vitamin A in cereals (Rice).

**25. What are the applications available for use of genetically modified mice?**

Transgenic mice can be addressed to many aspects of gene function and regulation. It is also used as a model for human diseases and the production of valuable pharmaceuticals. E.g. gene knocks out, but gain of function models. Analysis of thymidine kinase (TK) by mouse maltothionine-1 (MMT) gene.

**26. Give any 4 therapeutic products produced by animal cell culture.**

i. Erythropoietin - α, for Anemia
ii. Erythropoietin - β, for Anemia
iii. hGH – Human growth deficiency
iv. Somotropin – Chronic renal insufficiency, Turner's syndrome
v. Monoclonal Antibodies for therapeutic as anti-lipopolysachacide
vi. Vaccines – Malaria vaccines.

## PART – B

**1. What are the major strategies for gene transfer involved in plant cells?**

**There are four major strategies for gene transfer to plant cells**

As is the case for animal cells, gene transfer to plants can be achieved through four types of mechanism – viral transduction, bacterial gene delivery, and chemical and physical direct DNA transfer. Unlike the situation in animals, where bacterial gene transfer is a relatively new development, Agrobacterium-mediated transformation is the most widely used transformation method, particularly for dicotyledonous plants. Physical methods are the next most popular, especially particle bombardment for the transformation of monocotyledonous plants such as cereals. Chemical transfection methods are little used, and are compatible only with protoplasts, which behave in many ways analogously to animal cells. Many of the techniques used to transfect animal cells can therefore be applied to plant protoplasts, e.g. calcium phosphate transfection. All three of the above methods can be used for either transient expression or stable transformation. Another major difference between gene-transfer strategies in animal and plant cells is that no known plant viruses integrate their genetic material into the plant genome as part of the natural infection cycle. Therefore, plant viruses are used as episomal vectors rather than for stable transformation. However, while stable transformation cannot be achieved, plant viruses often cause systemic infections resulting in the rapid production of high levels of recombinant protein throughout the plant, and they can be transmitted through normal infection routes, or by grafting infected scions onto virus-free hosts.

**Agrobacterium-mediated transformation**

**Agrobacterium tumefaciens is a plant pathogen that induces the formation of tumors**

Gene transfer from bacteria to plants occurs naturally and is responsible for crown gall disease. This is a plant tumor that can be induced in a wide variety of gymnosperms and dicotyledonous angiosperms (dicots) by inoculation of wound sites with the Gram-negative soil bacterium A. tumefaciens. The involvement of bacteria in this disease was established nearly 100 years ago by Smith & Townsend (1907). It was subsequently shown that the crown gall tissue represents true oncogenic transofmration, since the undifferentiated callus can be cultivated in vitro even if the bacteria are killed with antibiotics, and yet retains its tumorous properties. These properties include the ability to form a tumor when grafted onto a healthy plant, the capacity for unlimited growth as a callus in tissue culture even in the absence of phytohormones necessary for the in vitro growth of normal cells, and the synthesis of opines, such as octopine and nopaline, which are unusual amino acid derivatives not found in normal plant tissue.

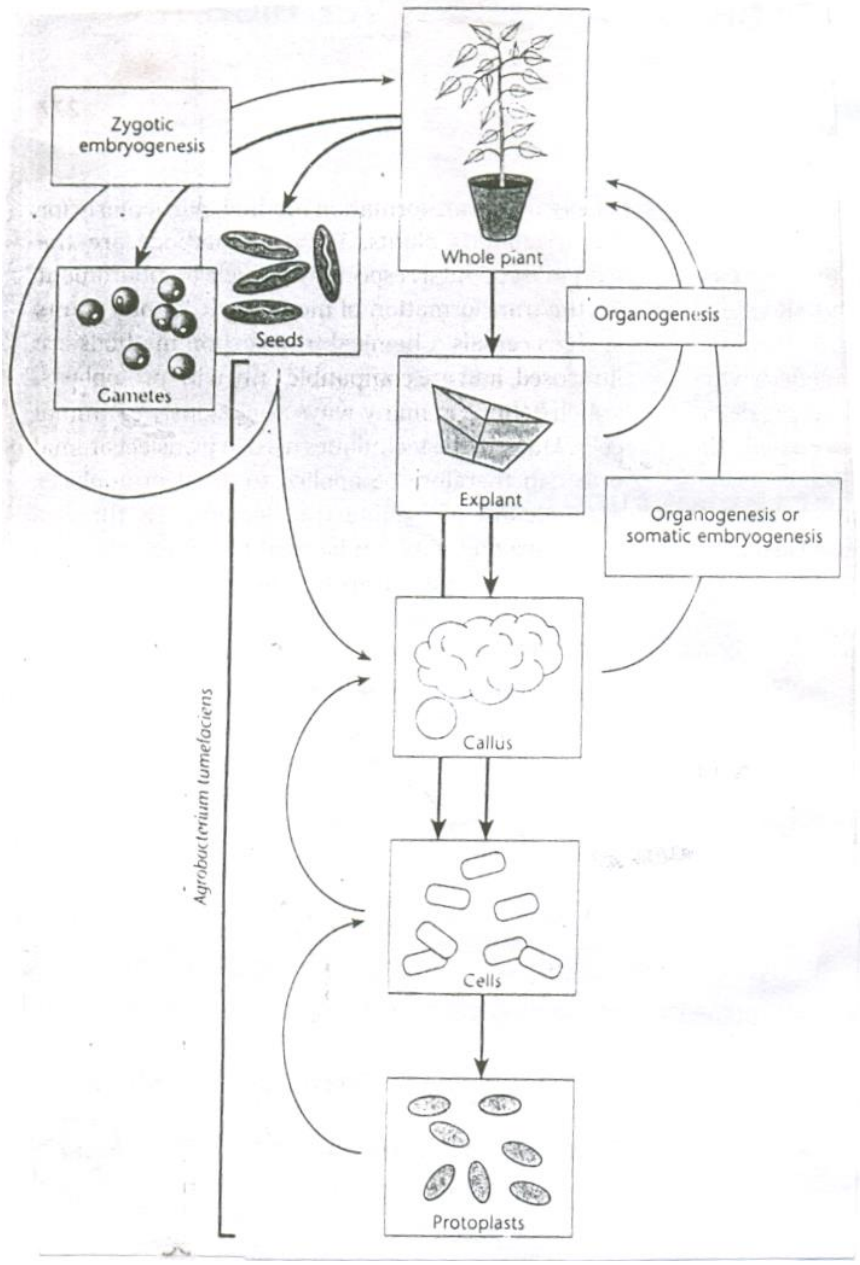



**Fig. Depending on species plant tissues are extremely phable and can be easily interconverted and regenerated in culture. This diagram shows some of the ways in which this flexibility can be exploited to facilitate gene transfer and the creation of transgenic plants.**

The metabolism of opines is a central feature of crown gall disease. Opine synthesis is a property conferred upon the plant cell when it is colonized by A. 'tumfaciens. The type of opine produced is determined not by the host plant but by the bacterial strain. In general, the bacterium induces the synthesis of an opine that it can catabolize and use as its sole carbon and nitrogen source. Thus, bacteria that utilize octopine induce tumors that synthesize octopine, and those that utilize nopaline induce tumors that synthesize nopaline (Bomhoff et al. 1976, Montaya et al. 1977)
**The ability to induce tumors is conferred by a Ti-plasmid found only in virulent**

*Agrobacterium strains*

Since the continued presence of Agrobacterium is not required to maintain plant cells in their transformed state, it is clear that some "tumor-inducing principle" is transferred from the bacterium to the plant at the wound site. Zaenen et al. (1974) first noted that virulent strains of A. tumefaciens harbor large plasmids (140-235 kbp), and experiments involving the transfer of such plasmids between various octopine – and

**Fig. Crown gall on blackberry cane**

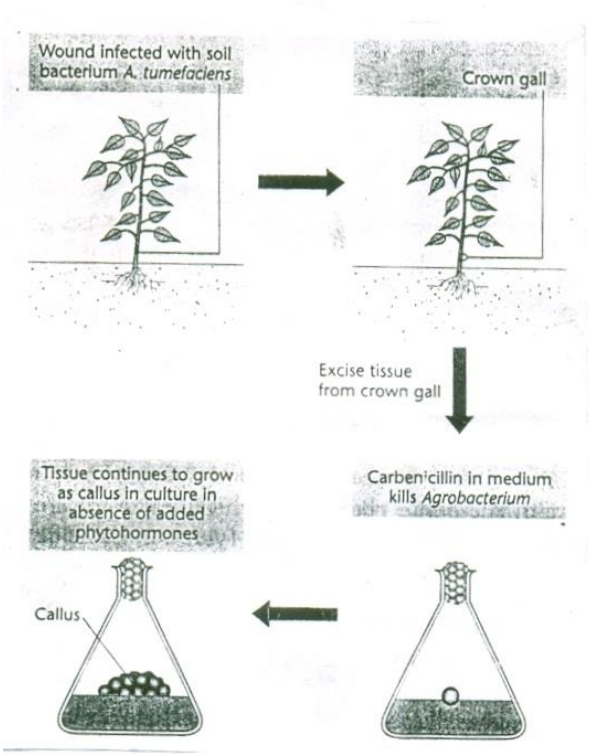



**Fig. A tumefaciens induces plant tumors, but is not required for the continuous proliferation of those tumors.**

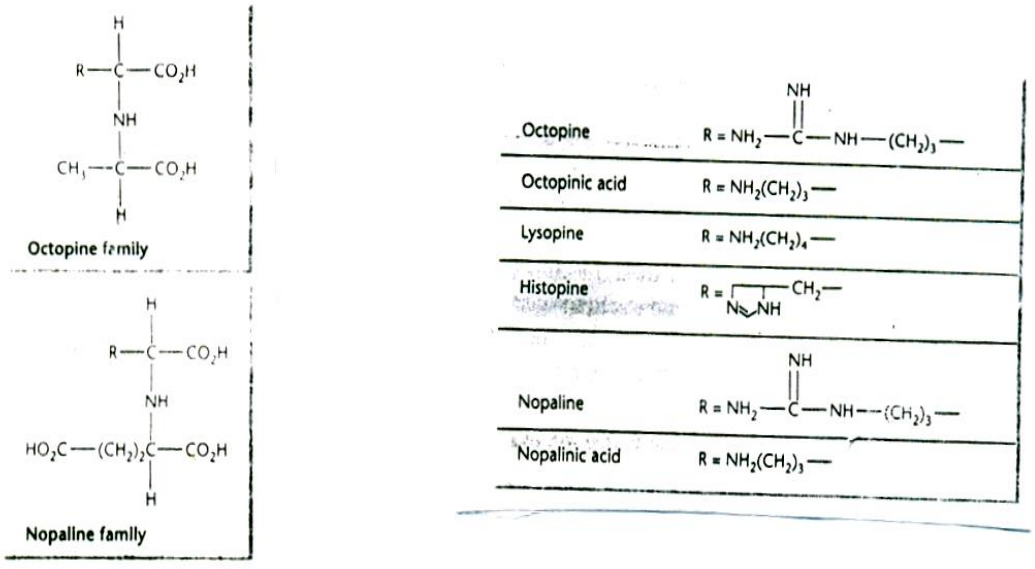



**Fig. Structures of some opines**

nopaline-utilizing strains soon established that virulence and the ability to use and induce the synthesis of opines are plasmid-borne traits. These properties are lost when the bacteria are cured of their resident plasmid (Van Larbeke et al. 1974. Watson et al.1975) but acquired by a virulent

strains when a virulence plasmid is reintroduced by conjugation (Bomhoff et al. 1976, Gordon et al. 1979). The plasmids therefore became known as tumor-inducing plasmids (Ti-plasmids).

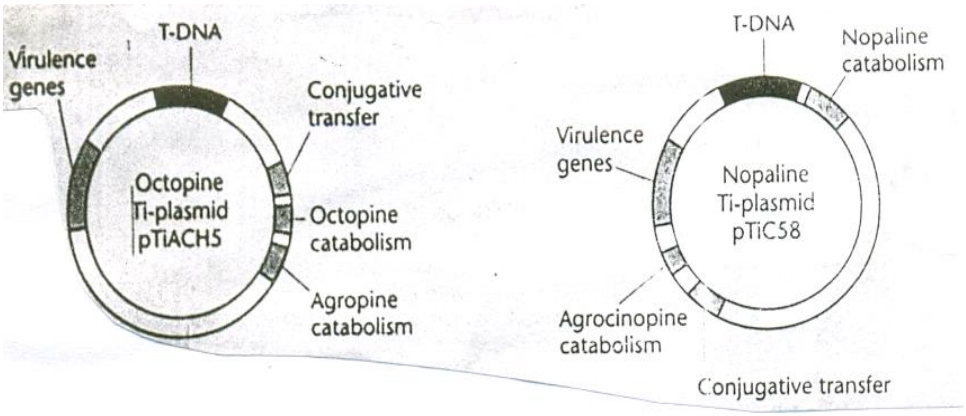



**Fig. Ti-plasmid gene maps**

Ti-plasmids specify the type of opine that is synthesized in the transformed plant tissue and the opine utilized by the bacterium. Plasmids in the octopine group are closely related to each other while those in the nopaline group are considerably more diverse. Between the groups, there are four regions of homology, including the genes directly responsible for tumor formation (Drummond & Chilton 1978, Engler et al. 1981). It should be noted that the presence of a plasmid in A. tumefaciens does not mean tat the strain is virulent. Many strains contain very large cryptic plasmids that do not confer virulence, and in some natural isolates a cryptic plasmid is present together with a Ti-plasmid.

**Direct DNA transfer to plants**

**Transgenic plants can be regenerated from transformed protoplasts**

Protoplast transformation has much in common with the transfection of animal cells. The protoplasts must initially be persuaded to take up DNA from their surroundings, after which the DNA integrates stably into the genome in a proportion of these transfected cells. Gene transfer across the protoplast membrane is promoted by a number of chemicals, of which polyethylene glycol has become the most widely used due to the availability of simple transformation protocols (Negrutiu et al. 1987). Alternatively, DNA uptake may be induced by electroporation (Shillito etal. 1985). As with animal cells, the introduction of a selectable marker gene along with the transgene of interest is required for the identification of stable transformants. This can be achieved using plasmid vectors carrying both the marker and the transgene of interest, but the use of separate vectors also results in a high frequency of co-transformation (Schocher et al. 1986). Putative transformants are transferred to selective medium where surviving protoplasts regenerate their cell walls and commence cell division, producing a callus. Subsequent manipulation of the culture

conditions then makes it possible to induce shoot and root development, culminating n the recovery of fertile transgenic plants. The major limitation of protoplast transformation is not the gene-transfer process itself, but the ability of the host species to regenerate from protoplasts. A general observation is that dicots are more amenable than monocots to this process. In species where regeneration is possible, an advantage of the technique is that protoplasts can be cryopreserved and retain their regenerative potential (DiMaio & Shillito 1989).

The first transformation experiments concentrated on species such as tobacco and petunia in which protoplast-to-plant regeneration is well documented. An early example is provided by Meyer et al. (1987), who constructed a plasmid vector containing the nptII market gene, and a maize cDNA encoding the enzyme dihydroquercetin 4-reductase, which is involved in anthocyanin pigement biosynthesis. The transgene was driven by the strong and constitutive CaMV 358 promoter. Protoplasts of a mutant, white-coloured petunia strain were transformed with the recombinant plasmid by electroporation and then selected on kanamycin-supplemented medium. After a few days, surviving protoplasts had given rise to microcalli, which could be induced to regenerate into whole plants. The flowers produced by these plants were brick red instead of white, showing that the maize cDNA had integrated into the genome and was expressed.

After successful experiments using model dicots protoplast transformation was attempted in monocots for which no alternative gene-transfer system was then available. In the first such experiments, involving wheat (Lorz et al. 2005) and the Italian ryegrass Lolium multiflorum (Potrykus et al. 1985a,b), protoplast transformation was achieved and transgenic callus obtained, but it was not possible to recover transgenic plants. The inability of most monocots to regenerate from protoplasts may reflect the loss of competence to respond to tissue culture conditions as the cells differentiate. In cereals and grasses, this has been addressed to a certain extent by using embryogenic suspension cultures as a source of protoplasts. Additionally, since many monocot species are naturally tolerant to kanamycin, the nptII market used in the initial experiments was replaced with alternative markers conferring resistance to hygromycin or phosphinothricin. With these modifications, it has been possible to regenerate transgenic plants representing certain varieties of rice and maize with reasonable efficiency (shimamoto et al. 1988, Datta et al. 1990, Omirulleh et al. 1993). However, the extended tissue culture step is unfavourable, often resulting in sterility and other phenotypic abnormalities in the regenerated plants.

Protoplast transformation was also the first method developed for gene transfer to the chloroplast genome of higher plants (Golds et al. 1993, O'Neill et al. 1993). In this context, plastid mutations conferring tolerance to antibiotics through alterations in ribosome structure can be used as an alternative to bacterial antibiotic resistance genes for the selection of plastid transformants (Kavanagh et al. 1999).

**Particle bombardment can be used to transform a wide range of plant species**

An alternative procedure for plant transformation was introduced in 1987, involving the use of a modified shotgun to accelerate small (1-4 μm) metal particles into plant cells at a velocity sufficient to penetrate the cell wall (~ 250 m/s). In the initial test system, intact onion epidermis was bombarded with tungsten particles coated in tobacco mosaic virus (TMV) RNA. Three days after bombardment, approximately 40% of the onion cells that contained particles also showed evidence of TMV replication (Sanford et al. 1987). A plasmid containing the cat reporter gene driven by the CaMV 358 promoter was then tested to determine whether DNA could be delivered by the same method. Analysis of the epidermal tissue three days after bombardment revealed high levels of transient CAT activity (Klein et al. 1987).

The stable transformation of explants from several plant species was achieved soon after these initial experiments. Early reports included the transformation of soybean (Christou et al. 1988), tobacco (Klein et al. 1988a), and maize (Klein et al. 1988b). In each case, the nptII gene was used as a selectable marker, and transformation was confirmed by the survival of callus tissue on kanamycin-supplemented medium. The ability to stably transform plant cells by this method offered the exciting possibility of generating transgenic plants representing species that were, at the time, intractable to other transformation procedures. In the first such report, transgenic soybean plants were produced from meristem (issue isolated from immature seeds (McCabe et al. 1988). In this experiment, the screenable marker gene gusA was introduced by particle bombardment and transgenic plants were recovered in the absence of selection by screening for GUS activity. Other early successes included cotton, papaya, maize and tobacco (Finer & McMullen 1991. Fitch et al. 1990, Gordon –Kamm et al. 1990. Fromm et al. 1990. Tomes et al. 1990: reviewed by Twyman & Christou 2004). Particle bombardment has also been pivotal in the development of chloroplast transformation technology.

There is no intrinsic limitation to the potential of particle bombardment since DNA delivery is governed entirely by physical parameters (Altpeter et al. 2005). Many different types of plant material have been used as transformation targets, including callus, cell suspension cultures, and organized tissues such as immature embryos, meristems, and leaves. The number of species in which transgenic plants can be produced using variants of particle bombardment has therefore increased dramatically over the last 10 years. Notable successes include almost all of the commercially important cereals, i.e. rice (Christou et. al.1991), wheat (Vasil et al. 1992), oat (Somers et al. 1992. Torbert et al. 1995), sugarcane (Bower & Birch 1992), and barley (Wan &Lemaux 1994, Hagio et al. 1995). Several literature surveys have been published documenting the range of species that have been transformed using this method (Christou 1996, Luthra et al. 1997, Twyman & Christou 2004).

The original gunpowder-driven device has been improved and modified resulting in greater control over particle velocity and hence greater reproducibility of transformation conditions. An apparatus based on electric discharge (McCabe & Christou 1993) has been used for the development of variety-independent gene-transfer methods for the more recalcitrant cereals and legumes. Several instruments have been developed where particle acceleration is controlled by pressurized gas. These include a pneumatic apparatus (Iida et al. 1990), a "particle inflow gun" using flowing helium (Takeuchi et al. 1992, Finer et al. 1992) and a device utilizing compressed helium (Sanford et al. 1991). Physical parameters such as particle size and acceleration (which affect the depth of penetration and the amount of tissue damage) as well as the amount and conformation of the DNA used to coat the particles, must be optimized for each species and type of explant (Finer et al. 1999, Twyman & Christou 2004). However, the nature of the transformation target is probably the most important single variable in the success of gene transfer.

**Other direct DNA transfer methods have been developed for intact plant cells**

There is a great diversity of approaches for gene transfer to animal cells and many of the same methods have been attempted in plants. Electroporation has been used to transform not only protoplasts but also walled plant cells, either growing in suspension or as part of intact tissues. In many cases, the target cells have been wounded or pre-treated with enzymes in order to facilitate gene transfer (e.g. D'Halluin et al. 1992, Laursen et al. 1994). However, immature rice, wheat and maize embryos can be transformed using electroporation without any form of pre-treatment (Kloti et al. 1993, Xu & Li 1994, Sorokin et al. 2000). Other transformation methods also involve perforation of the cell, including the use of silicon carbide whiskers (Thompson et al. 1995, Nagatani et al. 1997), ultrasound (Zhang et al. 1991), or a finely focused laser beam (Hoffman 1996). In most of these cases, only transient expression of the introduced DNA has been achieved, although transgenic maize plants have been recovered following whisker-mediated transformation (Frame et al. 1994). Finally, microinjection of DNA into plant cells can yield transformed cells or even transgenic plants, although as is the case for animal cells this method is not suitable for large-scale transformation (Crossway et al. 1986, Leduc et al. 1996, Holm et al. 2000).

**Direct DNA transfer is also used for chloroplast transformation**

So far, we have considered DNA transfer to the plant's nuclear genome. However, the chloroplast is also a useful target for genetic manipulation because thousands of chloroplasts may be present in photosynthetic cells and this can result in levels of transgene expression up to 50 times higher than possible using nuclear transformation. Furthermore, transgenes integrated into chloroplast DNA do not appear to undergo silencing or suffer from position effects that can influence the expression levels of transgenes in the nuclear DNA. Chloroplast transformation also provides a natural containment method for transgenic plants, since the transgene cannot be transmitted through pollen (reviewed by Maliga 1993).

The first reports of chloroplast transformation were serendipitous, and the integration events were found to be unstable. For example, an early experiment in which tobacco protoplasts were co-cultivated with Agrobacterium resulted in the recovery of one transgenic plant line in which the transgene was transmitted maternally. Southern blot analysis of chloroplast DNA showed directly that the foreign DNA had become integrated into the chloroplast genome (DeBlock et al. 1985). However, Agrobacterium is not an optimal system for chloroplast transformation because the T-DNA complex is targeted to the nucleus. Therefore, direct DNA transfer has been explored as an alternative strategy. Stable chloroplast transformation was first achieved in the alga Chlamydomonas reinhardtii, which has a single large chloroplast occupying most of the volume of the cell (Boynton et al. 1988). Particle bombardment was used in this experiment. The principles established using this simple organism were extended to tobacco allowing the recovery of stable transplastomic tobacco plants (Svab et al. 1990b). these principles included the use of vectors containing chloroplast homology regions, allowing targeted integration into the chloroplast genome, and use of the selectable marker gene aad (encoding aminoglycoside adenyltransferase) which confers resistance to strepiomycin and spectinomycin (Zoubenko et al. 1994). Efficient chloroplast transformation has been achieved both through particle bombardment (e.g. Staub & Maliga 1992a,b) and PEG mediated transformation (Golds et al. 1993, Koop et al. 1996). The use of a combined selectable-screenable marker (aad linked to the gene for green fluorescent protein) allows the tracking of transplastomic sectors of plant tissue prior to chlorophyll synthesis, so that transformed plants can be rapidly identified (Khan & Maliga 1999). It is now possible to transform the chloroplast genome and then eliminate selectable marker genes after transgene integration (Corneille et al. 2001, lamtham & Day 2000, Klaus et al. 2004).

Among crop plants, tobacco (Svab et al. 1990a,b), tomato (Ruf et al. 2001) and potato (Sidorov et al. 1999) chloroplasts have been transformed, as well as rapeseed and other brassicas (Hou et al. 2003, skarjinskaia et al. 2003). Most recently, soybean plastid transformation has been achieved (Dufourmantel et al. 2004). The major limitations in transforming the chloroplasts of other crop species, especially monocots, include a poor understanding of gene expression in non-green plastids, gene delivery methods for proplastids, and tissue culture conditions. Thus far, chloroplast transformation by particle bombardment has been achieved only in crops that allow direct organogenesis. Transplastomic plants have been developed with improvements in a number of key agronomic traits (Daniell et al. 2004), and there have been manyreports of chloroplasts expressing pharmaceutical proteins (summarized by Altpeter et. al. 2005).

**2. Explain the method of gene transfer by using Agrobacterium tumefaciens.**

**Agrobacterium-mediated transformation can be achieved using a simple experimental protocol in many dicots.**

Once the principle of selectable, disarmed T-DNA vectors was established, there followed an explosion in the number of experiments involving DNA transfer to plants. Variations on the simple general protocol of Horsch et al. (1985) have been widely used for dicot plants. In the original report, small disks (a few millimeters diameter) were punched from leaves, surface-sterilized, and inoculated in a medium containing A tumefaciens transformed with the recombinant disarmed T-DNA (as cointegrate or binary vector). The foreign DNA contained a chimeric neo gene conferring resistance to the antibiotic kanamycin. The disks were cultured for two days and transferred to medium containing kanamycin to select for the transferred neo gene, and carbenicillin to kill the Agrobacterium. After 2-4 weeks, developing shoots were excised from the callus and transplanted to root-inducing medium. Rooted plantlets were subsequently transplanted to soil, about 4-7 weeks after the inoculation step.

This method has the advantage of being simple and relatively rapid. It is superior to previous methods in which transformed plants were regenerated from protoplast-derived callus, the protoplasts having been transformed by co-cultivation with the Agrobacterium (De Block et al. 1984, Horsch et al. 1984). Contemporary protocols for the Agrobacterium mediated transformation of many solanaceous plants are variations on the theme of the leaf disk protocol, although the optimal explant must be determined for each species. Alternative procedures are required for the transformation of monocots, as discussed below.

**Monocots were initially recalcitrant to Agribacterium-mediated transformation, but it is now possible to transform certain varieties of many cereals using this method.**

Until the mid-1990s, most monocotyledonous plants (monocots) were thought to be outside the host range of Agrobacterium, prompting research into alternative transformation methods as discussed below. During the 1980s, limited evidence accumulated showing that some monocots might be susceptible to Agrobacterium infection (for example the discussion of agroinfection with maize streak virus DNA on p. 294).

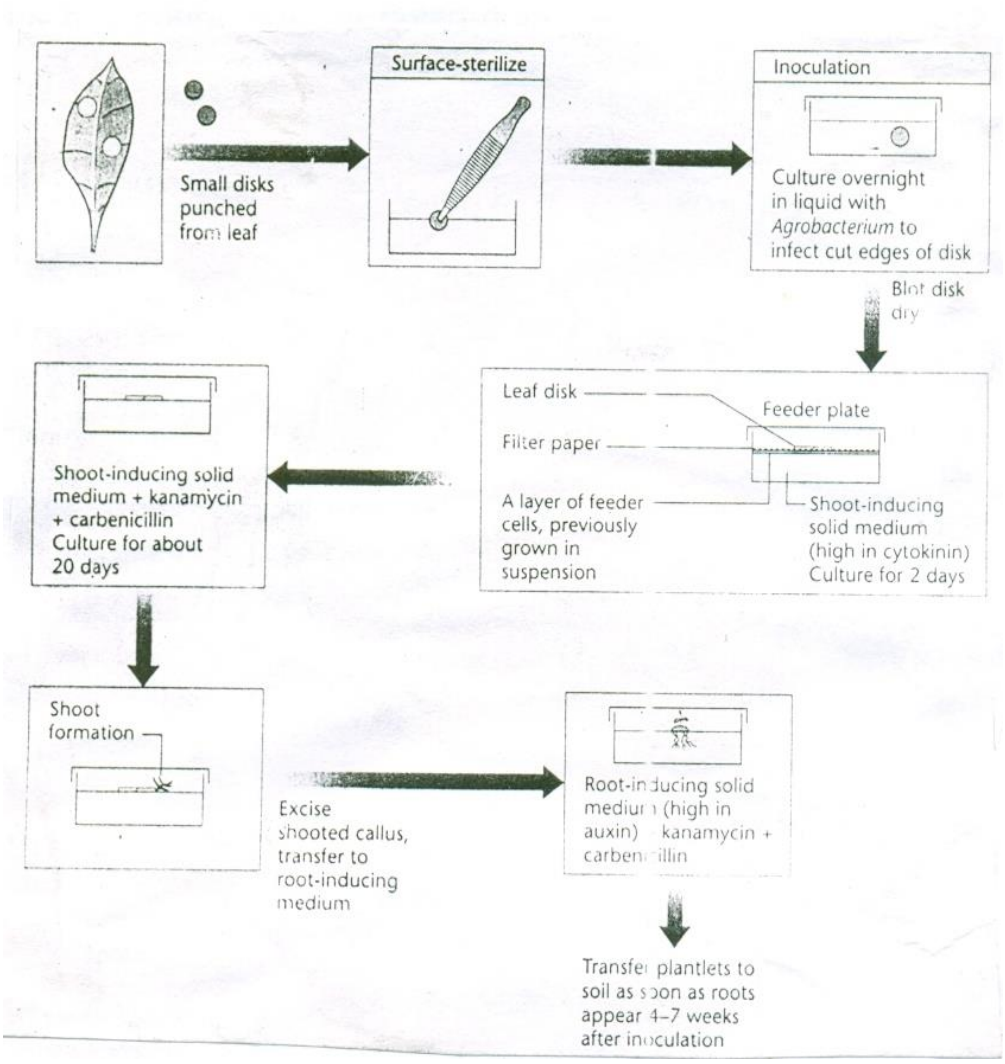



**Fig. Leaf-disk transformation by A. tumefaciens**

However, in most cases there was no convincing evidence for T-DNA integration into the plant genome. In the laboratory, it proved possible to induce tumors in certain monocot species such as asparagus (Hernalsteens et al. 1984) and yam (Schafer et al. 1987). In the latter case, an important factor in the success of the experiment was pre-treatment of the Agrobacterium suspension with wound exudates from potato tubers. It has been argued that Agrobacterium infection of monocots is inefficient because wounded monocot tissues do not produce phenolics such as acetosyringone at sufficient levels to induce virgene expression.

Eventually, however, researchers began to develop modified culture conditions and transformation procedures that worked with at least some monocots. Rice transformation was achieved in the early 1990s but the selection system (based on the nptII marker and selection with G418) interfered with regeneration, and only a small number of transgenic plants was produced (Raineri et al. 1990, Chan et al. 1992, 1993). The use of an alternative marker conferring resistance

to hygromycin allowed the regeneration of large numbers of transgenic japonica rice plants (Hiei et al. 1994), and the same selection strategy has been used to produce transgenic rice plants representing the other important subspecies, indica and javanica (Rashid et al. 1996, Dong et al. 1996). More recently, efficient Agrobacterium-mediated transformation has become possible for other important cereals, including maize (Ishida et al. 1996), wheat (Cheg et al. 1997), barley (Tingay et al. 1997), and sugarcane (Arencibia et al. 1998).

The breakthrough in cereal transformation using Agrobacterium reflected the recognition of a number of key factors required for efficient infection and gene transfer to monocots. The use of explants containing transfer to monocots. The use of explants containing a high proportion of actively dividing cells, such as embryos or apical meristems, was found to increase transformation efficiency greatly, probably because DNA synthesis and cell division favor the integration of exogenous DNA. In dicots, cell division is often induced by wounding, whereas wound sites in monocots tend to become lignified. This probably explains why traditional procedures such as the leaf disk method are inefficient in monocots. Hiei et al. (1994) showed that the co-cultivation of Agrobacterium and rice embryos in the presence of 100 mM acetosyringone was a critical factor for successful transformation. Transformation efficiency in increased further by the use of vectors with enhanced virulence functions. The modification of Agrobacterium for increased virulence has been achieved by increasing the expression of VirG (which in turn boosts the expression of the other vir genes) and/or the expression of VirE1, which is a major limiting factor in T-DNA transfer (reviewed by Sheng & Citovsky 196), resulting in so-called supervirulent bacterial strains such as AGL-1.. Komari et al. (1996) used a different strategy, in which a portion of the virulence region from the Ti-plasmid of supervirulent strain A281 was transferred to the T-DNA – carrying plasmid to generate a so-called superbinary vector. The advantage of the latter technique is that the superbinary vector can be used in any Agrobacterium strain.

**Binary vectors have been modified to transfer large segments of DNA into the plant genome**

A precise upper limit for T-DNA transfer has not been established. It is greater than 50 kbp (Herrera Estrella et al. 1983a,b), but using standard vectors it is difficult to transfer inserts larger than 30 kbp routinely due to instability in the bacterial host. The analysis of very large genes or the transfer of multiple genes (such as those encoding sequentially acting enzymes of a metabolic pathway) can now be achieved thanks to the development of high-capacity binary vectors based on the artificial chromosome type vectors used in E. coli. The first to be described was BIBAC2 (Hamilton 1997). This contains an F-plasmid origin of replication and is modeled on the bacterial artificial chromosome (BAC, p.79). The basic vector transforms tobacco with high efficiency, but the efficiency of transformation drops substantially when large inserts are used. This vector has been used to introduce 150 kbp of human DNA flanked by T-DNA borders into the tobacco genome, although virulence helper plasmids supplying high levels of VirG and VirE in trans were critical for successful DNA transfer (Hamilton et al. 1993). An alternative vector carrying a P1

origin of replication and modeled on the P1 artificial chromosome (PAC.p.79) was constructed by Liu et al. (1999). This transformation-competent bacterial artificial chromosome (TAC) vector was used to introduce upto 80 kbp of genomic DNA into Arabidopsis, and while there was some loss of efficiency, with the larger inserts, it was still possible to produce many transgenic plants. Both vectors contain a kanamycin resistance marker for selection in bacteria and hpt for hygromycin selection in transgenic plants. The hpt marker gene is laced adjacent to the right border T-DNA repeat. Both vectors also contain the Ri origin for maintenance in Agrobacterium, and within the T-DNA region, te sacB market for negative selection in E. coli interrupted by a polylinker for cloning foreign DNA.

One of the most attractive uses of high-capacity binary vectors is for the positional cloning of genes identified by mutation. The ability to introduce large segments of DNA into the plant genome effectively bridges the gap between genetic mapping and sequencing, allowing the position of mutant genes to be narrowed down by complementation. Genomic libraries have been established for several plant species in BIBAC2 and TAC vectors (Hamilton et al. 1999, Shibata & Lui 2000) and a number of novel genes have been isolated (e.g. Sawa et al. 1999, Kubo & Kakimoto 2001).

**Agrobacterium rhizogenes is used to transform plant roots and produce hairy-root cultures**

Agrobacterium rhizogenes causes hairy-root disease in plants, and this is induced by root-inducing (Ri) plasmids that are analogous to the Ti-plasmids of A.tumefaciens. The Ri T-DNA includes genes homologous to the iaaM (Tryptophan 2-monooxygenase) and iaaH (indoleacetamide hydrolase) genes of A. tumefaciens. Four other genes present in the Ri T-DNA are named rol for root locus. Two of these, rolB and rolC, encode P-glucosidases able to hydrolyze indole – and cytokine-N-glucosides. A. rhizogenes therefore appears to alter plant physiology by releasing free hormones from inactive or less active conjugated forms (Estruch et al. 1991 a,b).

Ri-plasmids are of interest from the point of view of vector development because opine-producing root tissue induced by Ri-plasmids in a variety of dicots can be regenerated into whole plants by manipulation of phytohormones in the culture medium. Ri T-DNA is transmitted sexually by these plants and affects a variety of morphological and physiological traits, but does not in general appear deleterious. The Ri-plasmids therefore appear to be already equivalent to disarmed Ti-plasmids (Tepfer 1984). Transformed roots can also be maintained as hairy-root cultures, which have the potential to produce certain valuable secondary metabolites at higher levels than suspension cultures, and are much more genetically stable (Hamil et al. 1987, Signs & Flores 1990). The major limitation for the commercial use of hairy-root cultures is the difficulty involved in scale-up, since each culture comprises a heterogeneous mass of interconnected tissue, with highly uneven distribution (reviewed by Giri & Narassu 2000).

Many of the principles explained in the context of disarmed Ti-plasmids are applicable to Ri-plasmids. A cointegrate vector system has been developed (Jensen et al. 1986) and applied to the study of nodulation in transgenic legumes. Van Sluys et al. (1987) have exploited the fact that Agrobacterium containing both a Ri -plasmid and a disarmed Ti-plasmid can frequently co-transfer both plasmids. The Ri-plasmid induces hairy-root disease in recipient Arabidopsis and carrot cells, serving as a transformation marker for the co-transferred recombinant T-DNA, and allowing regeneration of intact plants. No drug resistance marker on the T-DNA is necessary with this plasmid combination.

**3. (i) Agrobacterium tumefaciens – nature's smallest genetic engineer – How?**
**(ii) What are the limitations with Agrobacterium mediated transformation? How it is rectified?**

**The ability to induce tumors is conferred by a Ti-plasmid found only in virulent *Agrobacterium strains***

Since the continued presence of Agrobacterium is not required to maintain plant cells in their transformed state, it is clear that some "tumor-inducing principle" is transferred from the bacterium to the plant at the wound site. Zaenen et al. (1974) first noted that virulent strains of A. tumefaciens harbor large plasmids (140-235 kbp), and experiments involving the transfer of such plasmids between various octopine – and

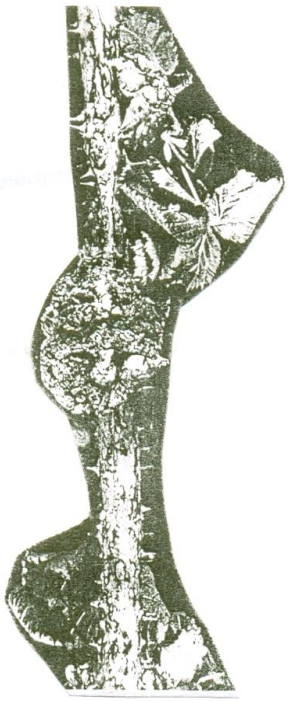


**Fig. Crown gall on blackberry cane.**

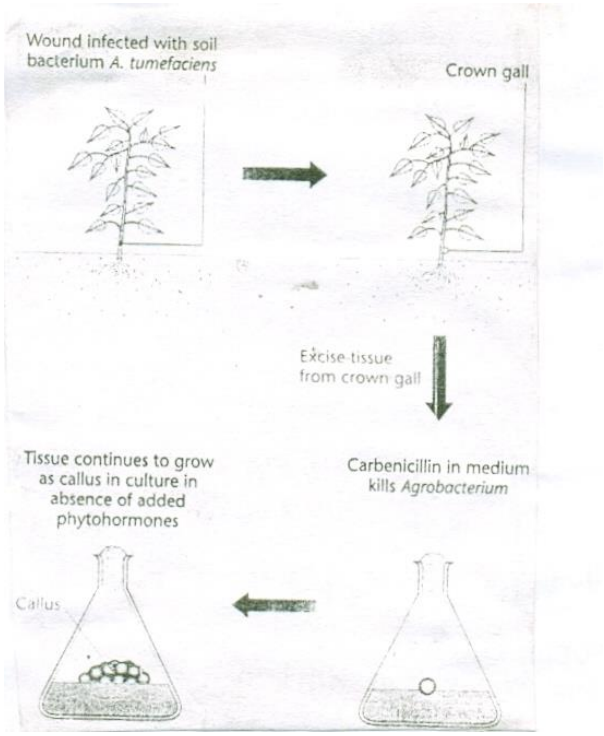



**Fig. A tumefaciens induces plant tumors, but is not required for the continuous proliferation of those tumors.**

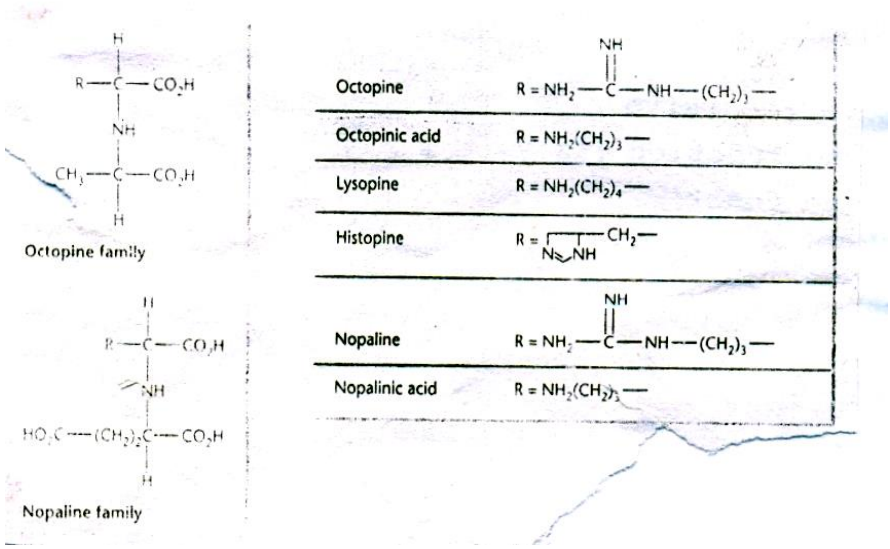



**Fig. Structures of some opines**

nopaline-utilizing strains soon established that virulence and the ability to use and induce the synthesis of opines are plasmid-borne traits. These properties are lost when the bacteria are cured of their resident plasmid (Van Larbeke et al. 1974. Watson et al.1975) but acquired by a virulent strains when a virulence plasmid is reintroduced by conjugation (Bomhoff et al. 1976, Gordon et

al. 1979). The plasmids therefore became known as tumor-inducing plasmids (Ti-plasmids).

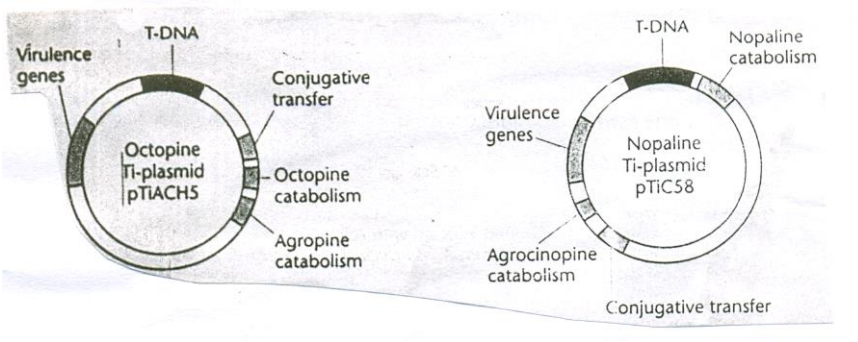



**Fig. Ti-plasmid gene maps**

Ti-plasmids specify the type of opine that is synthesized in the transformed plant tissue and the opine utilized by the bacterium. Plasmids in the octopine group are closely related to each other while those in the nopaline group are considerably more diverse. Between the groups, there are four regions of homology, including the genes directly responsible for tumor formation (Drummond & Chilton 1978, Engler et al. 1981). It should be noted that the presence of a plasmid in A. tumefaciens does not mean tat the strain is virulent. Many strains contain very large cryptic plasmids that do not confer virulence, and in some natural isolates a cryptic plasmid is present together with a Ti-plasmid.

**A short segment of DNA, the T-DNA, is transferred to the plant genome**

Complete Ti-plasmid DNA is not found in plant tumor cells but a small, specific segment of the plasmid, about 23 kbp in size, is found integrated in the plant nuclear DNA at an apparently random site. This DNA segment is called T-DNA (transferred DNA) and carries genes that confer both unregulated growth and the ability to synthesize opines upon the transformed plant tissue. However, these genes are non-essential for transfer and can be replaced with foreign DNA. The structure and organization of nopaline plasmid T-DNA sequences are usually simple, i.e. there is a single integrated segment. Conversely, octopine T-DNA comprises two segments, $T_L$ (which carries the genes required for tumor formation) and $T_R$ (which carries the genes for opine synthesis). The two segments are transferred to the plant genome independently and may be present as multiple copies. The significance of this additional complexity is not clear.

In the Ti-plasmid itself, the T-DNA is flanked by 25-bp imperfect direct repeats known as border sequences, which are conserved between octopine and nopaline plasmids. The border sequences are not transferred intact to the plant genome, but they are involved in the transfer process. The analysis of junction regions isolated from plant genomic DNA has shown that the integrated T-DNA end points lie internal to the border sequences. The right junction is rather

precise, but the left junction can vary by about 100 nucleotides (Yadav et al. 1982. zambryski et al. 1982). Deletion of the right-border repeat abolishes T-DNA transfer, but the left-hand border surprisingly appears to be non-essential. Experiments in which the right-border repeat alone has been used have shown that an enhancer, sometimes called the overdrive sequence, located external to the repeat is also required for high-efficiency transfer (Shaw et al. 1984, Peralta et al. 1986). The left-border repeat has little transfer activity alone (Jen & Chilton 1986).

The genes responsible for T-DNA transfer are located in a separate part of the Ti-plasid called the vir (virulence) region. Two of these genes, virA and virG, are constitutively expressed at a low level and control the plant-induced activation of the other virgenes. VirA is a kinase that spans the inner bacterial membrane, and acts as the receptor for certain phenolic molecules that are released by wounded plant cells. A large number of such compounds has been characterized, but one in particular, acetosyringone, has been the most widely used in the laboratory to induce vir gene expression (Stachel et al. 1985). Notably, phenolic compounds such as acetosyringone do not attract bacteria to wounded plant cells. Rater, the bacteria appear to respond to simple molecules such as sugars and amino acids, and the vir genes are induced after attachment (Parke et al. 1987, Loake et al. 1988). Many sugars also synergize the action of the phenolic signals to enhance vir gene expression (Shimada et al. 1990). Activated VirA transphosphorylates the VirG protein, which is a transcriptional activator of the other vir genes. The VirA and VirG proteins show similarities to other two-component regulatory systems common in bacteria (Winans 1992). In addition to VirG, further genes on the bacterial chromosome also encode transcription factors that regulate vir gene expression (reviewed by Kado 1998, Gelvin 2000, 2003).

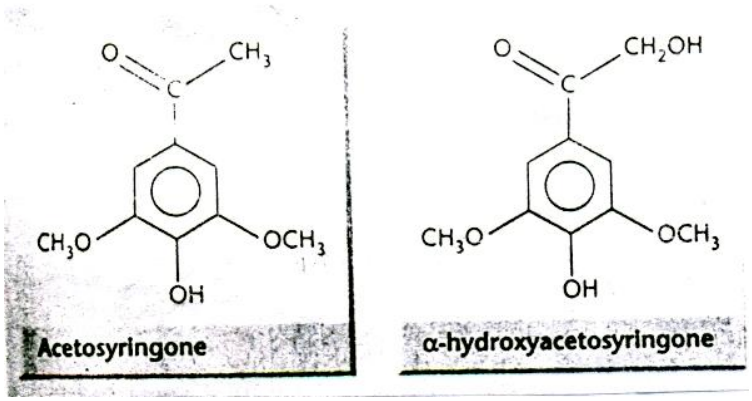



**Fig. Structures of signal molecules produced by wounded plant tissue, which activate T-DNA transfer by A tumefaciens.**

The induction of vir gene expression results in the synthesis of proteins that form a conjugative pilus through which the T-DNA is transferred to the plant cell. The components of the pilus are encoded by genes in the virB operon (reviewed by Lai & Kado 2000). DNA transfer itself is initiated by an endonuclease formed by the products of the virD1 and virD2 genes. This

introduces either single-strand nicks or a double-strand break at the 25 bp borders of the T-DNA, a process enhanced by the VirC12 and VirC2 proteins, which recognize and bind to the overdrive enhancer element. The VirD2 protein remains covalently attached to the processed T-DNA. Recent studies have suggested that the type of T-DNA intermediate produced (single-or double-stranded) depends on the type of Ti-plasmid, with double-stranded T-DNA favored by nopaline plasmids where the T-DNA is a single element) and single "T-strands" favored by octopine and succinopine plasmids, where the T-DNA is split into noncontiguous sections (Steck 1997), T-strands are coated with VirE2, a single-stranded DNA binding protein. The whole complex, sometimes dubbed the firecracker complex because of its proposed shape is then transferred through the pilus and into the plant cell. The VirD2 protein has been proposed to protect the T-DNA against nucleases, to target the DNA to the plant cell nucleus, and to integrate it into the plant genome. The protein has two distinct nuclear localization signals, with the C-terminal signal thought to play the major role in targeting the T-DNA (Tinland et al. 1992). It has been observed that the nucleus of wounded plant cells often becomes associated with the cytosolic membrane close to the wound site, suggesting that the T-DNA could be transferred directly to the nucleus without extensive exposure to the cytosol (Kahl & Schell 1982). Once in the nucleus, the T-DNA is thought to integrate through a process of illegitimate recombination, perhaps exploiting naturally occurring chromosome breaks (Tinland 1996, Tzfira et al. 2004).

The Agrobacterium gene-transfer system appears to be a highly adapted form of bacterial conjugation. Many broad-host-range plasmids can transfer from Agrobacterium to the plant genome using their own mobilization functions (Buchanan-Wollaston et al. 1987) and the vir genes encode many components that are common with broad-host-range plasmid conjugation systems (reviewed by Kado 1998). In addition to plants, Agrobacterium can transfer DNA to other bacteria, yeast, and filamentous fungi. Recently, anovel insight into the scope of this gene-transfer mechanism was provided by Citovsky and colleagues (Kunik et al. 2001) by demonstrating that gene transfer from Agrobacterium to cultured human cells was also possible! For the interested reader, T-DNA transfer has been discussed in several comprehensive reviews (Zupan et al. 2000, Tzfira & Citofsky 2000, 2002, Gelvin 2003, Valentine 2003).

**Disarmed Ti-plasmid derivatives can be used as plant gene-transfer vectors**

Genetic maps of T-DNA have been obtained by studying spontaneous and transposon-induced mutants that affect tumor morphology, generating tumors that are larger than normal, or that show "shooty" or "rooty" phenotypes. Although normal tumors can grow on medium lacking auxins and cytokinins, the tumor cells actually contain high levels of these hormones. Ooms et al. (1981) therefore proposed that the oncogenes carried on the T-DNA encoded products involved in phytohormone synthesis and that the

| *Gene* | *Product* | *Function* |
|---|---|---|

| | | |
|---|---|---|
| ocs | Octopine synthase | Opine synthesis |
| nos | Nopaline synthase | Opine synthesis |
| tms1 (iaaH, auxA) | Tryptophan-2-mono-oxygenase | Auxin synthesis |
| tms2 (iaaM, auxB) | Indoleacetamide hydrolase | Auxin synthesis |
| tmr (ipt, cyt) | Isopentyl transferase | Cytokinin synthesis |
| tml | Unknown | Unknown, mutations affect tumor size |
| frs | Fructopine synthase | Opine synthesis |
| mas | Mannopine synthase | Opine synthesis |
| ags | Agropine synthase | Opine synthesis |

**Table. Functions of some T-DNA genes in A. tumefaciens TI-plasmids**

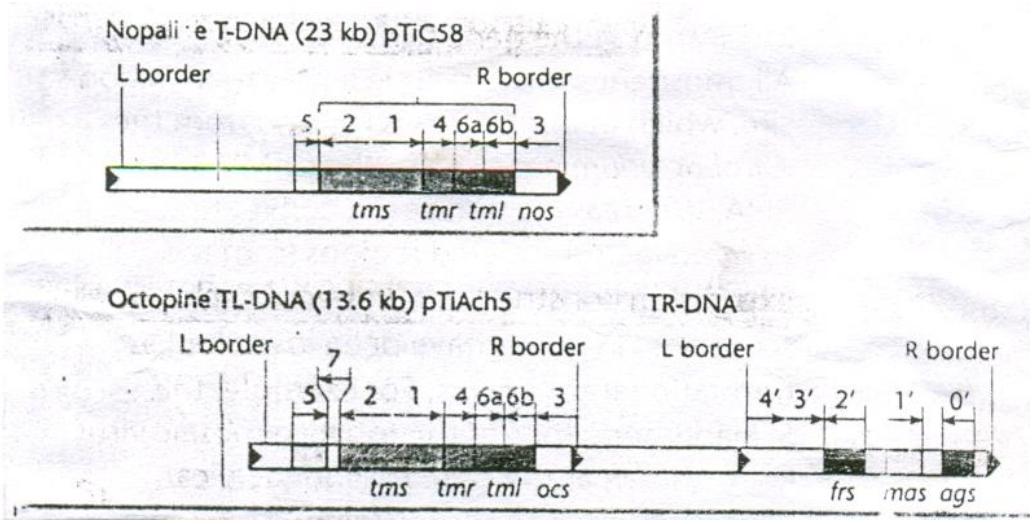



**Fig. Structure and transcription of T-DNA. The T-regions of nopaline and octopine Ti-plasmids have been aligned to indicate the common DNA sequence. The size and orientation of each transcript (numbered) is indicated by arrows. Genetic loci, delined by deletion and transposon mutagenesis, are shown as follows: nos, nopaline synthase: ocs, octopine synthase, tms, shooty tumor: tmr, rooty tumor.**

abnormal morphologies of T-DNA mutants were due to a disturbance in the balance of plant hormones in the callus. The cloning and functional analysis of T-DNA genes has confirmed that those with "shooty" mutant phenotypes encode enzymes for auxin biosynthesis, and those with "rooty" phenotypes are involved in cytokinin production (Weiler & Schroder 1987). Other genes have been identified as encoding enzymes for opine synthesis, while the function of some genes remains unknown. The transcript maps of T-DNAs from a nopaline plasmid (pTiC58) and an octopine plasmid (pTiAch5) are shown in figure (Willmitzer et al. 1982, 1983, Winter et al. 1984).

Interestingly, nucleotide sequencing has revealed that the T-DNA genes have promoter

elements and polyadenylation sites that are eukaryotic in nature (De Greve et al. 1982 a,b, Depicker et al. 1982, Bevan et al. 1983 a,b). This explains how genes from a bacterial plasmid come to be expressed when transferred to the plant nucleus. It is possible that the sequences may have been captured from plants during the evolution of the Ti-plasmid. The ability of Agrobacterium to induce tumors in a wide variety of plants suggested that T-DNA promoters such as those of the ocs (octopine synthase) and nos (nopaline synthase) genes could be useful for driving transgene expression. These and other promoters used for transgene expression in plants are discussed in Box 1.

We have seen that the Ti-plasmid is a natural vector for genetically engineering plant cells because it can transfer its T-DNA from the bacterium to the plant genome. However, wild-type Ti-plasmids are not suitable as general gene vectors because the T-DNA contains oncogenes that cause disorganized growth of the recipient plant cells. To be able to regenerate plants efficiently we must use vectors in which the T-DNA has been disarmed by making it non-oncogneic. This is most effectively achieved simply by deleting all of its oncogenes. For example, Zambryski et al. (1983) substituted pBR 322 sequences for almost all of the T-DNA of pTiC58 leaving only the left- and right- border regions and the nos gene. The resulting construct was called pGV3850.

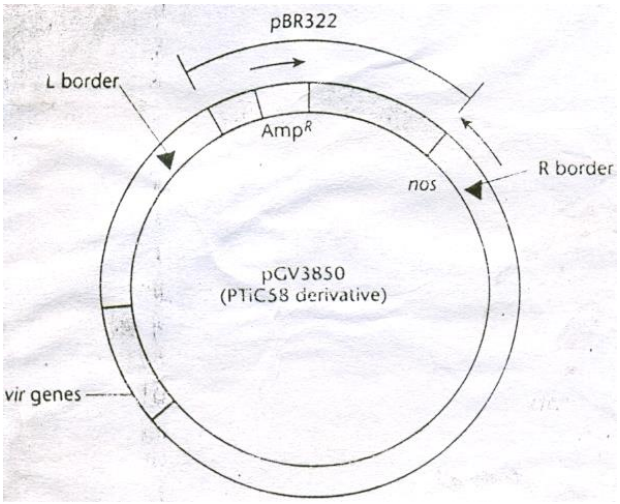



**Fig. Structure of the Ti-plasmid pGV3850, in which the T-DNA has been disarmed**

Agrobacterium carrying this plasmid transferred the modified T-DNA to plant cells. As expected, no tumor cells were produced, but the fact that transfer had taken place was evident when the cells were screened for nopaline production and found to be positive. Callus tissue could be cultured from these nopaline-psitive cells if suitable phytohormones were provided, and fertile adult plants were regenerated by hormone induction of plantlets.

The creation of disarmed T-DNA was an important step forward, but the absence of tumor

formation made it necessary to use an alternative method to identify transformed plant cells. In the experiment described above, opine production was exploited as a screenable phenotype, and the ocs and nos genes have been widely used as screenable marker (reviewed by Dessaux & Ptit 1994). However, there are several drawbacks associated with this system particularly the necessity to carry out enzymatic assays on all potential transformants. To provide a more convenient way to identify transformed plant cells, dominant selectable markers have been inserted into the T-DNA so that transformed plant cells can be selected on the basis of drug or herbicide resistance.

**Box 1. Control of transgene expression in plants**

**Promoters**

To achieve high-level and constitutive transgene expression in plants, a very active promoter is required. In dicots, promoters from the Agrobacterium nopaline synthase (nos), octopine synthase (ocs), and mannopine synthase (mas) genes have been widely used. These are constitutive and also moderately induced by wounding (An et. al. 1990, Langridge et al. 1989). The most popular promoter for transgene expression in dictors is the 35S RNA promoter from cauliflower mosaic virus (CaMV 35S). This is very active, but can be improved still further by duplicating the enhancer region (Rathus et al. 1993). These promoters have a much lower activity in monocots, and duplicating the CaMV 35S enhancer has little effect. Alternative promoters have therefore been sought for transgene expression in cereals (reviewed by McElroy & Brettel 1994). The rice actin-1 and maize ubiquitin-1 promoters have been widely used for this purpose (McElroy et al. 1995, Christensen & Quail 1996). As well as constitutive promoters, a large number of promoters have been used to direct transgene expression in particular tissues. In monocots, promoters from seed storage-protein genes, such as maize zein, wheat glutenin, and rice glutelins, have been used to target transgene expression to the seeds, which is beneficial for the accumulation of recombinant proteins (Wu et al. 1998; reviewed by Bilan et al. 1999). Promoters targeting transgene expression to green tissue are also useful (e.g. Graham et al. 1997, Datta et al. 1998).

**Other components of the expression vector**

As discussed for animal cells, other sequences in the expression vector also influence transgene expression. Generally, the presence of an intron in a plant expression cassette increases the activity of the promoter (Bilan et al. 1999). The insertion of a heterologous intron enhances the activity of the CaMV 35S promoter in monocots (e.g. see Mascarenhas et al. 1990, Vain et al. 1996) and constructs containing the actin or ubiquitin promoters generally include the first intron of the gene (McElroy et al. 1991). All transgenes must include a polyadenylation site, which in most cases is derived from the Agrobacterium nos gene or the CaMV 35S RNA. Whereas in animals it is conventional to remove untranslated regions from the expression construct, a number of such

sequences in plants have been identified as translational enhancers. For example, the 5′ leader sequence of the tobacco mosaic virus RNA, known as the omega sequence, can increase transgene expression up to 80-fold (reviewed by Futterer & Hohn 1996, Gallie 1996). As in animals, the translational start site should conform to Kozak's consensus (Kozak 1999) and the transgene should be codon-optimized for the expression host. A good example of the latter is the use of codon-optimized insecticidal toxin genes from Bacillus thuringiensis for expression in transgenic crops, leading to dramatically increased expression levels compared with the unmodified genes (Koziel et al. 1996). Also, the inclusion of targeting information in the expression cassette may be beneficial for the accumulation of recombinant proteins. For example, recombinant antibodies expressed in plants are much more stable if targeted to the endoplasmic reticulum (ER), since this provides favourable molecular environment for folding and assembly. Targeting is achieved using an N-terminal signal sequence to direct the ribosome to the ER and a C-terminal tetrapeptide retrieval signal, KDEL, which causes accumulation in the ER lumen Horvath et al. 2000).

**4. Discuss the uses of plant viruses as a cloning vectors in plant Genetic Engineering.**

**Plant viruses can be used as episomal expression vectors**

As an alternative to stable transformation using Agrobacterium or direct DNA transfer, plant viruses can be employed as gene transfer and expression vectors. There are several advantages to the use of viruses. First, viruses are able to adsorb to and introduce their nucleic acid into intact plant cells. However, for many viruses, naked DNA Or RNA is also infectious, allowing recombinant vectors to be introduced directly into plants by methods such as leaf rubbing. Second, infected cells yield large amounts of virus, so recombinant viral vectors have the potential for high-level transgene expression. Third, viral infections are often systemic. The virus spreads throughout the plant allowing transgene expression in all cells. Fourth, viral infections are rapid, so large amounts of recombinant protein can be produced in a few weeks. Finally, all known plant viruses replicate episomally, therefore the transgenes they carry are not subject to the position effects that often influence the expression of integrated transgenes. Since plant viruses neither integrate nor pass through the germline, plants cannot be stably transformed by viral infection and transgenic lines cannot be generated. However, this limitation can also be advantageous in terms of containment.

A complete copy of a viral genome can also be introduced into isolated plant cells or whole plants by Agrobacterium or direct DNA transfer. In this manner, it is possible to generate transiently transformed cell lines or transgenic plants carrying an integrated recombinant viral genome. In the case of RNA viruses, transcription of an integrated cDNA copy of the genome yields replication-competent viral RNA, which is amplified episomally, facilitating high-level transgene expression. Transgenic plants are persistently infected by the virus and can produce large amounts of recombinant protein. In the case of DNA viruses, Agrobacterium-mediated

transient or stable transformation with T-DNA containing a partially duplicated viral genome can lead to the "escape" of intact genomes, which then replicate episomally. The latter process, known as "agroinfection" or "agroinoculation" provides a very sensitive assay for gene transfer. More recently, the Agrobacterium-mediated delivery of viral genomes has been enhanced through a process called magnifection, in which amplification of the vector occurs in all infected leaves (Marrilonet et al. 2005).

As well as teir use for the expression of whole foreign proteins, certain plant viruses have recently been developed to present short peptides on their surfaces, similar to the phage display technology. Epitope-display systems based on cowpea mosaic virus, alfalfa mosaic virus, potato virus X, and tomato bushy stunt virus have been developed as a potential source of vaccines, particularly against animal viruses (reviewed by Lomonossoff & Hamilton 1999, Pogue et al. 2002. Yusibov & Rabindran 2004, Khalsa et al. 2004, Twyman et al. 2005).

**The first plant viral vectors were based on DNA viruses because of their small and simple genomes**

The vast majority of plant viruses have RNA genomes. However, the two groups of DNA viruses that are known to infect plants – the caulimoviruses and the geminiviruses – were the first to be developed as vectors because of the ease with which their small, DNA genomes could be manipulated in plasmid vectors.

The type member of the caulimoviruses is cauliflower mosaic virus (CaMV). The 8-kb dsDNA genome of several isolates has been completely sequenced, revealing an unusual structure characterized by the presence of three discontinuities in the duplex. A map fo the CaMV genome is shown in figure. There are eight tightly packed genes, expressed as two major transcripts: the 35S RNA (which essentially represents the entire genome) and the 19S RNA (which contains the coding region for gene VI). As discussed earlier in the chapter, the promoter and terminator sequences for both transcripts have been utilized in plant expression vectors, and the 35S promoter is particularly widely used.

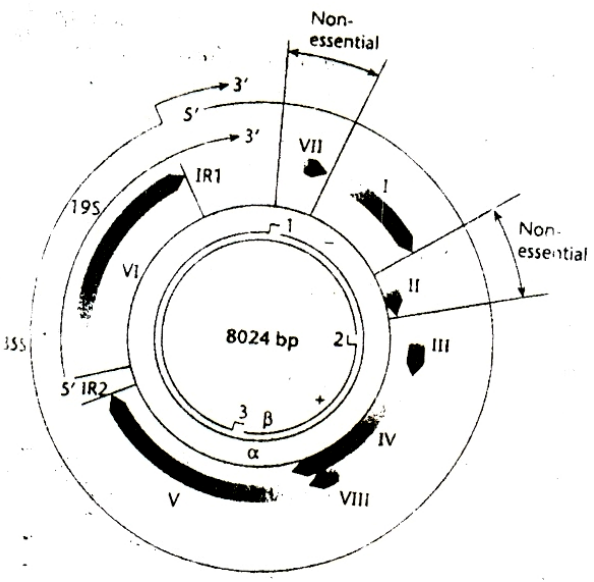


**Fig. Map of the cauliflower mosaic virus genome. The eight coding regions are shown by the thick gray arrows, and the different reading frames are indicated by the radial positions of the boxes. The thin lines in the center indicate the plus and minus) DNA strands with the three discontinuities. The major transcripts, 19S and 35S are shown around the outside.**

Only two of the genes in the CaMV genome are non-essential for replication (gene II and gene VII) and since CaMV has an icosahedral capsid, the size of the genome cannot be increased greatly without affecting the efficiency of packaging. The maximum capacity of the CaMV capsid has been defined as 8.3 kb, and with the removal of all non-essential genes, this represents a maximum insert size of less than 1 kb (Daubert et al. 1983). This restriction in the capacity for foreign DNA represents a major limitation of CaMV vectors. Thus far it has been possible to express a number of very small transgenes, such as the 240-bp bacterial dhfr gene (Brisson et al. 1984), the 200-bp murine metallothionein cDNA (Lefebvre et al. 1987) and a 500-bp human interferon cDNA (dc Zoeten et al. 1989). We describe how similar limitations were overcome for SV40, a virus that infects primate cells, through the development of replicon vectors and helper viruses or complementary cell lines supplying essential functions in trans. Unfortunately, such an approach is not possible with CaMV due to the high level of recombination that occurs, leading to rapid excision of the foreign DNA (Gronenborn & Matzeit 1989).

Geminiviruses are characterized by their twin (geminate) virions, comprising two partially fused icosahedral capsids. The small single-stranded DNA genome iscircular, and in some species is divided into two segments called DNA A and DNA B. Interest in geminivirus vector development was stimulated by the discovery that such viruses use a DNA replicative intermediate, suggesting they could be more stable than CaMV, whose RNA-dependent replication cycle is rather error-prone (Stenger et al. 1991). Of the three genera of geminiviruses, two have been developed as vectors. The begomoviruses have predominantly bipartite genomes: they are transmitted by the whitefly Bemisia tabaci and infect dicots. Species that have been developed as vectors include African cassava mosaic virus (ACMV) and tomato golden mosaic

virus (TGMV). The mastreviruses have monopartite genomes: they are transmitted by leafhoppers and predominantly infect monocots. Species that have been developed as vectors include maize streak virus (MSV) and wheat dwarf virus (WDV).

An important additional distinction between these genera is that mastreviruses are not mechanically transmissible. MSV, for example, has never been introduced successfully into plants as native or cloned DNA. Grimsley et al. (1987) were able to overcome this problem using Agrobacterium, and were the first to demonstrate the principle of agroinfection. They constructed a plasmid containing a tandem dimmer of the MSV genome. This dimmer was inserted into binary vector, and maize plants were infected with A. tumefaciens containing the recombinant T-DNA. Viral symptoms appeared within two weeks of inoculation. Agroinfection has been used to introduce the genomes of a number of different viruses into plants. It can be demonstrated that if the T-DNA contains partially or completely duplicated genomes, single copies of the genome can escape and initiate infections. This may be mediated by homologous recombination or a replicative mechanism (Stenger et al. 1991). The study of Grinsley et al. (1987) incidentally provided the first evidence that Agrobacterium could transfer T-DNA to maize. Agroinfection is a very sensitive assay for transfer to the plant cell because of the amplification inherent in the virus infection and the resulting visible symptoms.

A number of geminiviruses have been developed as expression vectors because of the possibility f achieving high-level recombinant protein expression as a function of viral replication (reviewed by Stanley 1993, Timmermans et al. 1994, Palmer & Rybicki 1997). A generally useful strategy is the replacement of the coat protein gene, since this is not required for replication and the strong promoter can be used to drive transgene expression. In the case of begomoviruses, which have bipartite genomes, the coat protein gene is located on DNA A along with all the functions required for DNA replication. Replicons based on DNA A are therefore capable of autonomous replication in protoplasts (e.g. Townsend et al. 1986). Geminivirus replicon vectors can facilitate the high-level transient expression of foreign genes in protoplasts. There appears to be no intrinsic limitation to the size of the insert, although larger transgenes tend to reduce the replicon copy number (e.g. Laufs et al. 1990, Matzeit et al. 1991). Generally, it appears tat mastrevirus replicons can achieve a much higher copy number in protoplasts than replicons based on begomoviruses. A WDV shuttle vector capable of replicating in both E.coli and plants was shown to achieve a copy number of greater than $3 \times 10^4$ in protoplasts derived from cultured maize endosperm cells (Timmermans et al. 1992), whereas the typical copy number achieved by TGMV replicons in tobacco protoplasts is less than 1000 (Kanevski et al. 1992). This may, however, reflect differences in the respective host cells rather than the intrinsic efficiencies of the vectors themselves.

Geminiviruses are also valuable as expression vectors in whole plants. In the case of the

mastreviruses, all viral genes appear to be essential for systemic infection, so coat protein replacement vectors cannot be used in this manner. In contrast, the coat protein genes of ACMV and TGMV are non-essential for systemic infection, but they are required for insect transmission (Briddon et al. 1990). Therefore, replicon vectors based on these viruses provide an in planta contained transient expression system. Note that viral movement functions are supplied by DNA B, so systemic infections occur only if DNA B is also present in the plant.

In an early study, Ward et al. (1988) replaced most of the ACMV AV1 gene with the cat reporter gene. In infected tobacco plants, high-level CAT activity was detected for up to four weeks. Interestingly, they found that deletion of the coat protein gene caused a loss of infectivity in plants, but this was restored upon replacement with cat, which is approximately the same size as the deleted gene. This and many subsequent reports indicated that, while there may be no intrinsic limit to the size of replicon vectors in protoplasts, systemic infection is dependent on preserving the size of the wild-type DNA A component. A further limitation to this system is that the transmissibility of the recombinant genomes is poor, probably because they are not packaged. One way in which this can be addressed is to generate transgenic plants in which recombinant viral genomes are produced in every cell. This is achieved by transforming plants with DNA constructs containing a partially duplicated viral genome (Meyer et al. 1992). Intact replicons can excise from the delivered transfer in the same way as the MSV genome escapes during agroinfection, and autonomously replicating episomal copies can be detected, Transgenic tobacco plants have also been produced carrying an integrated copy of DNA B (Hayes et al. 1988, 1989). In the presence of replication functions supplied by DNA A, the DNA B sequence is rescued from the transgene and can replicate episomally. DNA B can then provide movement functions to DNA A facilitating the systemic spread of the vector.

**Most plant virus expression vectors are based on RNA viruses because they can accept large transgenes than DNA viruses.**

Most plant RNA viruses have a filamentous morphology, so the packaging constraints affecting the use of DNA viruses such CaMV should not present a limitation in vector development. However, investigation into the use of RNA viruses as vectors lagged behind research on DNA viruses, awaiting the advent of robust techniques for the manipulation of RNA genomes.

A breakthrough was made in 1984, when a full-length clone corresponding to the genome of brome mosaic virus (BMV) was obtained. Infectious RNA could be produced from this cDNA by in vitro transcription (Ahlquist & Janda 1984, Ahlquist et al. 1984). The BMV genome comprises three segments: RNA1, RNA2, and RNA3. Only RNA1 and RNA2 are necessary for replication. RNA3, which is dicistronic, encodes the viral coat protein and movement protein. During BMV infection, a subgenomic RNA fragment is synthesized from RNA3, containing the coat protein

gene alone. It is therefore possible to replace the coat protein gene with foreign DNA and still generate a productive infection (Ahlquist et al. 1987). This was demonstrated by French et al. (1986) in an experiment where the coat protein gene was substituted with the cat reporter gene. Following the introduction of recombinant RNA3 into barley protoplasts along with the essential RNA1 and RNA2 segments, high-level CAT activity was achieved.

This experiment showed that brome mosaic virus was a potentially useful vector for foreign gene expression. However, to date, BMV has been used solely to study the function of genes from other plant viruses. Following the demonstration that infectious BMV RNA could be produced by in vitro transcription, the genomes of many other RNA viruses have been prepared as cDNA copies. Some of these viruses have been extensively developed as vectors for foreign gene expression (see comprehensive reviews by Scholthof et al. 1996, Porta & Lomonossoff 2001).

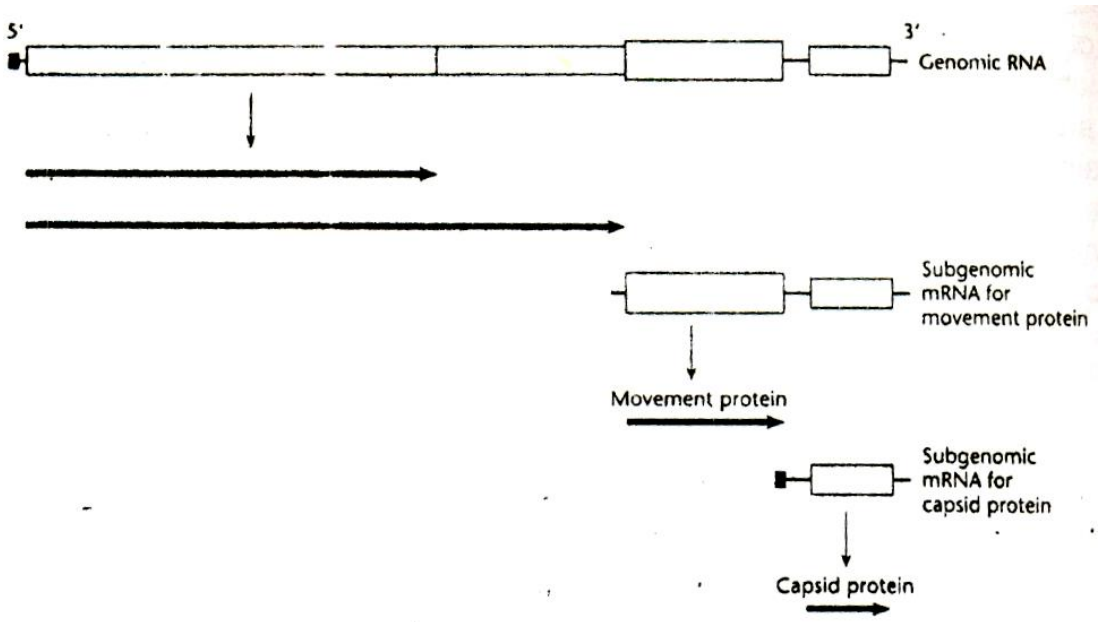



**Fig. Genome map and expression of tobacco mosaic virus**

Tobacco mosaic virus (TMV) is one of the most extensively studied plant viruses and was thus a natural choice for vector development. The virus has a monopartite RNA genome of 6.5 kb. At least four polypeptides are produced, including a movement protein and a coat protein that are translated from subgenomic RNAs. The first use of TMV as a vector was reported by Takamatsu et al. (1987). They replaced the coat protein gene with out, and obtained infected plants showing high level CAT activity at the site of infection. However, the recombinant virus was unable to spread throughout the plant because the coat protein is required for systemic infection.

Since there should be no packaging constraints with TMV, Dawson et al. (1989) addressed

the deficiencies of the TMV replacement vector by generating a replication-competent addition vector in which the entire wild-type genome was preserved. Dawson and colleagues added the bacterial cat gene, controlled by a duplicated coat protein subgenomic promoter, between the authentic movement and coat protein genes of the TMV genome. In this case, systemic infection occurred in concert with igh-level CAT activity, but recombination events in infected plants resulted in deletion of the transgene and the production of wild-type TMV RNA. Homologous recombination can be prevented by replacing the TMV coat protein gene with the equivalent sequence from the related Odontoglossum ringspot virus (Donson et al. 1991). This strategy has been used to produce a range of very stable expression vectors that have been used to synthesize a variety of valuable proteins in plants, such as ribosome-inactivating protein (Kumagai et al. 1993) and scFV antibodies (McCormick et al. 1999). It has also been possible to produce complete monoclonal antibodies by co-infecting plants with separate TMV vectors expressing the heavy and light immunoglobulin chains (Verch et al. 1998).

Potato virus X (PVX) is the type member of the Potexvirus family. Like TMV, it has a monopartite RNA genome of approximately 6.5 kb which is packaged in a filamentous particle. The genome map of PVX is shown in figure and contains genes for replication, viral movement, and the coat protein, the latter expressed from a subgenomic promoter. Reporter genes such as gusA and green fluorescent protein have been added to the PVX genome under the control of a duplicated coat protein subgenomic promoter and can be expressed at high levels in infected plants (Chapman et al. 1992), Baulcombe et al. 1995). As with the early TMV vectors, there is a tendency for the transgene to be lost by homologous recombination, but in the case of PVX, no alternative virus has been identified whose coat protein gene promoter can functionally substitute for the endogenous viral promoter. For this reason, PVX is generally not used for long-term expression, but has been widely employed as a transient expression vector. It has been used for the synthesis of valuable proteins such as antibodies (e.g. Hendy et al. 1999, Franconi et al. 1999, Ziegler et al. 2000) and for the expression of genes that affect plant physiology (.e.g. the fungal avirulence gene avr9, Hammond-Kosack et al. 1995).

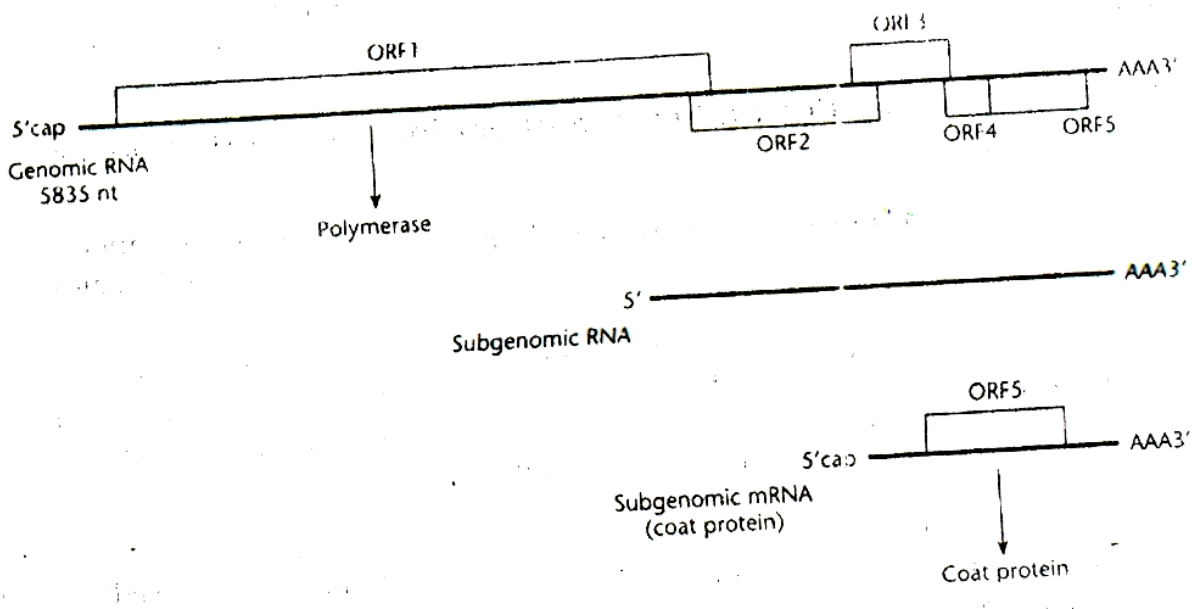


**Fig. Genome map and expression of potato virus X**

The stable transformation of plants with cDNA copies of the PVX genome potentially provides a strategy for extremely high-level transgene expression, because transcripts should be amplified to a high copy number during the viral replication cycle. However, instead of high-level expression, this strategy leads to potent and consistent transgene silencing, as well as resistance to viral infection (English et al. 1996, English & Baulcombe 1997). The basis of this phenomenon and its implications for transgene expression in plants. In terms of vector development, however, it is notable that PVX-based vectors are probably most widely used to study virus-induced gene silencing and related phenomena (Dalmay et al. 2000) and to deliberately induce silencing of homologous plant genes (reviewed by Baulcombe 1999).

**5. Write short notes on the following:-**

**i. Production of Herbicide resistance in transgenic plants**

**ii. Production of transgenic Bt cotton**

**i) Herbicide resistance is the most widespread trait in commercial transgenic plants**

Herbicides are used to kill weeds, and generally affect processes that are unique to plants, e.g. photosynthesis or amino acid biosynthesis. Both crops and weeds share these processes, and developing herbicides that are selective for weeds is very difficult. An alternative approach is to modify crop plants so that they become resistant to broad spectrum herbicides, i.e. incorporating selectivity into the plant itself rather than relying on the selectivity of the chemical. Two strategies to engineer herbicide resistance have been adopted. In the first, the target molecule in the cell either is rendered insensitive or is overproduced. In the second, a pathway that degrades or detoxifies the herbicide is introduced into the plant. An example of each strategy is considered

below.

**Table – Mode of action of herbicides and method of engineering herbicide-resistant plants**

| Herbicide | Pathway inhibited | Target enzyme | Basis of engineered resistance to herbicide |
|---|---|---|---|
| Glyphosate | Aromatic amino acid biosynthesis | 5-Enol-pyruvyl shikimate-3-phosphate (EPSP) synthase | Overexpression of plant EPSP gene or introduction of bacterial glyphosate-resistant aroA gene |
| Sulfonylurea | Branched-chain amino acid biosynthesis | Acetolactate synthase (ALS) | Introduction of resistant ALS gene. |
| Imidazolinories | Branched-chain amino acid biosynthesis | ALS | Introduction of mutant ALS gene |
| Phosphinothricin | Glutamine biosynthesis | Glutamine synthetase | Overexpression of glutamine synthetase or introduction of the bar gene, which detoxifies the herbicide |
| Atrazine | Photosystem II | $Q_B$ | Introduction of mutant gene for $Q_B$ protein or introduction of gene for glutathione-S-transferase, which can detoxify atrazines. |
| Bromoxynil | Photosynthesis | | Introduction of nitrilase gene, which detoxifies bromoxynil |

Glyphosate is a non-selective herbicide that inhibits 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase, a key enzyme in the biosynthesis of aromatic amino acids in plants and bacteria. A glyphosatetolerant Petunia hybrida cell line obtained after selection for glyphosate resistance was found to overproduce EPSP synthase as a result of gene amplification. A gene encoding the enzyme was subsequently isolated and introduced into petunia plants under the control of a CaMV promoter. Transgenic plantsexpressed increased levels of EPSP synthase in their chloroplasts and were significantly more tolerant to glyphosate (Shah et al. 1986). An alternative approach to glyphosate resistance has been to introduce a gene encoding a mutant EPSP synthase. This mutant enzyme retains its specific activity but has decreased affinity for the herbicide. Transgenic tomatoc plants expressing this gene under the control of an opine promoter were also glyphosate tolerant (Comai et al. 1985). Following on from this early research, several companies

have introduced glyphosate tolerance into a range of crop species, with soybean and cotton the first to reach commercialization (Padgette et al. 1996, Nida et al. 1996). Currently, nearly three-quarters of all transgenic plants in the world are resistant to glyphosate (James 2004).

Phosphinothricin (PPT) is an irreversible inhibitor of glutamine synthetase in plants and bacteria. Bialaphos, produced by Streptomyces hygroscopicus consists of PPT and two alanine residues. When these residues are removed by peptidases the herbicidal component PPT is released. To prevent self-inhibition of growth, bialaphos-producing strains of S. hygroscopicus also produce the enzyme phosphinothricin acetyultransferase (PAT), which inactivates PPT by acetylation. The bar gene that encodes the acetylase has been introduced into potato, tobacco, and tomato cells using Agrobacterium-mediated transformation. The resultant plants were resistant to commercial formulations of PPT and bialaphos in the laboratory (De Block et al. 1987) and in the field (De Greef et al. 1989). More recently, it has been shown that bialaphos-resistant transgenic rice plants which were inoculated with the fungi causing sheath blight disease and subsequently treated with the herbicide were completely protected from infection (Uchimiya et al. 1993). This agronomically important result depends on the observation that bialaphos is toxic to fungi as well as being a herbicide. PPT resistance is widely used in plants as a selectable marker, however, it has also been introduced into a number of different crops for weed control, including sugarcane and rice (Gallo-Meagher & Irvine 1996, Oard et al. 1996).

The benefit of herbicide-resistant transgenic crops is the increased yield and seed quality as competing weed species are eliminated. However, there was initial concern that this would come with an associated penalty of increased herbicide use, which could have a serious impact on the environment. Contrary to these predictions, the introduction of herbicide resistant plants has actually reduced chemical use by up to 80% in many areas, as farmers adopt better weed-control policies and switch to herbicides with low use rates. A further risk is that transgenes for herbicide tolerance could spread to weed species, resulting in a new breed of "superweeds" (Kling 1996). It is too early to say whether this will be a problem. Although a range of herbicide-resistant transgenic crops is being tested, only crops resistant to glyphosate or bromoxynil are currently grown on a commercial scale. The benefits and risks of herbicide-resistant crops have been reviewed (Gressel 1999, 2000). Although herbicide resistance is the most common strategy for weed control, several others are being evaluated including enhanced alelopathy, in which crop plants are modified to produce their own weed-inhibiting compounds (reviewed by Dukeet al. 2002). Typically, the primary crop is the target of modification, but such approaches can also be used to produce aggressive "cover crops" that inhibit weeds before the primary crop is planted. There has been recent interest in the use of suicide transgenes inducible by temperature or photoperiod cues to cause cover crops to self destruct at the correct time of year (Stanilaus & Cheng 2002). It is also possible to modify microbial pathogens so that they preferentially attack weeds (e.g. Amsellum et al. 2002).

**(ii) The bacterium Bacillus thuringiensis provides the major source of insect-resistant genes**

Insect pests represent one of the most serious biotic constraints to crop production. For example, more than one-quarter of all the rice grown in the world is lost to insect pests, at an estimated cost of nearly $50 billion. This is despite an annual expenditure of approximately $1.5 billion on insecticides for this crop alone. Insect-resistant plants are therefore desirable not only because of the potential increased yields, but also because the need for insecticides is eliminated and, following on from this, the undesirable accumulation of such chemicals in the environment is avoided. Typical insecticides are non-selective, so they kill harmless and beneficial insects as well as pests. For these reasons, transgenic plants have been generated expressing toxins that are selective for particular insect species.

Research is being carried out on a wide range of insecticidal proteins from diverse sources. However, all commercially produced insect-resistant transgenic crops express toxin proteins from the Gram-positive bacterium Bacillus thuringiensis (Bt) (Peferoen et al. 1997, de Maagd et al. 1999). Unlike other Bacillus species, B. turingiensis produces crystals during sporulation, comprising one or a small number of ~130 kDa protoxins called crystal proteins. These proteins are potent and highly specific insecticides. The specificity reflects interactions between the crystal proteins and receptors in the insect midgut. In susceptile species, ingested crystals dissolve in the alkaline conditions of the gut and the protoxins are activated by gut proteases. The active toxins bid to receptors on midgut epithelial cells, become inserted into the plasma membrane, and from pores that lead to cell death (and eventual insect death) through osmotic lysis. Approximately 150 distinct Bt toxins have been identified and each shows a unique spectrum of activity (van Frankenhuyzen & Nystrom 2002).

` Bt toxins have been used as topical insecticides since the 1930s, but never gained widespread use because they are rapidly broken down on exposure to daylight, and thus have to be applied several times during a growing season. Additionally, only insects infesting the exposed surfaces of sprayed plants are killed. These problems have been addressed by the expression of crystal proteins in transgenic plants. Bt genes were initially introduced into tomato (Fischhoff et al. 1987) and tobacco (Vaeck et al. 1987, Barton et al. 1987) and later cotton (Perlak et al. 1990) resulting in the production of insecticidal proteins that protected the plants from insect infestation. However, field tests of these plants revealed that higher levels of the toxin in the plant tissue would be required to obtain commercially useful plants (Delannay et al. 1989). Attempts to increase the expression of the toxin gene in plants by the use of different promoters, fusion proteins, and leader sequences were not successful. However, examination of the bacterial cry1Ab and cry1Ac genes indicated that they differed significantly from plant genes in a number of ways (Perlak et al. 1991). For example, localized AT rich regions resembling plan, introns, potential plant polyadenylation signal sequences. ATTTA sequences which can destabilize mRNA, and rare plant codons were all found. The elimination of undesirable sequences and modifications to bring codon usage into line with the host species resulted in greatly enhanced expression of the

insecticidal toxin and strong insect resistance of the transgenic plants in field tests (Koziel et al. 1993). By carrying out such enhancements, Perlak and colleagues expressed a modified cry3A gene in potato to provide resistance against Colorado beetle (Perlak et al. 1993). In 1995, this crop became the first transgenic insect-resistant crop to reach commercial production, as NewLeaf™ potato marketed by Monsanto. The same company also released the first commercial transgenic, insect-resistant varieties of cotton (Bollgard™, expressing cry 1Ac and protected against tobacco bollworm) and maize (YieldGard™ expressing cry 1 Ab and resistant to the European corn borer). Many other biotechnology companies have now produced Bt-transgenic crop plants resistant to a range of insects (reviewed by Schuler et al. 1998, de Maagd et al. 1999, Hilder & Boulter 1999, Liewellyn & Higgins 2002). Some of these have been extraordinarily successful: for example, Tu et al. (2000) showed that a Bt commercial hybrid variety expressing the cry 1Ab gene produced a 28% yield increase compared to wild-type plants in field trials in China.

Although Bt-transgenic plants currently dominate the market, there are many alternative insecticidal proteins under investigation. Two types of protein are being studied in particular: proteins that inhibit digestive enzymes in the insect gut (proteinase and amylase inhibitors) and lectins (carbohydrate-binding proteins). Research into these alternatives is driven in part by the fact that some insects are not affected by any of the known Bt crystal proteins. Homopteran insects, mostly sap-sucking pests such as planthoppers, fall into this category, but have been shown to be susceptible to lectins such as Galanthus nivalis agglutinin (GNA). This lectin has been expressed in many crops, including potato (Shi et al. 1994. Gatehouse et al. 1996), rice (Bano Maqbool & Christou 1999), tomato and tobacco (reviewed by Schuler et al. 1998).

**6. Explain the method of vitamin A in cereals (Rice) through transgenic technology.**

**The production of vitamin A in cereals is an example of extending an endogenous metabolic pathway**

Although the metabolic pathways for vitamin biosynthesis have been more fully elucidated in microbes than in plants, the engineering of plants for enhanced vitamin synthesis is now gaining much attention (Herbers 2003), Vitamin A, or 11-cis-retinal, is a dietary component required in all human cells but it is particularly important in the eye, where it functions as the lipid prosthetic group of the visual pigment opsin. Vitamin A deficiency is a significant health threat in the developing world, and is the most common (yet preventable) cause of blindness in developing countries. Humans usually obtain vitamin A directly from animal sources, but can synthesize it if provided with its immediate precursor, provitamin A (β-carotene), which is present at high levels in certain fruits and vegetables. The recommended daily allowance of vitamin A is expressed as retinol equivalents, and is equal to about 6 mg of β-carotene per day. There is very little β-carotene in cereal gains, which represent the staple diet for many of the word's poorest people.

The synthesis of carotenes in plants begins with the linkage of two geranylgeranyl

diphosphate molecules to form the precursor phytoene. The conversion of phytoene into β-carotene requires three further enzymatic steps. All four steps are absent in cereal endosperm tissue, so cereal grains accumulate geranylgeranyl diphosphate but not the downstream metabolic products in the pathway. The synthesis of β-carotene in cereals

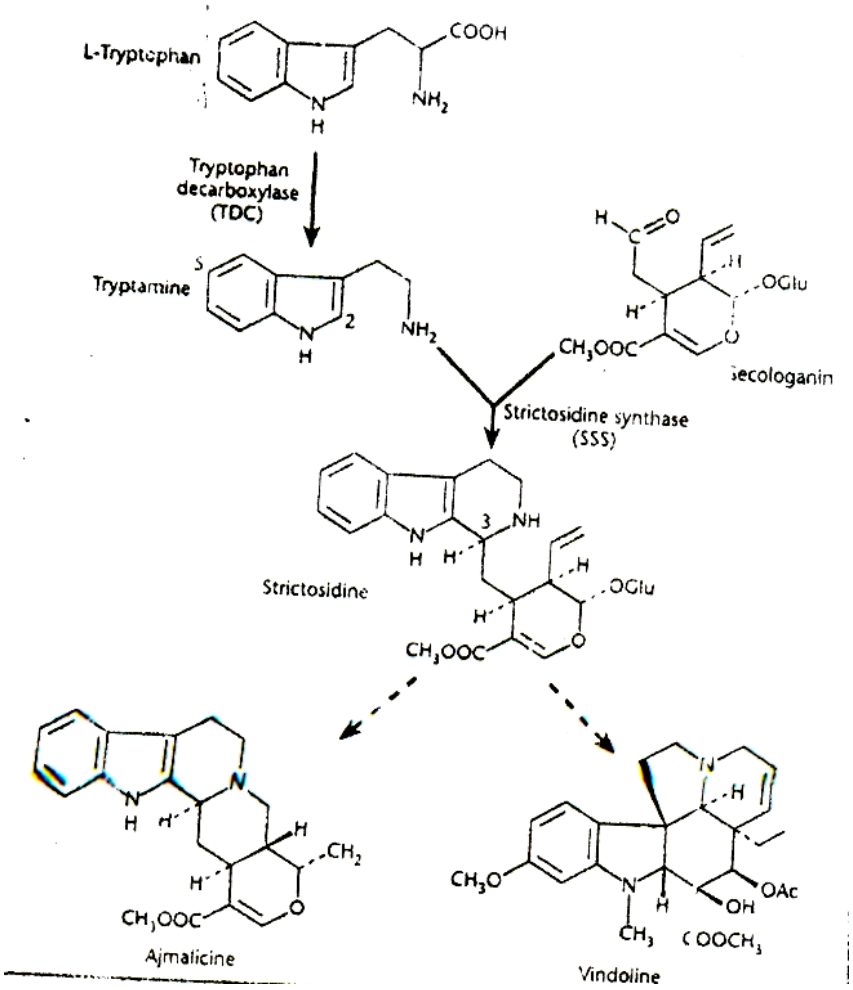



**Fig. Abbreviated pathway of terpene indole alkaloid biosynthesis, showing the conversion of Tryptophan into tryptamine by the enzyme Tryptophan decarboxylase, the condensation of tryptamine and secologanin into strictosidine, and the later diversification of strictosidine into valuable akaloids, such as ajmalicine and vindoline (a precursor of both vinblastine and vincristine).**

therefore represents an example of metabolic pathway extension, where novel enzymatic activities must be introduced into the plant and expressed in the endosperm to extend the pathway beyond its endogenous end point. The four enzyme activities in the β-carotene synthesis pathway missing in cereal grains are phytoene synthase, phytoene desaturase, ζ-carotene desaturase, and Iycopene β-cyclase. The first major breakthrough was the development of rice grains accumulating phytoene. Burkhardt et al. (1997) described rice plants transformed with the daffodil (Narcissus pseudonarcissus) phytoene synthase gene, which accumulated high levels of this metabolic

intermediate. Further work by the same group (Ye et al. 2000) produced transgenic rice plants expressing the daffodil genes encoding phytoene synthase and lycopene β-cyclase, and the crtl gene from the bacterium Erwinia uredovora, which encodes an enzyme with both phytoene desaturase and ζ-carotene desaturase activities. The daffodil genes were expressed under the control of the rice glutelin-1 promoter, which is endosperm specific, while the bacterial gene was controlled by the constitutive cauliflower mosaic virus (CaMV) 35S promoter. This ground-breaking, multi-gene engineering approach resulted in golden colored rice grains containing up to 2 μg7g of β-carotene, in which case a moderate rice meal of 100 g would represent about 10% of the RDA for vitamin A. Interestingly, similar results were achieved in rice plants containing phytoene synthase and the bacterial crtl gene but lacking lycopene β-cyclase (Beyer et al. 2002). This suggested either that rice gains contain a residual endogenous lycopene β-cyclase activity or that the endogenous enzyme is dormant in wild-type grains but induced in the transgenic grains by the high levels of metabolic intermediates.

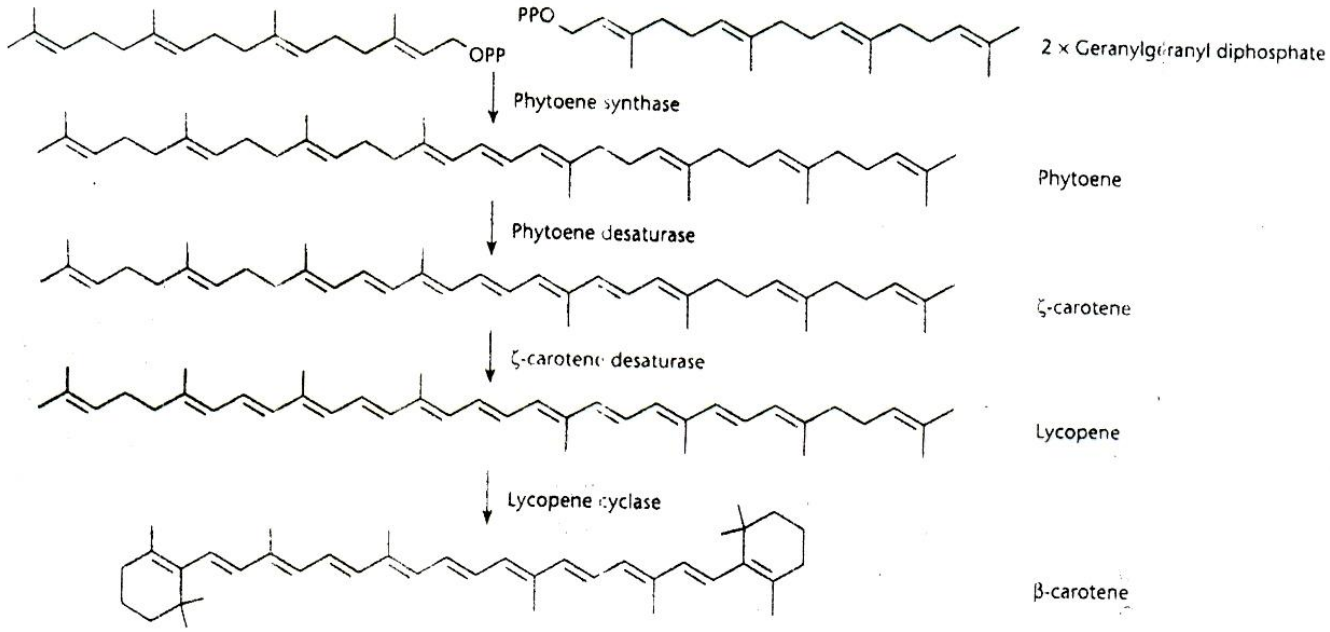



**Fig. Enzymatic steps and metabolic products in the β-carotene biosynthesis pathway which are missing in cereal grains**

The "golden Rice" project represented not just a technological breakthrough but also a model of humanitarian science that serves as an example for the deployment of other crops addressing food insecurity. From the beginning, the clear aim of the project organizers was to maintain freedom to operate and to provide the technology free of charge to subsistence farmers in developing countries, a feat that required careful negotiation over more than 100 intellectual and technical property rights (Potrykus 2001). Golden Rice fulfils an urgent need: it complements traditional interventions for vitamin A deficiency and provides a real opportunity to address a significant world health problem. It was developed to benefit the poor and disadvantaged, and

will be given away to subsistence farmers with no attached conditions. It requires no additional inputs compared with other rice varieties. To avoid biosafety concerns, Golden Rice lines have been generated with the innocuous metabolic selection marker mpl, which allows regenerating plants to grow when mannose is the only carbon source and avoids the use of antibiotics or herbicides for selection (Lucca et al. 2001).

While β-carotene synthesis in rice has the greatest potential to address real food security and health problems, experiments in other plants have revealed further useful information about this important metabolic pathway. Transgenic tomatoes have been described expressing E. uredovora phytoene synthase (crtB) (Fraser et al. 2000) and crtI (Romer et al. 2000) as well as a β-cyclase gene from Arabidopsis thaliana (Rosati et al. 2000). In the first case, fruit-specific expression of crtB was achieved using the tomato polygalacturonase promoter, and the recombinant protein was directed to the chromoplasts using the tomato phytoene synthase-1 transit sequence. Total fruit carotenoids were found to be two-to four-fold higher than in wild-type plants. Romer et al. (2000) expressed crtI constitutively, under the control of the CaMV 35S promote. This unexpectedly reduced the total carotene content by about 50% but the levels of β-carotene increased threefold to 520 μg/g dry weight. This probably reflects the existence of complex feedback mechanisms acting at several different levels, a possibility discussed in detail by Giuliano et al. (2000). Rosati et al. (2000) usedthe fruit-specific tomato phytoene desaturase promoter to express the A. thaliana β-lycopene cyclase gene and increased β-carotene levels in transgenic fruits to 60 μg/g fresh weight. Work is ongoing to determine how the β-carotene pathway is regulated and what steps need to be taken to overcome feedback control.

**7. Transgenic animals can be created as models of human disease – Discuss with example.**

**Transgenic animals can be created as models of human disease**

Mammals have been used as models for human disease for many years, since they can be exploited to carry out detailed analyses of the molecular basis of disease and to test newly developed therapeutics prior to clinical trials in humans. Before the advent of transgenic animal technology, however, models of inherited diseases (i.e. diseases with a genetic basis) were difficult to come by. Tey could be obtained as spontaneously occurring mutants, suitable mutant animals identified in mutagenesis screens, and susceptible animal strains obtained by selective breeding. Gene manipulation now offers a range of alternative strategies to create specific disease models (see reviews by Smithies 1993, Bedell et al. 1997, Petters & Sommer 2000).

Some of the earliest transgenic disease models were mice predisposed to particular forms of cancer because the germ line contained exogenously derived oncogenes (e.g. Sinn et al. 1987). This exemplifies so-called gain-of-function diseases, which are caused by a dominantly acting allele and can e modeled simply by adding that allele to the normal genome. E.g. by microinjection into

eggs. Other gain-of-function diseases that have been modeled in this way include Gerstmann-Straussler-Scheinker (GSS) syndrome, a neurodegenerative disease caused by a dominantly acting mutated prion protein gene. In one patient suffering from this disease, a mutation was identified in codon 102 of the prion protein gene. Transgenic mice were created carrying this mutant form of the gene in addition to the wild-type locus, and were shown to develop a similar neurodegenerative pathology to their human counterparts (Hsiao et al. 1990). Other examples of gain-of-function disease models include Alzheimer's disease, which was modeled by overexpression of the amyloid precursor protein (Quon et al. 1991), and the triplet repeat disorder spinocerebellar ataxia type 1 (Burright et al. 1995). Simple transgene addition can also be used to model diseases caused by dominant negative alleles, as shown for the premature aging disease. Werner'ssyndrome (Wang et al. 2000).

Recessively inherited diseases are generally caused by loss of function, and these can be modeled by gene knockout. The earliest report of this strategy was a mouse model for HRPT deficiency, generated by disrupting the gene for hypoxanthine-guanine phosphoribosyltransferase (Keuhn et al. 1987). A large number of genes has been modeled in this way, including cystic librosis (Snouwaert et al. 1992 Dorin et al. 1992), fragile-X syndrome (Dutch-Belgian Fragile X Consortium 1994), β-thallasemia (Skow et al. 1983, Ciavatti et al. 1995), and mitochondrial cardiomyopathy (Li et al. 2000). Gene targeting has been widely used to model human cancers caused by the inactivation of tumor suppressor genes such as TP53 and RBI (reviewed by Ghebranious & Donchower 1998, Macleod & Jacks 1999).

While the studies above provide models of single gene defects in humans, attention is now shifting towards the modeling of more complex diseases that involve multiple genes. This is a challenging area of research but there have been some encouraging early successes. In some cases, the crossing of different modified mouse lines has led to interesting discoveries. For example, undulated mutant mice lack the gene encoding the transcription factor I ax-1, and Patch mutant mice are heterozygous for a null allele of the platelet-derived growth factor gene. Hybrid offspring from a mating between these two strains were shown to model the humzan birth defect spina bifida occulta (Helwig et al. 1995). In other cases, such crosses have pointed the way to possible novel therapies. For example, transgenic mice over expressing human α-globin and a mutant form of the human β-globin gene that promotes polymerization provide good models of sickle cell anemia (Trudel et al. 1991). However, when these mice are crossed to those ectopically expressing human fetal hemoglobin in adulthood, the resulting transgenic hybrids show a remarkable reduction in disease symptoms (Blouin et al. 2000). Similarly, crossing transgenic mice over expressing the anti-apoptotic protein Bcl-2 to rds mutants that show inherited slow retinal degeneration resulted in hybrid off-spring in which retinal degeneration was strikingly reduced. This indicates that Bcl-2 could possibly be used in gene therapy to treat the equivalent human retinal degeneration syndrome (Nir et al. 2000).

The most complex diseases involve many genes and transgenic models would be difficult

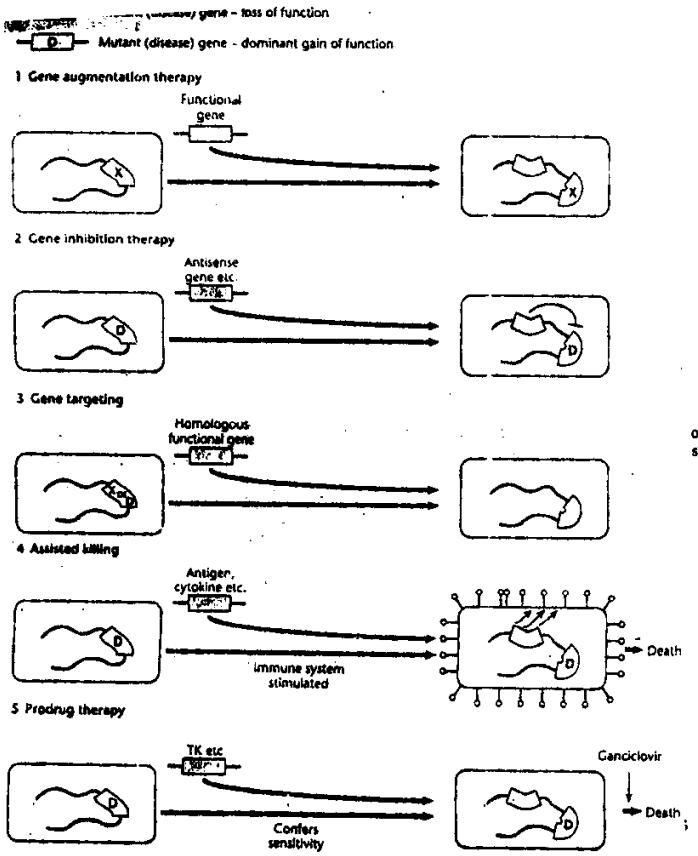
to create. However, it is often the case that such diseases can be reduced to a small number of "major genes" with severe effects, and a larger number of minor genes. Thus, it has been possible to create mouse models of Down syndrome, which in humans is generally caused by the presence of three copies of chromosome 21. Trisomy for the equivalent mouse chromosome 16 is a poor model because the two chromosomes do not contain all the same genes. However, a critical region for Down syndrome has been identified by studying Downs patients with partial duplications of chromosome 21. The generation of YAC transgenic mice carrying this essential region provides a useful model of the disorder (Smith et al. 1997) and has identified increased dosage of the Dyrk 1a (minibrain) gene as an important component of the learning defects accompanying the disease. Animal models of Down syndrome have been reviewed (Kola & Hertzog 1998, Reeves et al. 2001).

**Gene medicine is the use of nucleic acids to prevent, treat, or cure disease**

While disease modeling uses gene manipulation to create diseases in model organisms, gene medicine refers to the use of the same technology to ameliorate or even permanently cure diseases in humans. Gene medicine has a wide scope and includes the use of DNA vaccines, the targeted killing of disease cells (e.g. cancer cells), the use of oligonucleotides as drugs, and the use of gene transfer to correct genetic defects (gene therapy). Gene transfer can be carried out in cultured cells, which are then reintroduced into the patient, or DNA can be transferred to the patient in vivo, directly or by using viral vectors. The ex vivo approach can be applied only to certain tissues, such as bone marrow, in which the cells are amenable to culture. Gene therapy can be used to treat diseases caused by mutations in the patient's own DNA (inherited disorders, cancers), as well as infectious diseases, and is particularly valuable in cases where no conventional treatment exists, or that treatment is inherently risky. Strategies include:

- gene augmentation therapy (GAT), where DNA is added to the genome with the aim of replacing a missing gene product;
- gene targeting to correct mutant alleles;
- gene inhibition therapy using techniques such as antisense RNA expression or the expression of intracellular antibodies to treat dominantly acting diseases.;
- the targeted ablation of specific cells.

Therapeutic gene transfer effectively generates transgenic human cell clones, and for this reason only somatic cells can be used as targets. The prospect of germ-line transgenesis in humans raises serious ethical concerns, and with the rapid advances in technology allowing germ-line transformation and nuclear transfer in numerous mammals,




Overview of gene-therapy strategies.

**Fig. Overview of gene –therapy strategies**

these concerns will need to be addressed in the very near future (Johnson 1998). As an alternative to permanent gene transfer, transient gene therapy can be achieved using oligonucleotides, which can disrupt gene expression at many levels but do not permanently change the genetic material of the cell (Pollock & Gaken 1995).

The tools and techniques for gene therapy are essentially similar to those used for gene transfer to any animal cells. Transfection, direct delivery, or transduction can be used to introduce DNA into cells. Viral vectors are most popular because of their efficiency of gene transfer in vivo. However, extreme precautions need to be taken to ensure the safety of such vectors, avoiding potential problems such as the production of infectious viruses by recombination, and pathological effects of viral replication. A number of viral vectors have been developed for gene therapy, including those based on oncoretroviruses, lentiviruses, adenovirus, adeno-associated virus, herps virus and a number of hybrid vectors combining advantageous elements of different parental viruses (Robbins et al. 1998, Reynolds et al. 1999). The risks associated with viral vectors have promoted research into other delivery methods, the most popular of which include direct injection of DNA into tissues (e.g. muscle), the injection of liposome-DNA complexes into the blood, and direct transfer by particle bombardment or other methods. Although inherently much safer than viruses, such procedures show a generally low efficiency (Scheule & Cheng 1996, Tseng

& Huang 1998, Kay et al. 2001).

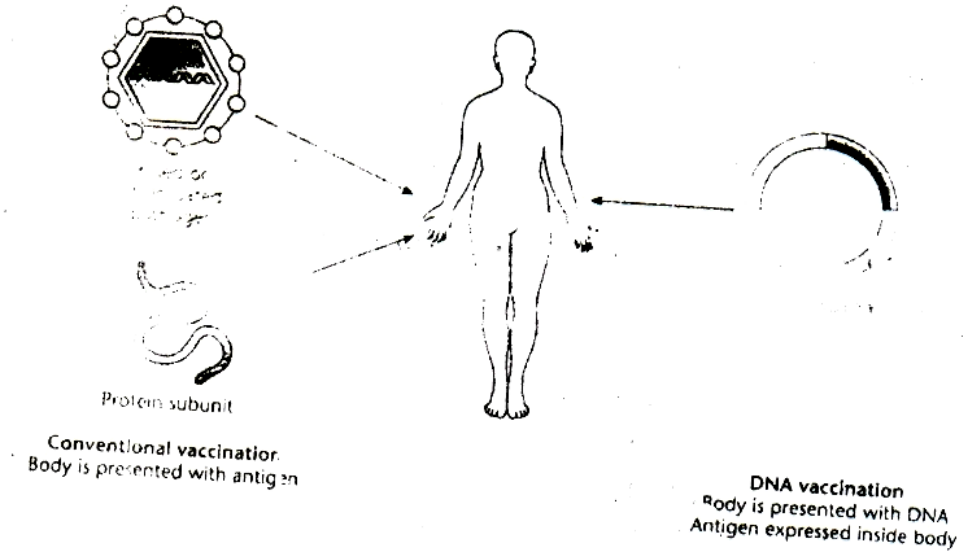



**Fig. A comparison of conventional vaccination and DNA vaccination**

**DNA vaccines are expression constructs whose products stimulate the immune system**

The immune system generates antibodies in response to the recognition of proteins and other large molecules carried by pathogens. With typical vaccines, the functional component of the vaccine introduced into the host is the protein that elicits the immune response. The introduction of DNA into animals does not generate an immune response against the DNA molecule, but, jf that DNA is expressed to yield a protein, that protein can stimulate the immune system (Reyes-Sandoval & Ertl 2001). This is the basis of DNA vaccination, as first demonstrated by Ulmer et al. (1993), DNA vaccines generally comprise a bacterial plasmid carrying a gene encoding the appropriate antigen under the control of a strong promoter that is recognized by the host cell. The advantages of this method include its simplicity, its wide applicability, and the ease with which large quantities of the vaccine can be produced. The DNA may be administered by injection, using liposome's or by particle bombardment. In the original demonstration, Ulmer and colleagues introduced DNA corresponding to the influenza virus nucleoprotein and achieved protection against influenza infection. Since then, many DNA vaccines have been used to target viruses (e.g. measles (Cardoso et al. 1996): HIV (Wang et al. 1993, Fuller et al. 1997, Hinkula et al. 1997); Ebola virus (Xu et al. 1998)), other pathogens (e.g. tuberculosis (Huygen et al. 1996)), and even the human cellular prion protein in mice (Krasemann et al. 1996).

The DNA –vaccination approach has several additional advantages. These include the following:-

- Certain bacterial DNA sequences have the innate ability to stimulate the immune system (see Klinman et al. 1997, Roman et al. 1997).

- Other genes encoding proteins influencing the function of the immune response can be co-introduced along with the vaccine (e.g. Kim et al. 1997).
- DNA vaccination can be used to treat diseases that are already established as a chronic infection (e.g. Mancini et al. 1996).

In principle, DNA vaccination has much in common with gene therapy, since both involve DNA transfer to humans, using a similar selection of methods. However, while the aim of gene therapy is to alleviate disease, by either replacing a lost gene or blocking the expression of a dominantly acting gene, the aim of DNA vaccination is to prevent disease, by causing the expression of an antigen that stimulates the immune system.

**Gene augmentation therapy for recessive diseases involves transferring a functional copy of the gene into the genome**

The first human genetic engineering experiment was one of gene marking rather than gene therapy, and was designed to demonstrate that an exogenous gene could be safely transferred into a patient and that this gene could subsequently be detected in cells removed from the patient. Both objectives were met. Tumor-infiltrating lymphocytes (cells that naturally seek out cancer cells and then kill them by secreting proteins such as tumor necrosis factor, TNF) were isolated from patients with advanced cancer. The cells were then genetically marked with a neomycin resistance gene and injected back into the same patient (Rosenberg et al.1990).

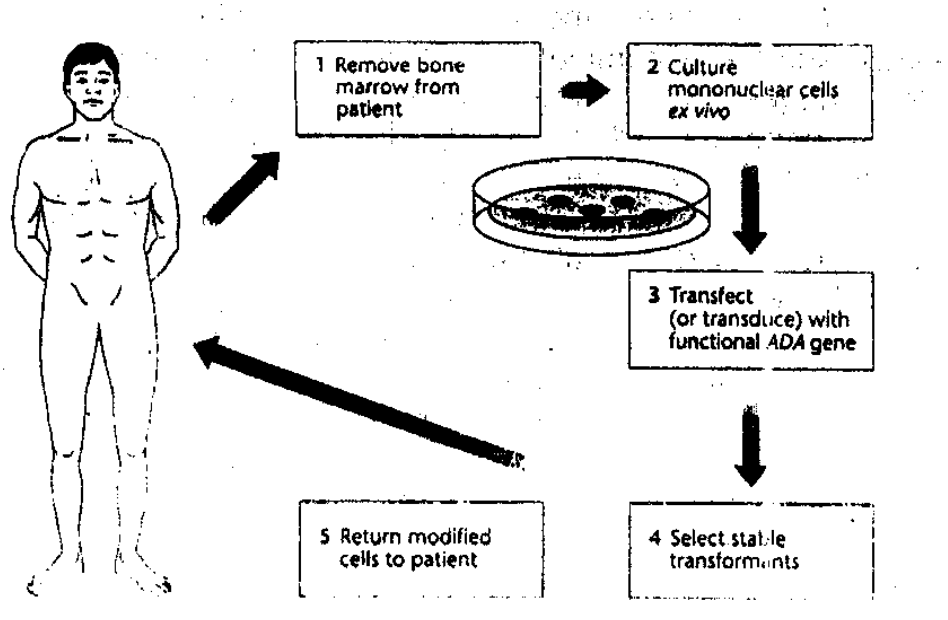



**Fig. Procedure for ex vivo gene therapy, based on the treatment of ADA deficiency**

The first clinical trial using a therapeutic gene-transfer procedure involved a four-year-old female patient. Ashanthi DeSilva, suffering from severe combined immune deficiency, resulting

from the absence of the enzyme adenosine deaminas (ADA). This disease fitted many of the ideal criteria for gene-therapy experimentation. The disease was life threatening (therefore making the possibility of unknown treatment-related side-effects ethically acceptable) but the corresponding gene had been cloned and the biochemical basis of the disease was understood. Importantly, since ADA functions in the salvage pathway of nucleotide biosynthesis cells in which the genetic lesion had been corrected had a selective growth advantage over mutant cells, allowing them to be identified and isolated in vitro. Conventional treatment for ADA deficiency involves bone marrow transplantation from a matching donor. Essentially the same established procedure could be used for gene therapy, but the bone marrow cells would be derived from the patient herself, and would be genetically modified ex vivo. Cells from the patient were subjected to leukopheresis and mononuclear cells were isolated. These were grown in culture under conditions that stimulated T-lymphocyte activation and growth and then transduced with a retroviral vector carrying a normal ADA gene as well as the neomycin-resistance gene. Following infusion of these modified cells, both this patient and a second, who began treatment in early 1991, showed an improvement in their clinical condition as well as in a battery of in vitro and in vivo immune function studies (Anderson 1992). However, the production of recombinant ADA in these patients is transient, so each must undergo regular infusions of recombinant T-lymphocytes. Research is ongoing into procedures for the transformation of bone marrow stem cells, which would provide a permanent supply of corrected cells.

Gene-augmentation therapies for a small number of recessive single-gene diseases are now undergoing clinical trials. We consider cystic librosis (CF) as an example (Davies et al. 2001). CF is a disorder which predominantly affects the lungs, liver, and pancreas. The disease is caused by the loss of a cAMP-regulated membrane-spanning chlorine channel. This results in an electrolyte imbalance and the accumulation of mucus, often leading to respiratory failure. CF is a recessive disorder, suggesting that the loss of function could be corrected by introducing a functional copy of the gene. Indeed, epithelial cells isolated from CF patients can be restored to normal by transfecting them with the cloned cystic fibrosis transmembrane regulator (CFTR) cDNA. Unlike ADA-deficiency, the cells principally affected by CF cannot be cultured and returned to the patient, so in vivo delivery strategies must be applied. Targeted delivery of the CFTR cDNA to affected cells has been achieved using adenoviral vectors, which have a natural tropism of the epithelial lining of the respiratory system. Recombinant viruses carrying the CFTR cDNA have been introduced into patients using an inhaler (Zabner et al. 1993, Hay et al. 1995, Knowles et al. 1995). The CFTR cDNA has also been introduced using liposomes (e.g. Caplen et al. 1995). While such treatments have resulted in CFTR transgene expression in the nasal epithelium, there were neither consistent changes in chloride transport nor reduction in the severity of CF symptoms: they have been largely ineffective.

**8. Explain in detail about the Gene augmentation therapy for recessive diseases through transfer of functional gene with suitable examples.**

**Gene augmentation therapy for recessive diseases involves transferring a functional copy of the gene into the genome**

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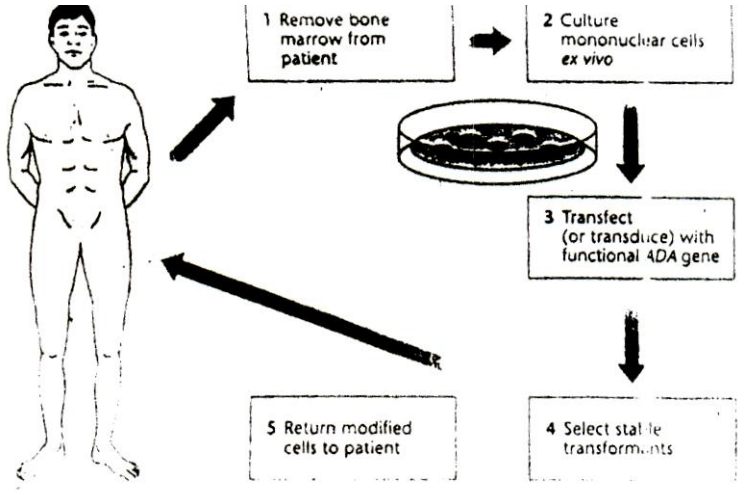


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**9. Discuss various methods developed for the production of transgenic mice.**

**Three major methods have been developed for the production of transgenic mice**

The ability to introduce DNA into the germline of mice is one of the greatest achievements of the twentieth century and has paved the way for the transformation of other mammals. Genetically modified mammals have been used not only to study gene function and regulation, but also as bioreactors producing valuable recombinant proteins, e.g. in their milk. Several methods for germline transformation have been developed, all of which require the removal of fertilized eggs or early embryos from donor mothers, brief culture in vitro and then their return to foster mothers, where development continues to term. These methods are discussed below and summarized in figure. Note that these methods have been developed with nuclear transgenesis in mind, but mitochondria have their own genome. Recently, methods have been developed for the production of mitochondrial transgenics (or transmitochondrial mice), which are considered in

Box 1.

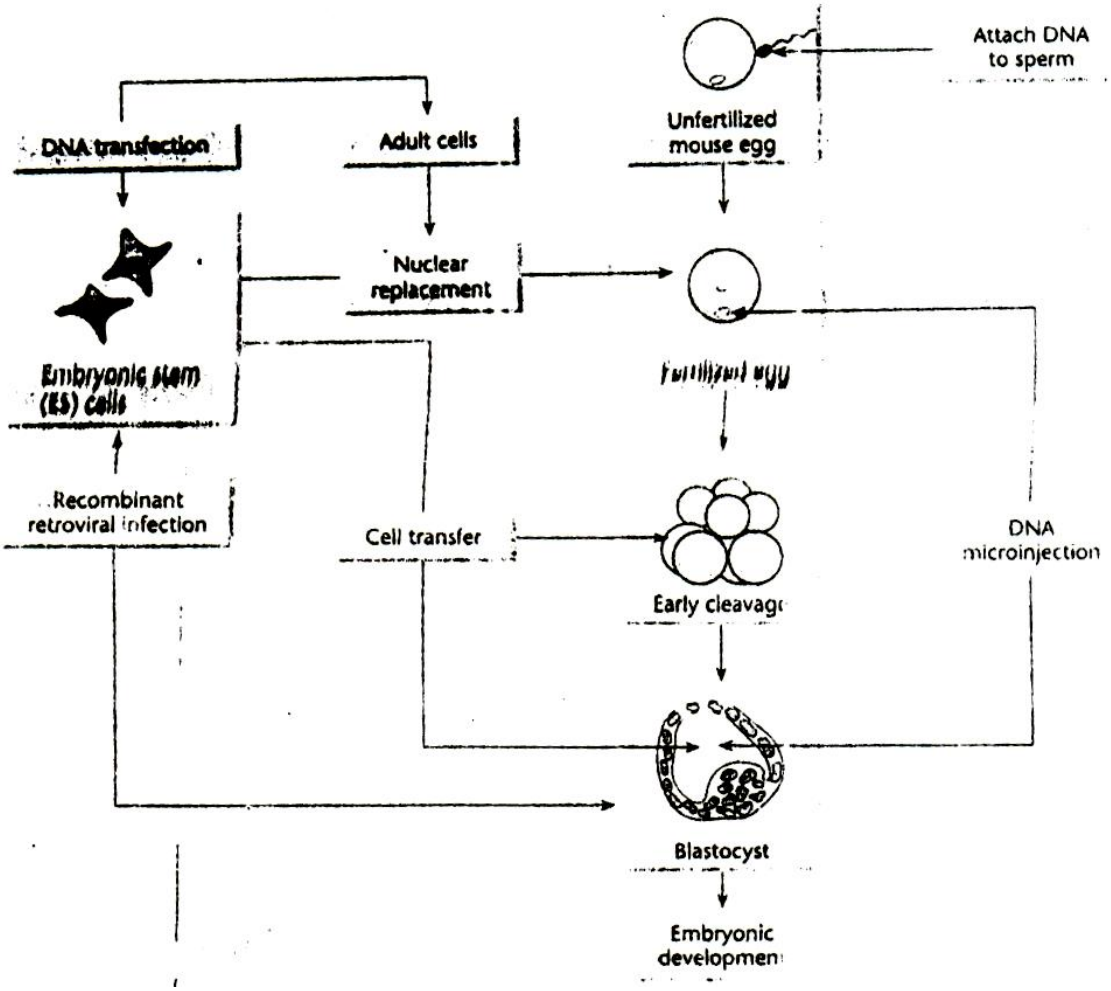


**Fig. Summary of methods for producing transgenic mammals**

**Pronuclear microinjection involves the direct transfer of DNA into the male pronucleus of the fertilized mouse egg**

Direct microinjection of DNA was the first strategy used to generate transgenic mice. Simian virus 40 (SV40) DNA was injected into the blastocoele cavities of preimplantation embryos by Jaenisch & Mintz (1974). The embryos were then implanted into the uteri of foster mothers and allowed to develop. The DNA was taken up by some of the embryonic cells and occasionally contributed to the germline, resulting in transgenic mice containing integrated SV40 DNA in the following generation. Transgenic mice have also been recovered following the injection of viral DNA into the cytoplasm of the fertilized egg (Harbers et al. 1981).

The technique that has become established is the injection of DNA into one of the pronuclei of the egg (reviewed by Palmiter & Brinster 1986). The technique is shown in figure. Just after

fertilization, the small egg nucleus (female pronucleus) and the large sperm nucleus (male pronucleus) are discrete. Since the male pronucleus is larger, this is usually chosen as the target for injection. About 2 pl of DNA solution is transferred into the nucleus through a fine needle, while the egg is held in position with a suction pipette. The injected embryos are cultured in vitro to the morula stage and then transferred to pseudopregnant foster mothers (Gordon & Ruddle 1981). The procedure requires specialized microinjection equipment and considerable dexterity from the handler. The exogenous DNA may integrate immediately or, less commonly, may remain extrachromosomal for one or more cell divisions. Thus the resulting animal may be transgenic or may be mosaic for transgene insertion. The technique is reliable, although the efficiency varies, so that 5-40% of mice developing from manipulated eggs contain the transgene (Lacy et al. 1983). However, once the transgene is transmitted through the germline, it tends to be stably inherited over many generations. The exogenous DNA tends to form head-to-tail arrays prior to integration, and the copy number varies from a few copies to hundreds. The site of integration appears random and may depend on the occurrence of natural chromosome breaks. Extensive deletions and rearrangements of the genomic DNA often accompany transgene integration (Bishop & Smith 1989).

**Box.1 Mitochondrial transgenesis in mice**

Several human genetic diseases are caused by mitochondrial mutations, and to model such diseases it is necessary to introduce DNA into the mitochondrial genome of suitable animals. This cannot be achieved using standard transgenesis techniques and a novel method has been developed in which donor cell lines carrying mitochondrial mutations are enucleated and then fused with recipient mouse zygotes. The resulting transmitochondrial mice carry the mutation in every cell and pass the mutation through the female germline, since mitochondria in the sperm only rarely contribute to the embryo.

The procedure for producing transmitochondrial mice is shown in figure. This involves five distinct steps. First, a cell line must be produced carrying mitochondria containing the appropriate transgene or mitochondrial mutation. There are many ways to achieve this, but one of the most efficient is fusion between cells depleted in mitochondrial DNA (mtDNA) and synaptosomes from aged mice which often contain mtDNA deletions and other mutations. The resulting cell lines are called cybrids because their cytoplasm is a hybrid of two cell lines. The next step is to remove the nucleus from the cybrid cells, so that they can act as mitochondrial donors without introducing additional nuclei into the egg. This is generally achieved by centrifugation in the presence of cytochalasin B. After washing the enucleated cybrids, zygotes are collected from donor females and a micromanipulator is used to drill a hole through the zona pellucida and place the cybrids in the perivitelline space. After a brief recovery period in culture, embryos containing cybrids are washed in a medium that promotes fusion and placed between the electrodes of a fusion chamber. Along AC pulse (225 V, 10 – 15s) is used to align the cytoplasts and oocytes, and then a brief DC

pulse (2500V, 20 ms) is used to induce fusion, which occurs within the next hour. After a brief recovery period in culture, the fused embryos are implanted into pseudopregnant foster mothers and raised to term.

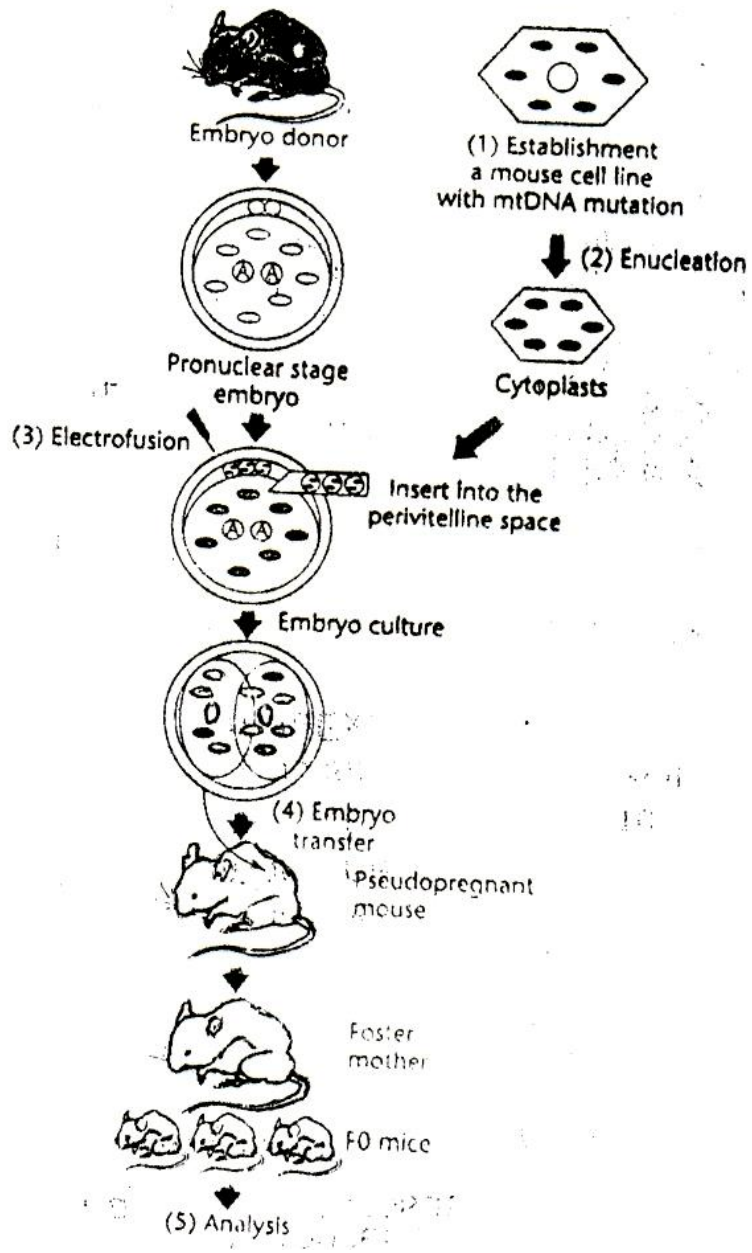


**Fig. Procedure for generating transmitochondrial mice.**

**Recombinant retroviruses can be used to transducer early embryos prior to the formation of the germline**

Recombinant retroviruses provide a natural mechanism for stably introducing DNA into the genome of animal cells. Retroviruses are able to infect early embryos (as well as ES cells), so

recombinant retroviral vectors can be used for germline transformation (Robertson et al. 1986). An advantage over the microinjection technique is that only a single copy of the retroviral provirus is integrated, and the genomic DNA surrounding the transgenic locus generally remains intact. The infection of preimplantation embryos with a recombinant retrovirus is technically straight forward and, once the infected embryos are implanted in the uterus of a foster mother, can lead to germline transmission of the transgene. However, there are also considerable disadvantages to this method, including the limited amount of foreign DNA that can be carried by the virus, the possible interference of viral regulatory elements with the expression of surrounding genes, and the susceptibility of the virus to de novo methylation, resulting in transgene silencing. The founder embryos are always mosaics with respect to transgene integration (reviewed by Jaenisch 1988). Retroviral transduction is therefore not favored as a method for generating fully transgenic animals, but it is useful for generating transgenic sectors of embryos. For example, the analysis of chicken-limb buds infected with recombinant retroviruses has allowed many of the genes involved in limb development to be functionally characterized (see review by Tickle & Eichele 1994).

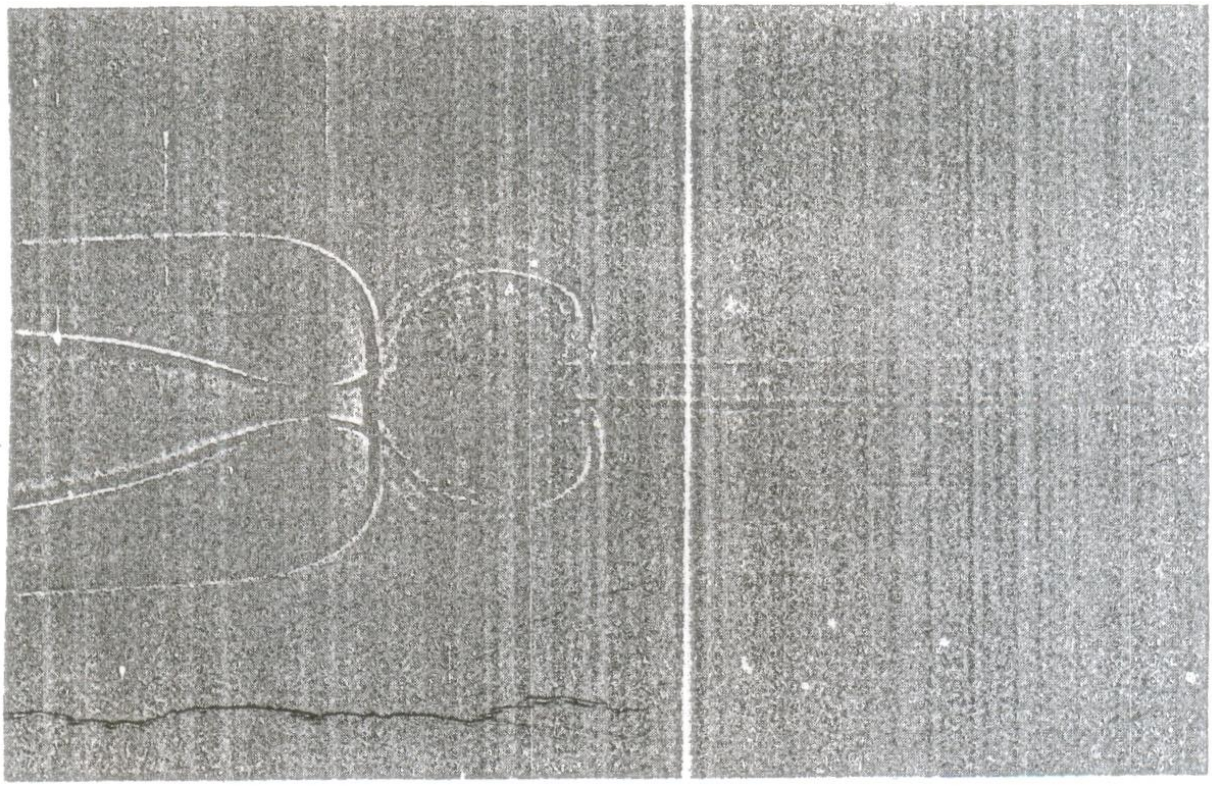

**Fig. Pronuclear microinjection of a fertilized mouse egg. The two pronuclei are visible, and the egg is held using a suction pipette. The DNA is introduced through a line glass needle.**

**Transgenic mice can be produced by the transfection of ES cells followed by the creation of chimeric embryos**

ES cells are derived from the inner cell mass of the mouse blastocyst and thus have the potential to contribute to all tissues of the developing embryo (Exans & Kaufman 1981, Martin 1981). The ability of ES cells to contribute to the germline was first demonstrated by Bradley et al. (1984) and requires culture conditions that maintain the cells in an undifferentiated state (Joyner 1998). Since these cells can be serially cultured like any other established cell line. DNA can be introduced by transfection or viral transduction and the transformed cells can be selected using standard markers. In contrast, since there is no convenient way to select for eggs or embryos that have taken up foreign DNA , each potential transgenic mouse generated by pronuclear microinjection must be tested by Southern blot hybridization or the PCR to confirm transgene integration.

Transfected ES that survive selection are introduced into the blastocoele of a host embryo at the blastocyst stage, where they mix with the inner cell mass. This creates a true chimeric embryo.[1] The contribution of ES cells to the germline can thus be confirmed using visible markers. Most ES cell lines in common use are derived from mouse strain 129 which has the dominant coat color agouti. A popular strategy is to use host embryos from a mouse strain such as C57BL/6J, which has a recessive black coat color. Colonization of the embryo by vigorous ES cells can be substantial, generating chimeras with patchwork coats of black and agouti cell clones. If the ES cells have contributed to the germline, mating chimeric males with black females will generate heterozygous transgenic offspring with the agouti coat color, confirming germline transmission of the foreign DNA. Most ES cells in use today are derived from male embryos, resulting in a large sex bias towards male chimeras (McMahon & Bradley 1990). This is desirable because male chimeras sire many more offspring than females. The procedure for generating transgenic mice using ES cells is shown in figure.

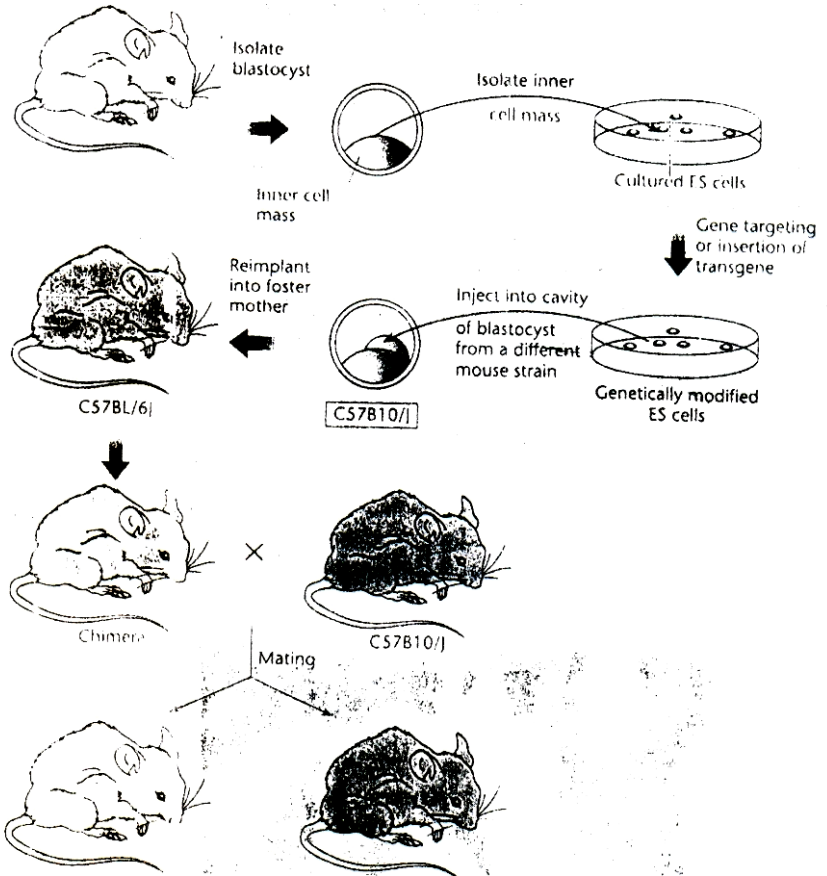

**Fig. Procedure for generating transgenic mice using transformed ES cells. The ES cells, which are manipulated in culture, contribute potentially to all the tissues of the embryo, including the germline. Germline transmission of the transgene can be confirmed by checking that offspring of chimeric embryos carry ES cell coat color markers.**

**10. Explain the Nuclear transfer technology to be used for cloning of Animals.**

**Nuclear transfer technology can be used to clone animal**

The failure of traditional transgenesis techniques to yield routine procedure for the genetic modification of mammals other than mice has driven researchers in search of other methods. Over fifty years ago, Briggs & King (1952) establihsed the principle of nuclear transfer in amphibians by tranplanting nuclei from the blastula of the frog Ranan pipens to an enucleated egg, obtaining a number of normal embryos in the process. In Xenopus laevis, nuclei from various types of cell in the swimming tadpole can be transplanted to an egg that has been UV-irradiated to destroy the peripheral chromosomes, and similar results are obtained (reviewed by Gurdon 1986, 1991). The important principle here is that, while animal cells become irreversibly committed to their fat as development proceeds, the nucei of most cells still retain all the genetic information required for the entire developmental progam and can, under appropriate circumstances, be reprogrammed by the cytoplasm of the gg ot recapitualte development. In all species, it appears that the earlier the developmental stage at which nuceic are isolated, the greater their potential to be reprogrammed. Nuclear transplantation can be used to generate clones of animals with the same genotype by

transplanting many somatic nuclei from the same individual into a series of enucleated eggs (King and Briggs 1956). This allows animals with specific and desirable traits to be propagated. If possible in mammals, this would have obvious applications in farming.

Nuclear transfer in mammals has ben practiced with success for the last decade, although rabbits and farm animals, such as sheept, pigs, and cows, are far more amenable to the process than mice. In each case, donor nuclei were obtained form he morula or blastocyst – stage embryo and transferred to an egg or oocyte from which the nucelus had been removed with a pipette (Smith & Wilmut 1989, Willadsen 1989, Collas & Robl 1990, Mc Laughlin et al. 1990). The donor nucleus can be introduced by promoting fusion between the egg and a somatic cell. A brief electic pulse is often used to achieve this, as it also activates embryonic development by stimulaating the mobilization of calcium ions.

A major advance was made in 1995, when two live lambs, Megan and Morag (fig.), were produced by nuclear transfer from culturd embroyonic cells (Campbell et al. 1996). This demonstrated the principle that mmalian nuclear transfer was possible using a cultured cell line. The same group later reported the birth of Dolly (fig.). following nuclear

**Fig. Megan and Morag, the first sheep produced by nuclear transfer from cultured cells. Reproduced by kind permission of the Roslin Instiute, Edinburgh.**

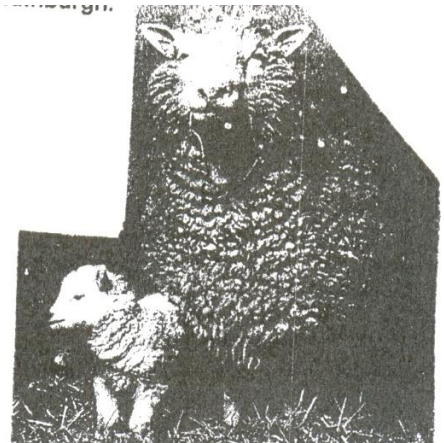

**Figure: Dolly and her lamb Bonnie, Dolly was the first mammal to be generated by nuclear transfer from an adult cell. Reproduced by kind permission of the Roslin institute, Edinburgh.**

Transfer from an adult mammary epithelial cell line Wilmut et. al 1997. This was the first mammal to reproduced by nuclear transfer from a differentiated adult cell, and aroused much debate among both scientists and the public concerning the possiblity of human cloning. It was suggested that a critical factor in the success of the experiment was the quiescent state of the cells in culture, allowing synchronization between the donor and recipient cell cycles (reviewed by Wilmut et. al 2002). For the production ofDolly, this was achieved by lowering the level of scrum in the culture medium, causing the cells to withdraw from the cell cycle due to lack of growth factors. However, the success rate was very low; only one of 250 transfer experiments produced a viable lamb, a phenomenon that has been blamed on a lack of fundamental understanding of the nuclear reprogramming events that occur following transplantation. Similar transfer experiments have since been carried out inmice, cows, pigs, goats, cats, dogs, rabbits, mules, and rats using variationson the transfer methodology developed by Wilmut and colleagues. Despitemajor efforts, there has been no success thus far in the production of a cloned primate although various individuals, nations, and religious sects have put forward as yet unsubstantiated claims to have produced the first cloned human being. There are also several ongoing projects looking at the feasibility of cloning rare animals or animals in captivity representing species that are extinct in the wild. For example, a cloned guar (a rare and endangered type of ox native to Asia) was born to a surrogate cow in 2001, although it died the same day from an infection.

The success of nuclear transfer in domestic mammals provides a new route for the production of transgenic animals. This involves the introduction of DNA into cultured cells, which are then used as a source of donor nuclei for nuclear transfer. Such a cell – based strategy has many advantages over traditional techniques, such as microinjection, including the ability to screen transformed cells for high – level transgene expression prior to the nuclear – transfer step. The production of a transgenic mammal by nuclear transfer from a transfected cell line was first achieved by Schnieke et. al (1997), who introduced the gene for human factor IX into fetal sheep fibroblasts and transferred the nuclei to enucleated eggs. The resulting sheep, Polly, produces the recombinant protein in her milk and can therefore be used as a bioreactor. McCreath et. al (2000) succeeded in producing a transgenic sheep by nuclear transfer from a somatic cell whose genome had been specifically modified by gene targeting. A foreign gene was introduced into the COLIAI

locus and was expressed at high levels in the lamb. Nuclear transfer was also used to produce the first "geneknockout" mammals other than nice: Denning produced cloned lambs with targeted disruptions of either the PRP gene, which encodes the prion protein, or the GGTAI gene, whch encodes an enzyme that adds carbohydrate groups to proteins that provoke the human immunesystem, one of the most significant obstacles to xenotransplantation, the transplantation of organs from animals such as monkey, sheep and pigs to human recipients. In 2002, two groups independently reported targeted distruption of the GGTAI gene in pigs, whose organs are similar in size to their human counter parts and are envisaged as the most likely source for xenotransplants. In each of these reports only one allele of the autosomal target gene was disrupted. The first homozygous GGTAI knockout pigs were produced by Phelps et. al (2003) using heterozygous cells derived from the earlier cloned pigs. The aim of the experiment was to disrupt the second GGTAI allele by gene targeting and then select cells lacking the gal – epitope using a fungal toxin. However, on further analysis it was established that the second allele had been produced by a spontaneous point mutation with in the GGTA1 gene, so in the strict sence the pigs were still heterozygous, although for two null alleles.

**Gene transfer to Xnopus can result in transient expression or germline transformation**

**Xenopus oocytes can be used as a heterologous expression system**

Gurdon et al. (1971) first showed that xenopus oocyttes synthesized large amounts of globin after they had been microinjected with rabbit globin mRNA. Since then, the Xenopus oocyte expression system has been a valuable tool for expressing a very wide range of proteins from plants and animals (Colman 1984). X. laevis is an Aftrican clawed fron. Oocytes can be obtained in large numbers by removal of the ovary of an adult female. Each fully grown oocyte is a large cell (0.8-1.2 mm diameter) arrrested at first meiotic prophase. This large cell has a correspondingly large nucelus (called the germinal vesicle). Which is located in the darkly pigmented hemisphere of the oocyte.

Due to large size of oocytes, mRNA – eiher natural or syntehsized by transcription in vitro, using phasge – T7 RNA polymerase (Melton 1987) – can be readily introduced into the cytoplasm or nucleus by microinjection. This is achieve using a finely drawn glass capillary as the injection needle, held in a simple micromanipulator. DNA can also be injected. The oocyte nucleus contains a store of the three eukaryotic RNA polymerases, enough to furnish the needs of the developing embryo at least until the 60,000-cell stage. The RNA polymerases are available for the transcription of injected exogenous DNA. Using this system, it has therefore been possible to express complementary DNAs (cDNAs) linked to a heat – shock promoter or to mammalian virus promoters (Ballivet et al. 1988, Ymer et al. 1989, Swick et al. 1992). In addition, vaccinia virus vectors (Chapter 12) can be used for gene expression in the cytoplasm (Yang et al. 1991).

An important aspect of the oocyte expression system is that recombinant proteins are usually correctly post – translationally modified and directed to the correct cellular compartment. For example, oocytes translate a wide variety of mRNAs encoding secretory proteins, modify them, and corrrectly secrete then (Lane et al. 1980), Colman et al. 1981). Foreign plasma – membrane proteins are generally targeted to the plasma membrane of the oocyte, where they can be shown to be functional. The fist plasma – membrane protein to be expressed in this system was the acetylcholine receptor ffom the electric organ of the ray. Torpedo marmorata (Sumikawa et al. 1981). Injected oocytes translated mRNA extracted from the electric organ and assembled functional multi – subunit receptor molecules in the plasma membrane (Barnard et al. 1982). Following this work, the oocyte has become a standard heterologous expression system for plasma – membrane proteins, including ion channels, carriers, and receptors. The variety of successfully expressed palsma membrane proteins is very impressiv however, the are examples of foreign channels and receptors being non – functional in oocytes. Either due to lack of coupling to second – messenger systems in the oocyte. Incorrect post – translatinal modification, or other reasons ( reviewed in Goidin 1991).

**B.E./B.TECH. DEGREE EXAMINATION, NOVEMBER/DECEMBER 2008.**

**FIFTH SEMESTER**

# BIOTECHNOLOGY

## BT 1305 - GENETIC ENGINEERING

## (REGULATION 2004)

## PART – A

1. Chromosomes are the cellular bearers of heredity. How?

2. Write a short note on translational control of protein synthesis by ribosomal proteins.

3. What is meant by shotgun cloning?

4. How DNA ligase in prokaryotes is different from eukaryotes?

5. What is invitro packaging?

6. What is meant by chromosome walking?

7. What is nested PCR? Give a situation in which you might use this.

8. How will you decide the annealing temperature of a PCR reaction?

9. What are the criteria to choose reporter gene?

10. What is animal pharming. Give an example.

## PART – B

11. (a) (i) What is RCGM and mention its role in biosafety regulation transgencies.
(b) (ii) What are ligases? Write notes on mode of action of DNA ligase?

Or

(b) Explain the role of the following in control of gene expression.

(i) promoters
(ii) Repressors
(iii) Attenuation
(iv) Nucleosomes

12. (a) (i) Explain briefly about lamba insertion and replacement vectors
(ii) Describe the main features of an extra chromosomal mammalian cell expression vector.

Or

(b) (i) Why sticky ends are preferred for cloning? How linkers and adaptors are used in rDNA technology?

(ii) What are the vectors suitable for insect cell lines? Explain its features with a schematic diagram.

13. (a) (i) Create a genomic DNA library using the Phase λ EMBL 3A vector.

(ii) Comment on the strategies for cDNA libraries

Or

(b) Explain different methods of screening of gene library.

14. (a) (i) Explain a case study that uses the application of PCR.

(ii) Discuss how PCR is used to synthesize a gene.

Or

(b) Comment on the following:

(i) Taqman assay
(ii) Molecular beacons
(iii) Kunkel's method
(iv) Transposon tagging.

15. (a) (i) Why the Ti plasmid in its natural form is not suitable for transgenics. List out the modifications required to develop it in to a transformation vector?

(ii) Enumerate the problems associated with genetically modified plants.

Or

(b) (i) What are knockout mice? How and why are they established?

(ii) How plant genes are cloned by transposable elements?

## B.E./B.TECH. DEGREE EXAMINATION, MAY/JUNE 2007.

## FIFTH SEMESTER

# BIOTECHNOLOGY

## BT 1305 - GENETIC ENGINEERING

## (REGULATION 2004)

## PART - A

1. Although all human cell do have the same genes, they are identical in their expression –Why?

2. Identify the DNA elements responsible for transcription of eukaryotic gene.

3. Write the principle features of pUC cloning system.

4. How is a fusion protein created?

5. Partial digestion of DNA is preferred for construction genomic libaries –why?

6. Explain the main feature of an extrachromosomal mammailian cell expression vector.

7. "Primer Walking"- What for?

8. Name any two method of synthesizing a 0.5- kb gene

9. How could you increase the activity of plant promoter?

10. What is nuclear cloning?

## PART – B

11. (a) Explain with suitable example (s), the differences among the four lands of restriction and modification enzymes.

Or

(b) (i) What criteria are followed by FDA for accepting a recombinant protein as a food or food additive?

(ii) Discuss the premise the recombinant DNA technology is a radical technology that violates the fundamental laws of nature.

12. (a) Explain four different ways of plasmid introduction into E.Coli.

Or

(b) (i) What is backmid? How is it used?

(ii) Enumerate the criteria used to decide, if a particular recombinant protein should be produced in a yeast, insect or mammalian cell system.

13. (a) Outline two different strategies employed to detect a cloned target gene within a library in E.Coli. What conditions must be satisfied for each type of detection.

Or

(b) (i) How would you use a bacteriophage λ or BAC as a cloning vector.

(ii) Plasmid pRIT 454 was cut with Pst I, Hind III and Eco RI. From the data given below,

determine the map. The frequent in Kb, and the name of the restriction enzyme used are given below:

| | | | | |
|---|---|---|---|---|
| Pst | 6.8 | 5.9 | | |
| Hind III | 6.5 | 6.2 | | |
| Eco RI | 9.2 | 3.5 | | |
| Pst + Hind III | 4.8 | 4.2 | 2.0 | 1.7 |
| Pst+ Eco RI | 5.4 | 3.8 | 3.0 | 0.5 |
| Eco RI + Hind III | 6.2 | 3.5 | 1.8 | 1.2 |

14. (a) (i) Why is it necessary to make DNA single stranded before determining its sequence?

(ii) Describe how bacteriophage M 13 is used for sequencing a cloned DNA frequent.

Or

(b) Write short note on:

(i) Molecular beacon

(ii) RACE

(iii) RAPD and Random Amplified Polymorphic DNA

(iv) Tagman assay.

15. (i) Differentiate binary front co-integrate Ti plasmid based vectors.

(ii) How would you produce a transgenic plant that does not contain a market gene?

Or

(b) (i) how and why are knockout mice established?

(ii) Discuss how transgenesis could be useful to improve organ transplantation and to

produce human monoclonal antibodies.

**B.E./B.TECH. DEGREE EXAMINATION, NOVEMBER/DECEMBER 2006.**

**FIFTH SEMESTER**

**BIOTECHNOLOGY**

**BT 1305 - GENETIC ENGINEERING**

**(REGULATION 2004)**

**PART – A**

1. m RNA is formed from Antisence strand of DNA – comment.
2. Why do molecular biotechnologists use many different biological systems?
3. Why cleaved plasmid DNA is treated with alkaline phosphatase prior to the ligation step, in cloning experiment?
4. What is a linker? How is it used?
5. What are inclusion bodies? How can their formation be avoided?
6. What is the tac- promoter and how is it regulated?
7. How is dideoxynucleotide used in the determination of DNA sequence?
8. Why is it necessary to make DNA single stranded before determining its sequence?
9. What are knockout mice? How they are established?
10. What is good about chloroplast transformation?

**PART – B**

11. (a) (i) How would you express a foreign protein on the surface of a bacterium? What you want to do this?

(ii) How would you clone multiple copies of a gene on a single plasmid?

Or

(b) (i) Although all human cells do have same chromosomal DNA not of them express the identical proteins? How it is achieved?

(ii) What are the advantage and disadvantages of using transgenic mice as model systems for human diseases?

12. (a) (i) Describe the differences and similarities between prokaryotes and eukaryotes- structural genes.

(ii) Discuss three different modes of regulation of prokaryotic transcription.

Or

(b) (i) What is the significance of TATA Box?

(ii) Describe the major DNA elements that responsible for the transcription of eukaryotic structural genes.

13. (a) (i) What is c-DNA library?

(ii) Why would you use a plasmid, bacteriophag-lambda, cosmid or BAC as a cloning vector?

Or

(b) (i) Explain- Screening of genomic libraries in by hybridization.
-Expressed proteins are identified by antibody.

(ii) An alternative strategy is the use of Guessmers probe- What is it?

14. (a) Although both 5'RACE and ''RACE protocols are known, the specificity of their amplification is not very high. However this is overcome by using nested primer- Explain.

Or

(b) (i) Site directed mutagenesis is achieved by means of PCR with 100% efficiency – How?

(ii) RAPD does not involve radioactivity, southern transfer, DNA hybridization – in distinguishing plant cultivars – substantiate with specific example.

15. (a) (i) Agrobacterium tumefaciens –nature's smallest genetic engineer- How?

(b) What is the limitation with agrobacterium mediated transformation ? How is it by passed?

Or

(b) Discuss how transgenesis is used

(i) To produce human monoclonal antibodies

(ii) To improve organ transplantations.

**B.E./B.TECH. DEGREE EXAMINATION, NOVEMBER/DECEMBER 2005.**

**FIFTH SEMESTER**

**INDUSTRIAL-BIO-TECHNOLOGY**

## IB 331 – GENETIC ENGINEERING

### PART – A

1. Write short notes on plasmid incompatibility.
2. What is meant by retrofitting?
3. What is the role of eIF – 4E in cDNA cloning?
4. What is meant by transgene silencing?
5. Give two examples for production of plant-derived vaccines.
6. What are the methods used to select a specific clone?
7. What are the vectors used as alternatives to cosmids?
8. What is meant by chromosome walking?
9. Give two examples of DNA viruses as expression vectors.
10. What is meant by HDGS?

### PART – B

11. (i) Explain at least two methods of transferring DNA molecules into a host.
    (ii) Write short notes on safety guidelines of creating recombinant DNA research.

12. (a) Explain the method to construct a cDNA library from a brain tissue.

Or

(b) What is PCR? Explain the different steps involved in the amplification of a DNA fragment by PCR.

13. (a) Describe at least four methods to identify a specific clone from a cDNA library.
    (b) Explain in detail the two methods involved in DNA sequencing.

14. (a) Write notes on the following:
    (i) Direct DNA transfer to plants.
    (ii) Functions of T – DNA genes.
    (iii) Control of transgenic expression in plants.

Or

(b) DNA sequences as diagnostic tools – Explain.

15. (a) Write an account on the applications of recombinant DNA technology in pharmaceutical industries to derive medicine and cure genetic diseases due to inborn errors of metabolism.

Or

(b) Discuss the steps involved in the creation of mammalian clone by nuclear transplantation. Discuss the phenotypic, genotypic and functional characteristic of mammals derive from the method.

**B.E./B.TECH. DEGREE EXAMINATION, APRIL/MAY 2004.**

**FIFTH SEMESTER**

**INDUSTRIAL BIOTECHNOLOGY**

**IB 331 – GENETIC ENGINEERING**

**PART – A**

1. What is meant by triplet binding assay?
2. Comment on polynucleotide phosphorylase.
3. A linear DNA molecule is cut with ECORI and yields fragments of 3 kb, 4.2 kb and 5 kb. What are the possible restriction maps?
4. What is chromosome walking? When is it used?
5. For gene cloning experiments, why are plasmids often treated with alkaline phosphatase prior to the ligation step?
6. What are isoschizomers? Give examples.
7. What is PCR assisted DNA sequencing?
8. What are the requirements for an efficient prokaryotic expression vector?
9. Comment on the significance of selectable markers for inclusion in T-DNA?
10. Define Embryo sexing and its application.

## PART – B

11. (i) Describe the ways in which a DNA fragment can be covalently attached to a vector DNA.

(ii) How are gene specific probes obtained?

12. (a) (i) Define restriction enzyme. Add a note on their classification and nomenclature.

(ii) Why type II restriction endonucleases are important for recombinant DNA technology?

OR

(b) Outline the methods that could be used to detect cloned target gene within a clone bank in E. coli. Add a note on the conditions to be satisfied for each type of assay.

13. (a) Describe Basic PCR and the variants of basic PCR. Discuss their utility with specific examples.

OR

(b) What is dideoxynucleotide? How is it used to determine the sequence of a DNA molecule?

14. (a) What is site-directed mutagenesis? Detail the procedure for oligonucleotide directed mutagenesis with M13 DNA. Add a note on its advantages and disadvantages.

OR

(b) (i) Describe the structure of Ti plasmid and explain how different sequences help in infection and T-DNA transfer?

(ii) Describe co-integrate construction using Ti plasmid of Agrobacterium sp.

15. (a) Describe Wilmuts cloning experiment through nuclear transplantation technique.

OR

(b) Write an account on the use of recombinant DNA technology in medicine.

**B.E./B.TECH. DEGREE EXAMINATION, NOVEMBER/DECEMBER 2004.**

**FIFTH SEMESTER**

**INDUSTRIAL BIO-TECHNOLOGY**

## IB 331 – GENETIC ENGINEERING

### PART – A

1. What is a triplet codon? Given an example for stop codon.
2. How is the host cellular DNA protected from restriction endonucleases in bacteria?
3. Define transformation efficiency.
4. Write the principle involved in blue-white screening method.
5. What is the use of phosphatase in the construction of a foreign gene into a vector?
6. What is primer-dimer in the PCR product?
7. What is mean by klenow fragment of DNA polymerase?
8. What are the enzymes used in nick translation and end labeling?
9. How can a site-directed mutation happen with UV irradiation?
10. How can DNA fingerprinting solve paternity cases?

### PART – B

11. (i) Describe the method of preparing a genomic DNA.
    (ii) How will you isolate a gene of your interest from the genomic DNA?

12. (a) Describe the safety guidelines for the preparation, use, and disposal of the products of recombinant DNA research.

    OR

    (b) Explain different bacteriophage vectors and their applications in DNA cloning.

13. (a) What is a PCR? Describe the PCR cycle and the requirements needed for PCR in detail.

    OR

    (b) Compare the Maxam and Gilbert chemical method with Sanger's enzymatic method of DNA sequencing.

14. (a) Describe the steps involved in the Southern blotting – using radio – labeled probe and non – radioactive probe to identify a DNA fragment. Discuss the major differences between the Southern and Western blots.

OR

(b) Explain the following vectors for genetic engineering:

(i) Shuttle vectors.
(ii) Mammalian expression vectors.

15. (a) Explain the following approaches with examples:

(i) Assisted reproduction
(ii) Gene knockout animals.

OR

(b) Discuss the applications of recombinant DNA technology to increase the plant nutritional value, to derive a strain for high yield or harsh environment, and to use plant parts as a bioreactor and as a vaccine carrier.

**B.E./B.TECH. DEGREE EXAMINATION, NOVEMBER/DECEMBER 2003.**

**FIFTH SEMESTER**

**INDUSTRIAL BIO-TECHNOLOGY**

**IB 331 – GENETIC ENGINEERING**

**PART – A**

1. Name two restriction enzymes that produce cohesive ends.
2. What is a "Multiple Cloning Site" in a plasmid vector? Given an example.
3. What is the function of a promoter in a vector? Give an example.
4. Write brief notes on λgt11 phase vector.
5. What is a concatamer?
6. Give four utilities of genomic databases.
7. What is Polymerase Chain Reaction?
8. Where the DNA fragment having EcoRI end and blunt end can be cloned into a plasmid having the MCS BamHI-EcoRI-Hind III-SmaI-NotI?

9. Write the principles involved in gene-gun technique.

10. Give two examples of plant viruses that can be used as plant expression vectors.

## PART – B

11. Explain in detail at least four methods that can be used in the selection/screening of recombinant clones.

12. (a) Describe in detail the construction of a cDNA library from total mRNA to clone the gene specific for the expression of the protein "X".
    (b) Describe in detail the construction of a genomic library to get a clone that expresses the protein "X".

13. (a) Describe the various components of a plasmid vector using an example.

OR

(b) Write an essay on the safety guideline for the preparation, use, and disposal of recombinant materials.

14. (a) Explain at least four different methods that are used in the transduction of DNA into animal and/or plant cells.

OR

(b) Describe atleast four utilities of PCR methods using appropriate examples.

15. (a) Describe the manual and the automated gene sequencing methods.

OR

(b) Describe the use of transgenic plants and animals for the society.

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# UNIT 1 HUMAN GENETICS

**Contents**



**Learning Objectives**

Once you have studied this unit, you should be able to understand

- how a single gene or genes form the physical hereditary link between generations, from parent to offspring;
- the determination of sex and the relation of sex to inheritance, and sex related human traits; and
- the important factors of human diversity and ethnic variation.

## 1.1 INTRODUCTION

The field of anthropology is basically concerned with both biological and social/ cultural evolution as well as diversity of human population throughout the ages. For understanding human genetics one needs to understand about physical anthropology or biological anthropology which deals with the study of human biology, evolution of the human organism, the relation between environment and human organism, and genetic variations between individuals and groups. The field of human genetics (of anthropological interest may be referred to as anthropological genetics which is concerned with human population variation study) uses pattern of genetic similarity and differences among different human populations throughout the human ancestry to infer the history of human evolution, migration, admixture and diversity. This would enable the scientists in explaining how the modern *Homo sapiens* evolved through the stages of *Homo habilis* and *Homo erectus* through the millennia to the modern man and the reasons behind many of the biological differences that we observe in different ethnic groups of the world.

Genetics is a branch of biology that deals with heredity or inherited variation of genetic traits. The science of genetics studies the phenotypic (visible) differences between individuals and attempts to relate them to underlying genic or chromosomal differences. The hereditary units that are transmitted through parent to offspring are called genes.

The word 'gene' is used frequently in genetics as a designation for each of the small units of heredity within a cell. Genetics has proved to have numerous practical applicatons because man has learned to use the discoveries in many diferent fields. It is being used in such diverse areas as plant and animal breeding, medical diagnosis, and genetic counseling, and even in cases of law. Genes are biochemical instructions that are supposed to determine those inherited traits that reside in the long molecules of deoxyribonucleic acid or DNA. Long polynucleotide molecules of *deoxyribonucleic acid*, called DNA, are intimately assocaited with chromosmes and are found exclusively in chromosomes. The chemical composition of chromosomes includes histones, proteins and deoxyribonecleic acid. The DNA is found only in chromosomes and is double stranded. The genes are then, sections of the DNA ladder-like molecules; different genes are different because they contain different sequence of the "letters' A, T, C, and G. DNA in conjunction with protein matrix may form nucleoprotein and becomes organised as chromosomes that are found in the nucleus of the cell. DNA is a stable molecule, however, on rare occasions a change or heritable alteration may occur spontaneously, is called mutation which is the lead sources of biological variation. In the study of heredity, we must clearly distinguish between 'genes'and 'traits'. Genes are at the bottom of developemnt. On the other hand, traits, such as hair colour, eye colour, size, shape, etc. are end products of development. They require both the proper genes and proper environment for their development.

## 1.2 HISTORY, DEFINITION AND SCOPE

Human Genetics, as the name indicates, describes the study of inherited variation as it occurs in human beings. The inheritance of many traits, including human traits is at present fairly well known. The biochemical studies on the constituents of the chromosomes have given essentially the correct picture how heredity really works at the molecular level. Genes can be the common factor of the most inherited traits. Genes have become prominent in the nature versus nurture debate. Study of human genetics can be useful as it can answer questions about human nature, behaviour as well as understand the diseases and disease treatment, and genetics of human life.

The science of genetics emerged from the famous work of Father Gregor Mendel (1822-1884) while working at the Augustinian monastery of St Thomas at Bruno in Moravia with the common garden pea. He published the results of his studies in 1866 and thereby laid the foundation of modern genetics. In his paper, Mendel proposed some basic genetic principles — the law of segregation, and the law of independent assortment. The first one states that each parent contains two copies of a unit of inheritance (later called gene), however, any one of two genes (called allele) can be transmitted to the offspring through the gamete. Which allele in a parent's pair of alleles is inherited is a matter of chance. The second principle states that the segregation of such gene pair (allele) occurs independently in respect of other gene pair, i.e. the paired genes (allelic pairs) separate from one

another and are distributed to different sex cells. The result is that new combinations of genes present in neither parent are possible. However, during his (Mendel) lifetime very few people realised the importance of his path breaking research. In 1901, Hugo de Vries, Carl Correns and Erich von Tschermak realised that Mendel's observations, conclusions and hypothesis have great importance in the field of genetics. During early 1900s, researchers noted that chromosomes behave like Mendel's traits and also inherited in random combinations. In 1909 Wilhelm Johansen renamed Mendel's characters as 'gene' and William Bateson coined the term 'genetics' to study genes. Thereafter researchers repeated and confirmed Mendel's hypothesis and his (Mendel) ideas on the inheritance of traits became more widely accepted and is now termed as Mendel's laws of inheritance.

Another milestone in the field of genetics is the discovery of the model for the structure of DNA as a genetic material by J.D. Watson and F.H.C. Crick in 1953. This was probably the key stone that unlocked an explosion in the field of human genetics as a form of molecular revolution.

Following are some of the fields where human genetics may contribute its knowledge for the betterment of the human society.

- To understand basic principles of inherited variation in man and to understand application of genetics in human life, and
- To answer questions about human nature, understand the diseases and development of effective disease treatment and health care.

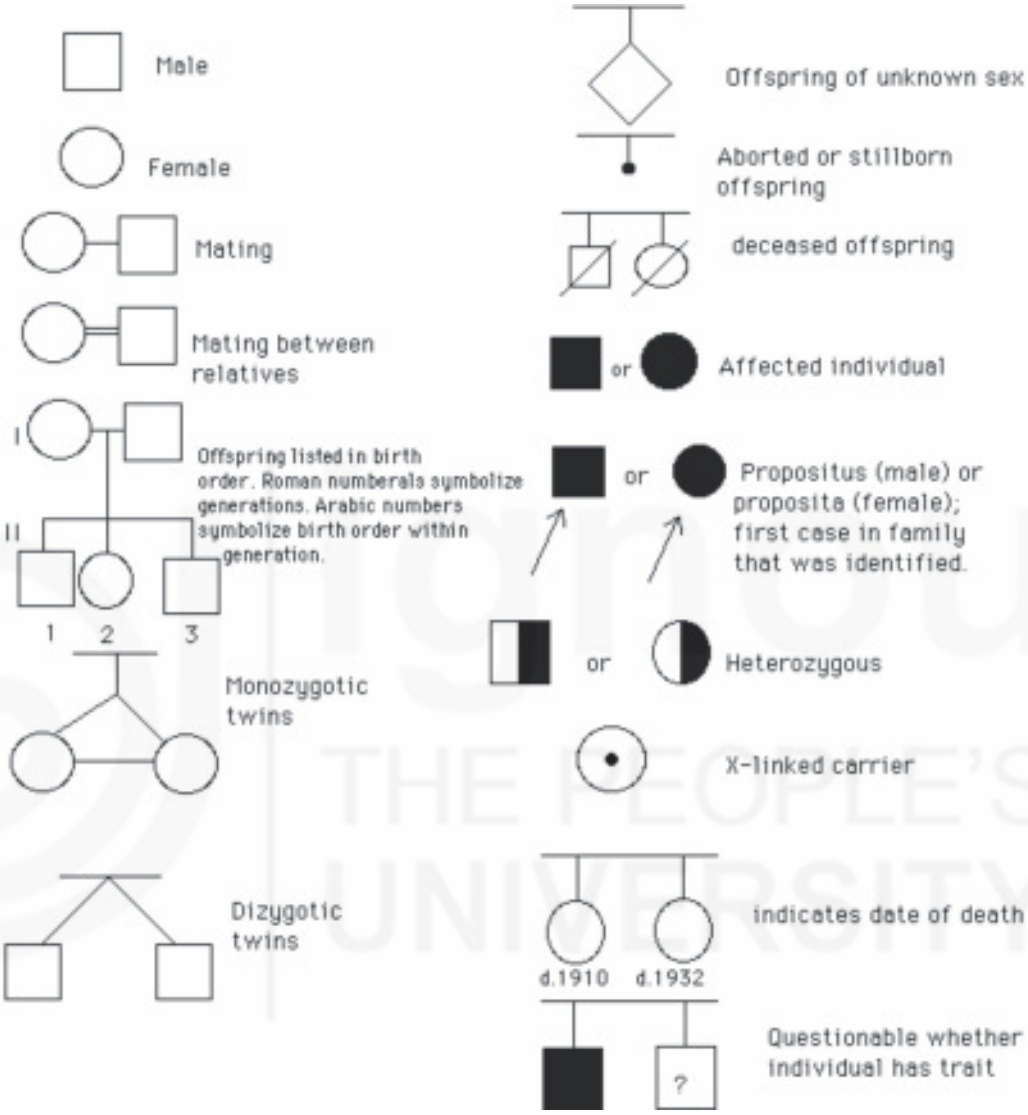
## 1.3 MENDELIAN INHERITANCE IN MAN

Mendelian traits or traits of simple inheritance are mainly discrete in nature and are controlled by alleles at single genetic locus. Therefore, in humans, traits or disorders that a single gene specifies are said to be Mendelian traits. Currently more than 4500 human traits are said to be inherited as per Mendelian principles; and another large conditions are suspected to be Mendelian traits. Many of the known Mendelian traits may be classified as disorders as per physical or mental disability. However, the most prevalent Mendelian disorders are very rare, usually affecting 1 in 10000 births or even less than that.

Human geneticist unlike others, who carried out experiment on plant or animal, can't have an access over experimental or controlled breeding. Hence they have to confine their study by observing the mode of inheritance in a pedigree. A Pedigree is a systematic drawing of the ancestral line of a given individual (both father and mother side) or family tree of a large number of individuals that depict blood relationship and transmission of inherited traits. A Pedigree can help to determine the genetic basis of a particular trait, especially in human, where experimental mating is not possible.

The term 'pedigree' (line of ancestors) is derived from French word '*pie de grue*' means crane's foot. The diagram of pedigree of large families with parents linked by curved lines to their offspring often resembled a bird's foot. You can tell a mode of inheritance just by looking at a pedigree. Pedigree is built of shapes connected by lines, vertical lines represent generations, horizontal lines that connect two or more shapes at their centers represent parents and vertical lines joined horizontally above them represent siblings. Matings are shown as

horizontal lines between two or more individuals. In case of shapes, *square* indicate male, *circles* indicate female and *diamonds* for unknown sex. Different shades or colours can be added to the symbols to identify different phenotype — full coloured shapes for individuals who express the trait under study and half-filled for carriers. Each generation is listed on a separate row labeled with Roman numerals, whereas, individuals within a generation labeled by Arabic numerals.




**Source:** www.bio.classes.UCSC.edu

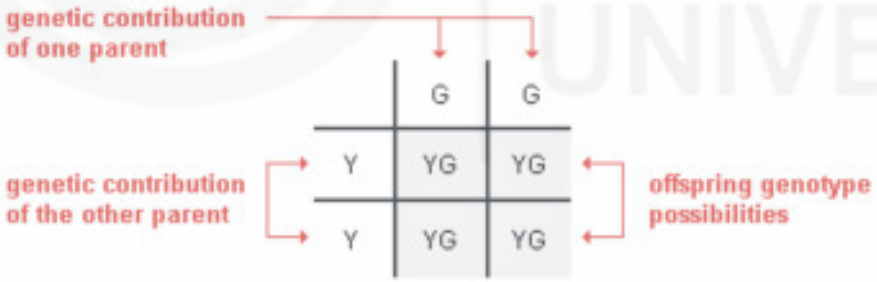
## 1.4 TYPES OF INHERITANCE

The patterns, in which Mendelian traits appear or transmitted in families, are called modes of inheritance. On the basis of chromosome where genes are located, you can find two types of inheritance - autosomal i.e. located on autosomes; and sex-chromosomal i.e. located on sex chromosomes, X or Y. Both autosomal and sex chromosomal inheritance may be subdivided as dominant or recessive inheritance on the basis of expression of alleles. However in respect of Y chromosome, there is no such subdivision like that described earlier. Hence, we have five modes of inheritance — autosomal recessive inheritance, autosomal dominant inheritance, X-linked recessive inheritance, X-linked dominant inheritance and Y-linked inheritance.

Mendel's observation of two different expressions of an inherited trait in a single locus (e.g. short or tall in respect of pea plant) narrates the facts that a gene can exist in alternate forms, usually called allele. An individual having two identical alleles is called homozygous, whereas the one with two different alleles is called heterozygous. Hence an individual may be homozygous either by two dominant alleles or two recessive alleles.

The allele that masks the effect of the other allele is called dominant (specifically completely dominant) and the masked one is called recessive. Whether the trait is dominant or recessive mostly depends upon the particular nature of the phenotype. Sometimes the heterozygous behave like an intermediate or a mix between homozygous dominant and homozygous recessive. Recessive disorders, in many cases, tend to be more severe or lethal and produce symptoms at an earlier age than dominant disorders.

If the genetic basis of a trait is known one can predict the outcomes of crosses. These are Punnett square method, forked line method and probability method. The ratios predicted from Mendel's law, apply to a new allele combination to each newly conceived offspring i.e. 50% chance of inheriting the allele, no matter what was the previous combination. You can compare the situation with tossing of coins; for first one the possibility of its being the head (or tail) is 50%. The same is true for second or any subsequent tossing. Therefore, if there is a 25% chance for a recessive disorder and first child is affected, there is no guaranty that next three will not be affected. The best way to calculate the probability of inherited traits was invented by Reginald Punnett and is called Punnett square. This is a simple graphical way to calculate all potential combinations of genotype for each time. You can start the same by drawing a grid of perpendicular lines. Now put the genotype of one parent across the top and other one down the left side. At last you can fill all the boxes by copying row and column letters (alleles).

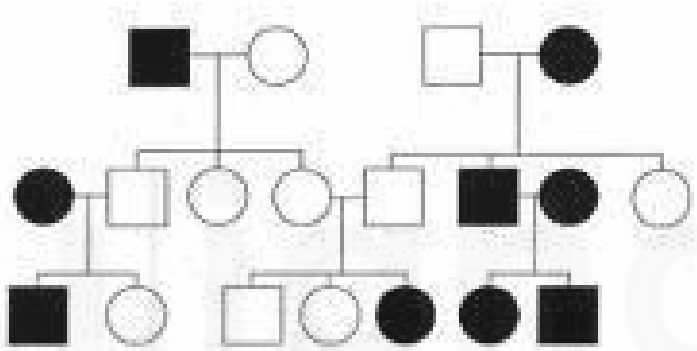



## 1.4.1 Autosomal Recessive Inheritance

Autosomal recessive trait can affect both sexes in equal proportions and can (but not necessarily) skip generation. The gene is carried on autosomes. For expression of recessive trait to be displayed, two copies of trait or allele needs to be present, which indicate that both the parents must be at least carrier for the specific traits. Therefore, a recessive trait can remain hidden for several generations without displaying the phenotype or diseases. The trait characteristically appears only in sibs, not in their parents, offspring or other relatives.

Sometimes a rare autosomal recessive trait may occur in families where the parents are close (blood) relatives, who are supposed to inherit the allele from a common ancestor. The situation is called consanguinity. Marriages between relatives - "consanguineous marriages", as they are often called, are important genetically.

Because closely related individuals have a higher chance of carrying the same alleles than less closely related individuals. The children from consanguineous marriages are more frequently homozygous for various alleles than are children from other marriages. In some ancient societies like the Pharaohs of ancient Egypt and the Incas of Peru favoured marriages of brothers and sisters of the ruling dynasties, to keep the 'royal blood' pure. These are extreme cases of consanguineous marriages. In some societies, more common types of close consanguinity are observed in cousin marriages. Examples of other consanguineous relations are those between uncle or aunt and nephew or niece (third degree), between cousins (fourth degree) and between second cousins (sixth degree). Consanguinity relations are identified by the number of steps from a common ancestor to only one of the related individuals, namely, the one more remote from him.



**Source:** www.migeneticsconnection.org

Some important characteristic features are:

- Occurrence and transmission is not influenced by sex;.
- Traits can express only in homozygous condition;
- In a pedigree you can find the trait only in siblings, not in their parents;
- On average ¼ th of the sibs of the proband are affected;
- In the instance of a rare disease, affected individuals have normal parents;
- Ratio of affected, carrier and non-affected is 1:2:1 (in sibs); and
- Parents of an affected child, in many cases, are close blood relatives.

Results from each of the six possible crosses are summarized in Table 1.1

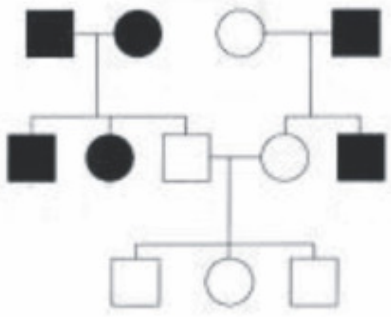
**Table 1.1: Summary of Autosomal Recessive inheritance**

| Parents | Offspring |
|---|---|
| One parent homozygous Normal<br>Other parent homozygous Normal | All the offspring will be homozygous normal |
| One parent homozygous Normal<br>Other parent heterozygous Normal (Carrier) | 50% probability that offspring will be homozygous normal<br>50% probability that offspring will be heterozygous normal (Carrier) |

| | |
|---|---|
| One parent heterozygous Normal (Carrier)<br>Other parent heterozygous Normal (Carrier) | 25% probability that offspring will be homozygous normal<br>50% probability that offspring will be heterozygous normal (Carrier)<br>25% probability that offspring will be affected |
| One parent homozygous Normal<br>Other parent affected | All the offspring will be heterozygous normal (Carrier) |
| One parent heterozygous Normal (Carrier)<br>Other parent affected | 50% probability that offspring will be heterozygous normal (Carrier)<br>50% probability that offspring will be affected |
| One parent affected<br>Other parent affected | All the offspring will be affected |

### 1.4.2 Autosomal Dominant Inheritance

Autosomal dominant trait, like autosomal recessive traits, can affect both sexes in equal proportions; the gene is carried on autosomes but unlike previous one does not skip generations. If no offspring inherits the trait in any generation its transmission stops. The trait is called "dominant" because a single copy of the trait, inherited from either parent, is enough to cause this trait to appear; the dominant allele masks the recessive one. Hence both homozygous dominant and heterozygous individual can express the trait. This often means that at least one parent must have the trait to transmit; otherwise it may appear because of mutation. Unaffected family members do not transmit the trait to their children. Dominance and recessiveness are obviously developmental phenomena resulting from genic action. They refer to the effect of a combination of differing alleles as compared to the effect of a homozygous combination.



**Source:** www.migeneticsconnection.org

Some important characteristic features are:

- Occurrence and transmission is not influenced by sex;
- Traits can express in both homozygous and heterozygous condition;
- You can find the trait in every generation of a pedigree;
- Affected individuals are usually born of normal parents;
- Affected individuals are always the product of a parent carrier of the same character;

- Trait always transmitted by an affected person (if heterozygous he/she is supposed to transmit the trait to half of the children and if homozygous to all the children); and
- All children of a normal individual will be normal i.e. unaffected family members do not transmit the trait to their children.

Results from each of the six possible crosses are summarized in Table 1.2

**Table 1.2: Summary of Autosomal Dominant inheritance**

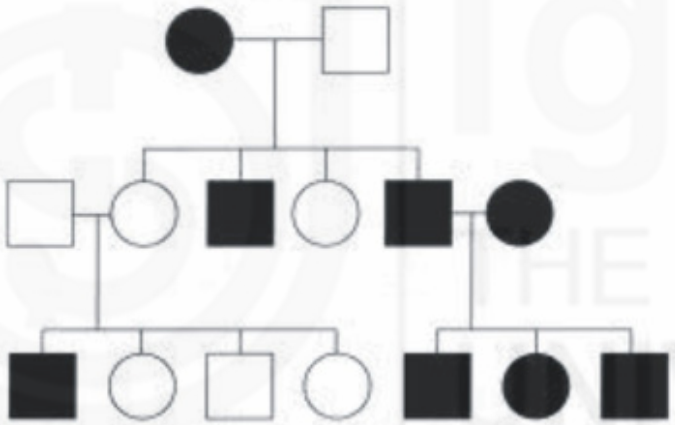
| Parents | Offspring |
|---|---|
| One parent homozygous affected<br>Other parent homozygous affected | All the offspring will be homozygous affected<br>50% probability that offspring will be homozygous affected<br>50% probability that offspring will be heterozygous affected |
| One parent homozygous affected<br>Other parent heterozygous affected | 25% probability that offspring will be homozygous affected<br>50% probability that offspring will be heterozygous affected<br>25% probability that offspring will be normal |
| One parent heterozygous affected<br>Other parent heterozygous affected | All the offspring will be heterozygous affected |
| One parent homozygous affected<br>Other parent normal<br>One parent heterozygous affected<br>Other parent normal | 50% probability that offspring will be heterozygous affected<br>50% probability that offspring will be normal |
| One parent normal<br>Other parent normal | All the offspring will be normal |

**Sex-Linkage**: In the human species, the sex-chromosomes contain many more genes than those concerned with sex-determination. These affect the widest range of characters and bear no relation to sex. Genes carried in the same chromosome are said to be 'linked' because they are assorted together. Haemophilia is due to the operation of a recessive sex-linked gene. A woman, heterozygous for it is therefore unaffected, since she carries the haemophilia gene (*h*) in one X-chromosome, and its normal allelomorph (*H*) in the other. Normal women can transmit haemophilia while a normal man cannot do so.

**Sex-linked Inheritance**: Colour blindness is an example of sex-linked inheritance in man. Women are much less often colour blind than men. But if a woman does happen to be colour blind, and if she marries a normal man, all of her sons are colour blind but none of her daughters are.

### 1.4.3 X-linked Recessive Inheritance

Sex-linkage was first discovered by Thomas H. Morgan (father of modern genetics) in 1910. Sex-linked traits affect male and female differently. As human male is hemizygous for X-linked traits, any gene on a male's X chromosome is expressed in his phenotype because there is no such second allele to mask its expression. Therefore, the condition of dominant and recessive trait is limited to female only. Females express X-linked traits or disorders when they are homozygous for the disorder and become carriers when they are heterozygous. Therefore female can transmit the trait as affected if her father is affected and mother at least carrier. However male can transmit the trait if any of the parents is affected or carrier (for mother). Therefore, the incidence is much higher in males than females. These patterns of inheritance are also called crisscross inheritance or skip generation inheritance, in which a character is inherited to the second generation through the carrier of first generation. X-linked (both recessive and dominant) traits are always passed on by the X chromosome from mother to son or from either parent to daughter. The trait never passed from father to son. The human male is hemizygous in respect of X-linked inheritance as they have single copy of X chromosome.



**Source:** www.migeneticsconnection.org

Some important characteristic features are:

- Occurrence and transmission is influenced by sex; males are more affected than females;
- Affected male does not transmit the trait to his sons but always transmits to all his daughters;
- Carrier female can transmit the trait to half of her children of either sex;
- The trait is transmitted from affected male through all his daughters to half of his grandsons; and
- The trait may be transmitted through a series of carrier females; carrier shows variable expression of the trait.

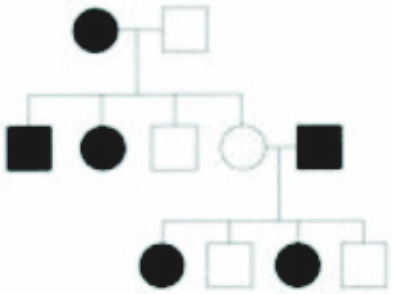
Results from each of the six possible crosses are summarized in Table 1.3

**Table 1.3: Summary of X linked Recessive inheritance**

| Parents | Offspring |
|---|---|
| Mother homozygous Normal<br>Father Normal | All the offspring will be homozygous normal |
| Mother homozygous Normal<br>Father affected | All the daughters will be heterozygous normal (carrier)<br>All the sons will be normal |
| Mother heterozygous Normal (carrier)<br>Father Normal | 50% probability that daughter will be homozygous normal<br>50% probability that daughter will be heterozygous normal (carrier)<br>50% probability that son will be normal<br>50% probability that son will be affected<br>50% probability that daughter will be heterozygous normal (carrier)<br>50% probability that daughter will be affected |
| Mother heterozygous Normal (carrier)<br>Father affected | 50% probability that son will be normal<br>50% probability that son will be affected |
| Mother affected<br>Father normal | All the daughters will be heterozygous normal (carrier)<br>All the sons will be affected |
| Mother affected<br>Father affected | All the offspring will be affected |

### 1.4.4 X-linked Dominant Inheritance

X-linked dominant inheritance shows the same phenotype as a heterozygote and homozygote. Incase of an X-linked dominant inheritance, male to male transmission is not there. This also makes it distinct from autosomal traits. X linked dominant cannot be distinguished from Autosomal Dominant by progeny of affected females, but only from the progeny of affected males. Affected females are more common than affected males (but heterozygous females have milder expression); on the other hand the traits (especially disorder) are more severe in males than their female counterparts.



**Source:** www.migeneticsconnection.org

Some important characteristic features are-

- Occurrence and transmission is influenced by sex; females are more affected than males but may be with variable expressions;
- Homozygous female transmitted the trait to all the children;
- Male transmitted the trait to all the daughters but never to a son;
- Affected males have no normal daughter;
- Affected heterozygous females transmit the trait to half of their children of either sex. Affected homozygous females transmit the trait to all their children; and
- X linked dominant cannot distinguish from Autosomal Dominant by progeny of affected females, but only from the progeny of affected males.

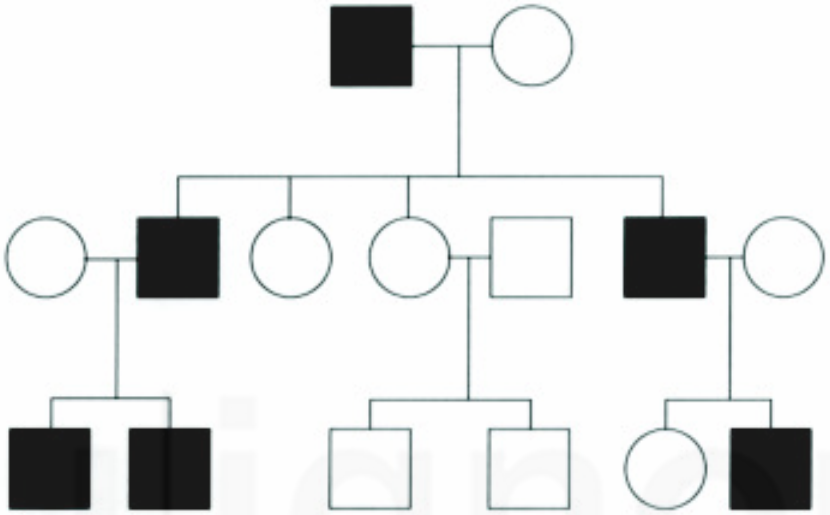
Results from each of the six possible crosses are summarized in Table 1.4

**Table 1.4: Summary of X linked Dominant inheritance**

| **Parents** | **Offspring** |
|---|---|
| Mother homozygous affected<br>Father affected | All the offspring will be homozygous affected |
| Mother homozygous affected<br>Father normal | All the daughters will be heterozygous affected<br>All the sons will be affected |
| Mother heterozygous affected<br>Father affected | 50% probability that daughter will be homozygous affected<br>50% probability that daughter will be heterozygous affected<br>50% probability that son will be affected<br>50% probability that son will be normal |
| Mother heterozygous affected<br>Father normal | 50% probability that daughter will be heterozygous affected<br>50% probability that daughter will be normal<br>50% probability that son will be affected<br>50% probability that son will be normal |
| Mother normal<br>Father affected | All the daughters will be heterozygous affected<br>All the sons will be normal |
| Mother normal<br>Father normal | All the offspring will be normal |

### 1.4.5 Y-linked Inheritance

The genes located on the Y chromosome, whose alleles are absent on the X chromosome are Y-linked genes or holandric genes (also hemizygous). Y-linked inheritance occurs when a gene is transmitted through the Y chromosome. Since Y chromosomes can only be found in males, hence Y linked genes are only passed on from father to son and never appear in females. Therefore, there is no skipping of generation and affected males have all affected sons, no females are said to be affected for the trait (www.sakshieducation.com).



**Source:** www.migeneticsconnection.org

Some important characteristic features are-

- In pedigree, only males are affected;
- Affected male transmitted the trait to all his sons but never to his daughter; and
- No skipping of generations.

Results from each of the two possible crosses are summarized in table 1.5

**Table 1.5: Summary of Y-linked inheritance**

| Father | Offspring |
|---|---|
| Father affected | All the sons will be affected |
| Father normal | All the sons will be normal |

## 1.5 EXAMPLES

| Traits/ Description | Autosomal Recessive | Autosomal Dominant |
|---|---|---|
| Albinism: is a form of hypopigmentary congenital disorder, characterised by a partial or total lack of melanin pigment in the eyes, skin and hair (or more rarely the eyes alone). | Albinism | Normal pigmentation |

| | | |
|---|---|---|
| Thalassemia: Human haemoglobin molecule consists of two alpha and two beta globin chains conjugated with heme. In alpha Thalassemia alpha chain is missing or defective and in beta Thalassemia beta chain is absent which leads to iron overload and anemia. | Thalassemia | Normal |
| Cystic Fibrosis: Cystic fibrosis is an inherited disease that causes thick, sticky mucus to be built up in the lungs and digestive tract. It is one of the most common chronic lung diseases in children and young adults, and may result in early death. | Cystic Fibrosis | Normal |
| Tay-Sachs disease: Tay-Sachs disease is caused by a mutation on chromosome 15. Tay-Sachs disease occurs when the body lacks hexosaminidase A, a protein that helps break down a chemical found in nerve tissue called gangliosides. | Tay-Sachs disease | Normal |
| Xeroderma pigmentosum: A disruption of affected person's DNA's ability to repair damage caused by ultraviolet radiation of sunlight. | Xeroderma pigmentosum | Normal |
| Hitchhiker's thumb: More formally known as "distal hyper extensibility of the thumb" can extend the top of the thumb backwards nearly 90° when the thumb is extended in a "thumbs-up" position. | Hitchhiker's thumb | Straight thumb |
| Dentinogenesis imperfecta: Pulp chambers and root canals of teeth are obliterated with abnormal dentin. There is also an increased constriction and junction between the crowns and the roots of the molar. | Normal teeth | Dentinogenesis imperfecta |
| Cleft Chin: It is a Y-shaped fissure on the chin with an underlying bony peculiarity. | No Cleft Chin | Cleft Chin |
| Brachydactyly: Better known as clubbed thumb, is a condition where the thumbs are shorter and stubbier than normal. Brachydactyly is a genetically inherited trait that is often dominant. | Normal thumb | Brachydactyly |

| | | |
|---|---|---|
| PTC taste sensitivity: Phenylthiocarbamide also known as PTC is anorganosulfur thiourea containing a phenyl ring. A crystalline compound, $C_6H_5NHCSNH_2$, that tastes intensely bitter to people with a specific dominant gene and tasteless to others. | Non-taster | Taster |
| Achondroplasia: Characterized by prominent forehead, low nasal root, redundant folds in arms and legs accompanied by short-limbed dwarfism. | Normal | Achondroplasia |
| Familial Hypercholesterolemia: Characterized by high LDL in blood resulting to deposition of cholesterol in arteries, tendons, skin, etc., which may leads to coronary artery diseases. | Normal | Familial Hypercholesterolemia |

| **Traits/ Description** | **X-linked Recessive** | **X-linked Dominant** |
|---|---|---|
| Duchene muscular dystrophy: It is an inherited disorder that involves rapidly worsening muscle weakness. | Affected | Normal |
| Haemophilia A: Occurs due to the deficiency of factor VIII in blood. Affected persons are unable to produce a factor needed for blood clotting, therefore the cuts, wounds, etc., of haemophilic persons continue to bleed and sometimes (if not stopped by clotting factors) leads to death. | Haemophilia A | Normal |
| Red green color blindness: Colour perception is mediated by light absorbing protein in the cone cells of the retina in the eye. Colour blindness is caused by an abnormality in any of the receptor protein. Red green colour blindness is the ability to perceive the colour green and red. | Red green color blindness | Normal vision |
| G6PD deficiency: It is an inherited disorder in which the body doesn't have enough enzyme glucose-6-phosphate dehydrogenase, or G6PD, which helps red blood cells (RBCs) function normally, and deficiency may cause hemolytic anemia. | G6PD deficiency | Normal |

| | | |
|---|---|---|
| Incontinentia pigmenti: Incontinentia pigmenti is a genetic defect on X chromosome which leads to unusual blistering and changes in skin color. | Normal | Incontinentia pigmenti |
| Fragile X syndrome: Fragile X syndrome is a genetic condition involving changes in part of the X chromosome resulting in mental retardation. | Normal | Fragile X syndrome |
| Congenital generalized hypertrichosis: Person has more hair follicles which lead to dense and more abundant terminal hair. Generally, it causes excess facial and upper body hair that covers extensive areas of skin. | Normal | Congenital generalized hypertrichosis |

| **Y-linked traits** |
|---|
| Hypertrichosis of ear: growth of hair on the rim of pinna |
| Testis determining factor (TDF) |

**Note:** You can find out more examples from NCBI databases OMIM: Online Mendelian Inheritance in Man

**Sex-limited and Sex-controlled Traits**

So far we have been discussing about sex-linked traits, but there are some such genes which are sex-limited in their effect, that is, they are expressed phenotypically in one sex only. In man sex-limited expression of genes occurs in uterine (in female) and prostate (in male) cancer. Anatomical and physiological properties of the female sex, such as width of pelvis or age of onset of menstruation is a sex-limited expression. Similarly, sex-limited male characters such as type of beard growth or amount and distribution of body hair, probably depend on genes common to both sexes, but the penetrance and expressivity of the genes are more limited to males. Sex-limitation is only the extreme example of control of the expression of certain genotypes by sex.

When a genotype is expressed in both sexes but in a different manner in each, we speak of sex-controlled, or sex-modified, genic expression. Sex-controlled dominance has been suggested as an explanation of the pattern of inheritance of baldness in man. Both sexes may be affected, but the high relative frequency of affected males is notable. Some of these traits are controlled by the sexual constitution of the individual and thus are under the influence of sex hormones.

## 1.6 SUMMARY

Mendel considered a single gene to be responsible for a single trait, but after the discovery of other types of non-Mendelian inheritance it is now clear that many genes may be involved for the production of single or many traits. Mendel's

laws also incorporate many of the modern discoveries which enriched these laws. For example, chromosome or meiosis was discovered after Mendel's work. Now we can correlate that Mendel's first law i.e. law of segregation is about anaphase-I where homologous chromosomes segregate from each other. Similarly as per second law, segregation of alleles for one character follow independently of the segregation of allele of other character because each pair of homologous behaves like an independent unit during meiosis. Again, especially after modern discovery, we can understand that it is the gene and not the trait (as per Mendel) that are inherited.

Since Mendel's time, understanding of the mechanisms of genetic inheritance has grown immeasurably. The simple rules of Mendelian inheritance do not apply in elucidating many of the inheritance patterns, and are understood to be non-Mendelian inheritance patterns.

## 1.7 GLOSSARY

**Allele** : an alternate form of gene that determine alternate traits or characteristics.

**Autosomal dominant** : the inheritance pattern of a dominant allele on autosomes.

**Autosomal recessive** : the inheritance pattern of a recessive allele on autosomes.

**Autosomes** : a non-sex determining chromosome. Human has 22 pairs of autosomes.

**Carriers** : a heterozygous individual who possess a deleterious recessive allele which is suppressed by dominant normal allele.

**Chromosome** : a structure within a cell's nucleus that carries gene and consists of a continuous molecule of DNA and proteins.

**Consanguineous** : relating to or denoting people descended from the same ancestor.

**Dominant trait** : the trait that is expressed in the $F_1$ generation.

**DNA** : a long linear polymer found in the nucleus of a cell, formed from nucleotides and shaped like a double helix; generally associated with the transmission of genetic information.

**Gene** : a sequence of DNA that instructs a cell to produce a particular protein.

**Genetics** : branch of biology that concerned with heredity and variation.

**Heterozygous** : having two different alleles of a gene at a single locus and produces different kinds of gametes.

**Homozygous** : having two identical alleles of a gene single locus and produces only one kind of gamete.

**Mutant** : an allele that differs from wild type allele, altering the phenotype.

**Mutation** : any event that changes genetic structure; any alteration in the inherited nucleic acid sequence of the genotype of an organism.

**Pedigree** : a chart consisting of symbols for individuals connected by lines that depict blood relationships and transmission of inherited traits.

**Probability** : probability is a way of expressing mathematical knowledge that an event will occur or has occurred.

**Proband** : proband, or propositus, is a term used most often in genetics to denote a particular subject (person in human genetics) being studied or reported on.

**Protein** : a type of macromolecule that is the direct product of genetic information.

**Recessive trait** : the trait that is masked in the $F_1$ hybrids.

**Sex cells** : sex cells are the cells that give rise to the gametes of organisms that reproduce sexually.

**Sex Chromosome** : a chromosome containing genes that specify sex.

**Sex linked** : genes that are part of a sex chromosome.

**Variable expression** : a genotype producing phenotype that varies among individuals.

**X linked dominant** : the inheritance pattern of a dominant allele on X chromosome.

**X linked recessive** : the inheritance pattern of a recessive allele on X chromosome.

**Y linked** : the inheritance pattern of a gene on Y chromosome.

**Suggested Reading**

Cummings, M. R. 1997. *Human Heredity: Principles and Issues*. Belmont, Wadsworth.

Gardner, E. J., Simmons, M.J. and Snustad, D.P. 1991. *Principles of Genetics*. New York, John Willey & Sons.

Lewis, R. 2003. *Human Genetics: Concepts and Applications 5th edition*. Boston, WCB McGraw Hill.

Stern, C. 1960. *Principles of Human Genetics* (2nd Edition). San Francisco and London, W. H. Freeman and Company.

Strickberger, M. W. 2003. *Genetics 3rd edition*. New Delhi, Prentice Hall.

**Website**

Mendel Web: www.mendelweb.org

National Center for Biotechnology Information: Online Mendelian Inheritance in Man: www.ncbi.nih.gov

## Sample Questions

1) What is autosomal recessive trait? From marriages between normally pigmented carrier people and albinos what proportion of children would be expected to be albino and normal? What is the chance in a family of three children that one would be normal and two albinos?

2) What do you mean by X linked recessive trait? From marriage between carrier female and affected male what proportion of children would be expected to be Haemophilic?

3) What do you mean by Human Genetics? Write a brief note on Mendelian genetics in Man.

4) What are X linked traits? How does X linked dominant trait is differentiated from Autosomal dominant trait?

5) What is Pedigree? Draw a pedigree of X linked recessive traits in Man.

# UNIT 2 HARDY-WEINBERG EQUILIBRIUM

**Contents**



**Learning Objectives**

After reading this unit, you will be able to:

- define what is Hardy-Weinberg Equilibrium or Law;
- depict the importance of HW Equilibrium and the field of population genetics;
- explain the method how to estimate the genotype and phenotype frequencies from HW theorem and to calculate in empirical situation; and
- evaluate the theory behind the deviations from H-WE, especially the gene frequency changes with respect to Mutation, Genetic drift, Selection, Gene flow and how to investigate them in empirical situations in human populations.

## 2.1 INTRODUCTION

Living organisms are endowed with unique abilities, traits that allow them to survive in a given environment. These traits or abilities may show or exhibit enormous variations within species and across species. Some of these traits are unique to that species; some traits are common within and across species with little variation, these are adaptive characters and gives survival advantage.

These traits are the '*phenotypic*' forms that can be observed as a quantitative trait (or measurable) or classified as types or categories. These traits are hereditary and transmitted across generations: either in the same form or in slight variable form. At times some new traits or variations of the trait appear among the offspring. Some of these traits are governed by '*genes'* or located in the '*genome'* of an organism. The nature of heredity of some of these traits could be complex and/or it could follow some simple principles of transmission.

Human population genetics deals with how these traits or variation change in a population over space and time (generations)? What are the factors that influence the variation of these traits in the population? To what extent these traits are hereditary and are influenced by environment? Can we understand them by simple theoretical models? Can we study how different forces operate differentially in different populations to give a characteristics distribution of gene and genotype frequencies?

Population genetics is the study of gene and genotype frequencies in populations of interbreeding organisms (small or large, natural or artificial) and predicting the way these frequencies are maintained or changed under the combined influence of various factors. It is concerned with applying models of gene frequency change involving different factors in the context of Mendelian genetics to examine evolution in a quantitative manner. In order to understand the pattern of allele frequencies we need to have a defined population, in this case a '*Mendelian population*'. Dobzhansky (1951) defined it as the reproductive community of individuals which share a common gene pool. Evolutionary studies involve reconstructing past demographic events that have led to the present day diversity patterns. Use of various models allows one to examine interplay of various factors and make inferences about the past based on present day data. But one has to be careful about interpreting results obtained from any model considering that all models have some assumptions inherent to them.

## 2.2 HARDY-WEINBERG EQUILIBRIUM

During the early 1900s people were interested to validate the Mendelian laws of genetics to other organisms, including Man. Are there Mendelian traits in Man?

**Mendelian Genetics in Man**

**BOX 2.1**

Brachydactyly **in a population:** All the mating types are:

The mating types include individuals Normal and Brachydactyly (a Dominant Mendelian trait)

N – Normal and B –Brachydactyly

a. Both parents are normal — All the offspring are N

b. One parent is N and the other B (heterozygous)

c. One parent is N the other B

d. Both parents are brachydactyly B (heterozygous)

e. Both parents are brachydactyly B (homozygous)

a NN – NN = All the offspring N
b NB – NN = ½ : ½ = N : B
c BB – NN = all are B
d NB – NB = 1 N : 1 B: 2 B
e BB – BB = All the offspring B

Of the 5 possible combinations of parental mating types 4 types of matings results brachydactyly offspring.

Therefore, B are more frequent than N in a population as per Mendelian expectation. However Normal individuals are more frequent than Brachydactyly in a population.

# there is apparent contradiction between what is observed and what is expected (Mendelian)!

At Cambridge one research scholar was studying 'brachydactyly' – a trait characteristic of small or short digital length ('*brachy*' and '*dactyl*' in Greek

means 'short' and 'digit' respectively) than the normal type. The trait runs in some families. Does 'brachydactyly' follow Mendelian principles? The results of the study showed that '*brachydactyly*' is dominant Mendelian trait and the pedigrees showed 3:1 ratio of brachydactyly to normal offspring. This has invoked an important and interesting question? If it is a dominant trait, there will be more and more brachydactyly individuals in the population, but normal individuals are more frequent than brachydactyly individuals (See Box 2.1)

GH Hardy has solved the puzzle theoretically and published the theorem in Science (Hardy, 1908). GH Hardy's proof illustrates that the gene (or allele) frequency, — here in this case, frequency of brachydactyly individuals in a population, — will not increase over generations, but remain the same, under equilibrium conditions or in the absence of confounding variables. In 1908, Dr. W. Weinberg independently also published similar results (Weinberg, 1908) and is called as HWEqulibirium**.** (See Box 2.2)

**BOX 2.2: Historical anecdotes: HWEqulibrium/Law**

In 1908, a German physicist Dr. Weinberg published similar results on Mendelian genetics in a German journal. It was discovered by Dr. Curt Stern (publication in Science 1943), and the Hardy theorem was rightfully referred as HW Law or HWEquilibrium. However, in 1903, at least five years earlier, two scientists have considered similar such possibility of change in gene frequency. They are: WE Castle 1903 in America and Karl Pearson 1903 in England. These two papers considers the question of equilibrium state of gene frequency and change in gene frequency partially with respect to some factors.

a) **What is H-W EQUILIBRIUM/LAW?**

HWE states that in a randomly mating population of sufficiently large size, and in the absence of the influencing factors such as; mutation, migration, selection, genetic drift and inbreeding, the gene and genotype frequencies will remain constant from generation to generation.

The mathematical proof of invariance of gene frequency under given assumptions, require:

a) simple knowledge of school algebra and

b) basic concepts of Mendelian genetics (See Box 2.3).

The proof in case of autosomal '*biallelic*' trait is given in Box 2.4. (for further reading see references)

**BOX 2.3: Basic concepts**

**Phenotype :** A trait or a character that is observed as types or measurable and is transmitted from parents to offspring. Some phenotypes are complex with unknown genotypes, and some are directly governed by hereditary units (genes).

**Gene :** The causative factor of hereditary transmission of traits (phenotypes) and are located in the chromosomes (the hereditary materials in cell nucleus and in mitochondria).

**Allele** : Genes, the causative factor of hereditary transmission can exist or express in different forms and are referred as 'alleles'.

**Codominant:** Where both the alleles are equally expressive in the offspring.

**Recessive** : The alleles whose expression is suppressed at phenotypic level. The heterozygote offspring of a recessive allele will express the phenotype of the dominant allele.

**Haploid** : Organisms which carry one set of chromosomes.

**Haplotype** : It is short form of 'Haploid genotype'**.** Refers to genetic markers located on one chromosome. A haplotype can be identified by SNP (single nucleotide polymorphism).

**Diploid** : Organisms which carry two sets of chromosomes, each set derived from either of the parent. Man is diploid and carries two sets of chromosome (2N).

A diploid individual can carry two copies (alleles) of the gene in each of the chromosome that he or she gets from his or her parents. The two copies could be of the same type (form/status) or of different type (form/status).

**Homozygous:** The two alleles that an individual carries are of the same or identical types.

**Heterozygous:** The two alleles that an individual carries are of different type.

**Genotype** : Is the combination of alleles that a diploid individual can carry in each of the chromosomes.

For example, in case of a 'biallelic' gene say A, B two forms (alleles) of the gene that occur in each of the two sets of the chromosomes. There could be three different genotypes: AA, AB, BB.

AA and BB : two different homozygotes (genotype).

AB = BA : heterozygote (genotype).

The box shows the "Punnet's square" – method of scoring different combination of genotypes based on the male and female gametes or mating types. This can be extended to multiple alleles.

**Polymorphism:** If a gene exists in more than one form or morph (alleles) and that occurs in stable frequency in a population.

**Punnet's square**

| Female gamete | Male Gamete A | Male Gamete B |
|---|---|---|
| A | AA | BA |
| B | AB | BB |

**Genotype**

**BOX 2.4: Hardy-Weinberg theorem or principle: Proof**

In case of genetic trait that has positive family history in a population, let us assume that the gene is biallelic and therefore the two alleles are: B1 and B2 and let

'p' is the frequency of 'B1 allele' and

'q' is the frequency of B2 allele,

N is the total individuals and

So that (p + q = 1) or p = (1 – q) or q = (1 – p)

An individual in the population can have three types of genotypes: B1B1, B1B2, and B2B2. And let the frequency of the above three genotypes in the parental population are: P, H and Q respectively.

| | Gene (alleles) | | Genotypes | | |
|---|---|---|---|---|---|
| | B1 | B2 | B1B1 | B1B2 | B2B2 |
| Frequencies | p | q | P | H | Q |

If there are total N individuals in the sample, there will be

P individuals with genotype B1B1 type,

H individuals with genotype B1B2 type and

Q individuals with genotype B2B2 type

So that sum of (P +H + Q) = N,

Assuming all the individuals of the three genotypes are equally fertile, then given the genotypes, one can calculate the frequencies 'p' and 'q' in the population, by gene counting method:

The gene (allele) frequency 'p' = [P + ½ (H)]1/N = (B1B1)/N + ½ (B1B2/N), and

The gene (allele) frequency 'q'= [Q + ½ (H)]1/N = (B2B2)/N + ½ (B1B2/N)

This is the gene (allele) frequencies of 'p' and 'q', which are also the gametes produced in the population.

Only some of the gametes form zygotes that will eventually become individuals in the next generation. The allele (gene) frequency in the zygote is unchanged provided there is no reproductive advantage of either of the allele and the zygotes formed represent a large sample of the parental gametes.

Random mating between individuals is equivalent to random union among their gametes. Therefore, in the next generation, the genotype frequencies among the zygotes (fertilized eggs) are the result of random union of two types of gametes. The genotype frequencies among the progeny are therefore can be worked out by Punnet's square. Or it is the multiplication of the frequencies of the gametic types produced by the parents. Viz.,

| | Genotype | | |
|---|---|---|---|
| | **B1B1** | **B1B2** | **B2B2** |
| **Frequency** | **$p^2$** | **2pq** | **$q^2$** |

| Allele | B1 | B2 | |
|---|---|---|---|
| Genotype | B1B1 | 2B1B2 | B2B2 |
| Frequency | $p^2$ | 2pq | $q^2$ |
| Absolute freq. | P | H | Q |

## BOX 2.4 (Contd.)

### Hardy-Weinberg theorem or principle: Proof

In a population there will be three different types of genotypes among males and females, who will mate randomly and they will give rise to their offspring who will represent the same genotypes in the next generation. We will have to work out the frequencies of offspring genotypes given the three genotypes among the male and female parents. This is worked out easily by Punnet's square: the frequencies of different mating types among the male and female genotypes in the population and different possible genotypes among the offspring are.

**Frequency of different mating types and the offspring genotypes**

| | Male Parent — Genotype | | | |
|---|---|---|---|---|
| Female Parent | | B1B1 | B1B2 | B2B2 |
| | Freq. | $p^2$ | $2pq$ | $q^2$ |
| B1B1 | $p^2$ | $p^4$ | $2p^3q$ | $p^2q^2$ |
| B1B2 | $2pq$ | $2p^3q$ | $2p^2q^2$ | $2pq^3$ |
| B2B2 | $q^2$ | $p^2q^2$ | $2pq^3$ | $q^4$ |

Once we know the possible offspring genotypes as a result of random mating among the three parental genotypes we can calculate the expected frequencies among the offspring genotypes for different combination of mating types in the population. Given the three genotypes six possible mating types are possible in the population and each mating type will give rise to offspring of different possible combination of genotypes. These are worked out in the following table and this gives the allele frequencies in the offspring population in the next generation:

**Frequency of different mating types and the offspring genotypes**

| Parent Female X Male (Genotype) | Freq. | Offspring — Genotype B1B1 | B1B2 | B2B2 |
|---|---|---|---|---|
| B1B1 X B1B1 | $p^2$ | $p^4$ | — | — |
| B1B1 X B1B2<br>B1B2 X B1B1 | $4p^3q$ | $2p^3q$ | $2p^3q$ | — |
| B1B1 X B2B2<br>B2B2 X B1B1 | $2p^2q^2$ | — | $2p^2q^2$ | — |
| B1B2 X B1B2 | $4p^2q^2$ | $p^2q^2$ | $2p^2q^2$ | $p^2q^2$ |
| B1B2 X B2B2<br>B2B2 X B1B2 | $4pq^3$ | — | $2pq^3$ | $2pq^3$ |
| B2B2 X B2B2 | $q^4$ | — | — | $q^4$ |
| | 1 | $p^2 (p^2 + 2pq + q^2)$ | $2pq (p^2 + 2pq + q^2)$ | $q^2 (p^2 + 2pq + q^2)$ |
| | | $p^2$ | $2pq$ | $q^2$ |

Thus the genotypic frequencies in the offspring remain the same in two successive generations, assuming that allele frequencies are not influenced by selection, mutation and mating is random and there is no differential fertility and mortality and the population is large.

The above is true for *autosomal loci* and can be extended for multiple loci. It is also true for sex-linked trait. Here the gene frequencies will oscillate (by ½) between two sexes in successive generation and will soon reach to equilibrium.

### 2.2.1 Importance and Implications of HWE

What are the implications and why it is so important? In brief, it is the fundamental theorem of population genetics.

- **Methodology:** Tells us how to calculate (or estimate) the allele frequency or genotype frequency from observed phenotypes in an empirical situation. It can help us to investigate how many alleles are governed by a phenotypic trait.

- **Evolution:** It is quantitative way of understanding the mechanism of evolutionary factors and its influences. Evolution is a dynamic and complex phenomenon and it is hardly possible to study evolution in the laboratory conditions. It gives insights into the inter-relationship between the forces and how to study the effects of each of these forces and the gene frequency. (See the box 2.5 for the relationship between gene frequencies and genotype frequencies).

- It is the benchmark criterion to test whether a new trait is in equilibrium or if not how to test the reasons for the deviations.

- It helps us in genetic counselling to expect the likelihood of a child being homozygous for a recessive deleterious trait given the parental genotype. It helps in forensic science in cases like identification of suspects, parent-offspring disputes etc.

- **Quantitative Genetics**: HWE helps us to investigate complex genetic traits, to estimate the role of environment and genetic components, spatial distribution of gene frequency etc.

Further implications of this principle are as under:

- In case in a population a particular trait or character is in HWE, (the converse) it does not mean that the assumptions are satisfied. (The theoretical proof is complicated and it is available).

- The allele frequencies remain constant from generation to generation. This means that hereditary mechanism itself does not change allele frequencies. It is possible for one or more assumptions of the equilibrium to be violated and still not produce deviations from the expected frequencies that are large enough to be detected by the goodness of fit test.

- When an allele is rare, there are many more heterozygotes than homozygotes for it. Thus, rare alleles will be impossible to eliminate even if there is selection against homozygosity for them.

- For populations in HWE, the proportion of heterozygotes is maximal when allele frequencies are equal ($p = q = 0.50$), and when this happens the heterozygote frequency will be 0.50 (2x0.50x0.50). Unless HWE is violated (as in selective loss of homozygotes), heterozygosity can never be more than 0.50 at any biallelic locus. The relationship between gene frequency and genotype frequency is illustrated in Box 2.5.

**BOX 2.5**

**The relationship between gene frequency and genotype frequency**

It is interesting to know the relation between the gene and genotype frequency for a biallelic loci which is under H-W equilibrium. The graph shows the changes in the three genotype frequencies as against the change in allele frequencies A1 and A2 from 0 to 1 in Cartesian coordinates (drawn on x and y axis).

A1A1/A2A2 – homozygotes, A1A2 – heterozygotes

**Relationship between genotype frequency and gene frequency - biallelic GENE**

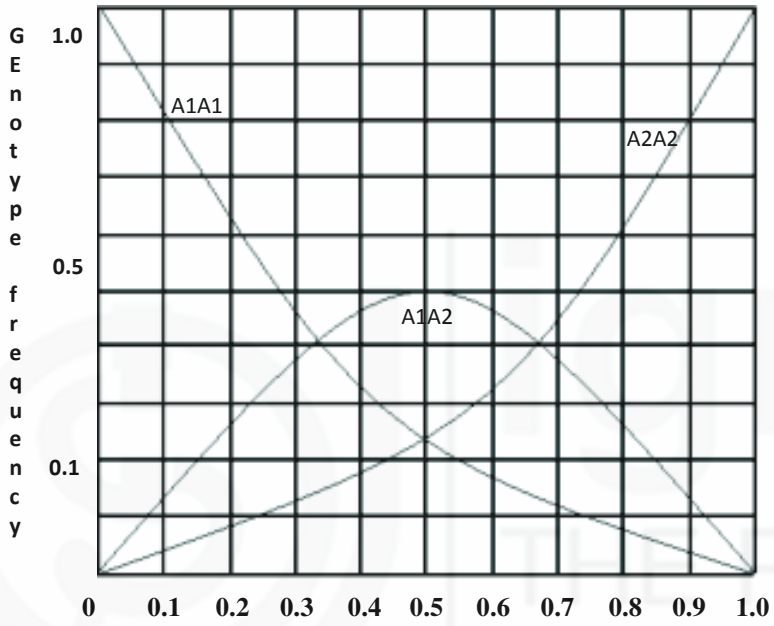



Fig: Left top curve – A1A1, Right top curve – A2A2, Lower curve – A1A2

The graph shows two interesting properties of the HWE:

- ✓ The frequency of the heterozygotes can reach to a maximum of 50%
- ✓ And this can occur when the gene frequency of '*p*' = '*q*' = 50%
- ✓ When one of the gene frequencies of an allele is low, the rare allele predominantly occurs as heterozygotes and there will be few heterozygotes.

- An application of HWE is that when the frequency of an autosomal recessive disease (e.g., sickle cell disease, hereditary hemochromatosis, congenital adrenal hyperplasia) is known in a population and unless there is reason to believe HWE does not hold in that population, the gene frequency of the disease gene can be calculated. Likewise, the carrier rate may be calculated for autosomal recessive disorders if the disease gene frequency is known. For example, phenylketonuria (PKU) occurs in 1/11,000 ($q^2$), which gives a heterozygote carrier frequency of approximately 1/50 [ 2xq(1-q) ]. If the diseased individuals ($q^2$) are deducted from the whole population, the carrier rate in normal individuals approximates to [ 2q/1+q ].

- It has to be remembered that when HWE is tested, mathematical thinking is necessary. When the population is found in equilibrium, it does not necessarily mean that all assumptions are valid since there may be counterbalancing forces. Similarly, a significant deviance may be due to sampling errors (including Wahlund effect), misclassification of genotypes, measuring two or more systems as a single system, population substructure, failure to detect rare alleles and the inclusion of non-existent alleles. The Hardy-Weinberg laws rarely holds true in nature (otherwise evolution would not occur). Organisms are subject to mutations, selective forces and they move about, or the allele frequencies may be different in males and females. The gene frequencies are constantly changing in a population, but the effects of these processes can be assessed by using the Hardy-Weinberg law as the starting point.

- The direction of departure of observed from expected frequency cannot be used to infer the type of selection acting on the locus even if it is known that selection is acting. If selection is operating, the frequency of each genotype in the next generation will be determined by its relative fitness (W). Relative fitness is a measure of the relative contribution that a genotype makes to the next generation. It can be measured in terms of the intensity of selection (s), where $W = 1 - s$ $[0 < s < 1]$. The frequencies of each genotype after selection will be $p^2 W_{AA}$, $2pq W_{Aa}$, and $q^2 W_{aa}$. The highest fitness is always 1 and the others are estimated proportional to this. For example, in the case of heterozygote advantage (or overdominance), the fitness of the heterozygous genotype (Aa) is 1, and the fitnesses of the homozygous genotypes negatively selected are $W_{AA} = 1 - s_{AA}$ and $W_{aa} = 1 - s_{aa}$. It can be shown mathematically that only in this case a stable polymorphism is possible. Other selection forms, underdominance and directional selection, result in unstable polymorphisms. The weighted average of the fitnesses of all genotypes is the mean fitness. It is important that genetic fitness is determined by both fertility and viability. This means that diseases that are fatal to the bearer but do not reduce the number of progeny are not genetic lethal and do not have reduced fitness (like the adult onset genetic diseases: Huntington's chorea, hereditary hemochromatosis). The detection of selection is not easy because the impact on changes in allele frequency occurs very slowly and selective forces are not static (may even vary in one generation as in antagonistic pleiotropy).

- All discussions presented so far concerns a simple biallelic locus. In real life, however, there are many loci which are multiallelic, and interacting with each other as well as with the environmental factors. The Hardy-Weinberg principle is equally applicable to multiallelic loci but the mathematics is slightly more complicated. For multigenic and multifactorial traits, which are mathematically continuous as opposed to discrete, more complex techniques of quantitative genetics are required.

## 2.3 APPLICATION IN HUMAN POPULATION GENETICS

The behaviour of HW principle under different assumptions is the discipline of 'population genetics', which describes, primarily, the changes in gene frequency that are influenced by demographic factors, population structure variables,

historical, random events, sampling fluctuations and evolutionary factors of selection and mutation. In simple the four main factors that influence the gene frequency in a population are: mutation and genetic drift (non-systematic factors), migration and selection (systematic factors). The genetic drift is effective, more specifically, in populations whose size is small or limited e.g., an isolate or an island population or a small endogamous population. These are described in detail below:

- For example, HWE has helped us to find out to investigate the number of alleles of ABO locus and how to calculate the gene frequency of ABO locus (e.g., Bernstein has given the method of correction ) (see Box 2.6)
- We were able to understand how HbS despite its deleterious effect it maintains its equilibrium in the population.
- HWE helps to understand the some of the health problems in some isolated populations, whose propagation is the result of genetic drift, and selection or inbreeding etc.
- HWE has forensic applications in solving problems related to disputed paternity, to provide evidence in case of crime to detect the culprit, property or biological inheritance cases.
- It helps in understanding the complex genetic disorders, to be able to estimate the contribution of genetic versus environmental effects.
- HWE helps to understand to investigate the human origins, the role of selection versus demographic effects on the genetic diversity in a population.

**BOX 2.6**

**HWE – Gene frequency estimation: Gene counting method**

Given the information about the genotypes, HWE helps us to estimate the allele frequency by 'gene counting method' (how many alleles a genotype contains). For example,

- As each homozygote carries two alleles and each
- Heterozygote carries one allele, therefore, estimate of an allele frequency in a population of size N individuals (or 2N alleles) will be
  = (2 homozygotes + heterozygote) *(1/2N)
- In a population there will be three genotypes and their absolute frequency will be say N1, N2 and N3 (where N1 + N2 + N3 = N). If there are two alleles say 'A' and 'a' with a frequency 'p' and 'q' respectively (where p + q = 1).
- By gene counting method assuming HW law the gene (allele) frequencies of p = (1/2N) * (2N1 + N2), q = (1/2N) * (2N3 + N2) and p = (1 – q)

## 2.4 DEPARTURE FROM HWE

In general, the factors that are assumed to be non-operative under HWE are hardly realised in the living systems. The living system (populations or organisms) are structured (non-random entities) and are influenced by multiple and interactive factors that operate through space and time. With the help of HW equilibrium it is possible to investigate and estimate the effect of these individual forces that change gene frequency in human populations.

### 2.4.1 Factors Affecting Change in Gene Frequency

The four aspects of the H-W assumptions are:

i) Demographic:
   a) Size, mating, fertility and mortality, and migration

ii) Evolutionary:
   a) Mutation, selection, gene flow

iii) Population structure:
   a) Social and Cultural factors
      i) Matings and Marriage specifications that regulate the marriage or mating type in a population.
      ii) Non-random mating – Sexual selection of mates

iv) Ecological:
   a) Population bottle-neck events:
      i) Pandemic: disease, earthquake etc.
      ii) Historical: wars etc.,

Of the above factors, for the present academic purpose, we will be dealing a few factors and examine how these factors change or influence the gene frequency in a population and how to estimate them in empirical situation.

#### 2.4.1.1 Mutation

Mutation is a random change in phenotypic or genotypic forms that occur once a while in a population. The probability or likelihood of occurrence, in a population, in general, is of the order of one over several lakhs or tens of thousand of individuals.

For example, several of the Mendelian syndromes and disorders that have been discovered in human populations are the result of mutation. In general, it is observed to be a single mutation or point mutation. At molecular level, mutation primarily refers to changes in the DNA sequences (or SNPs — Single Nucleotide Polymorphism) in the genome of an individual (population) with phenotypic manifestations resulting to non-normal cases, some of them are clinically or medically identified as diseases or a syndrome. If one can search *web* resources, there is a data base created by Hopkins institute and or by NIH (America) on a list of Mendelian syndromes, which can be found by a search criterion OMIM (Online Mendelian Inheritance in Man). One can also find such data bases from a variety of sources.

Some examples will help us to get an idea of mutation and its effects. Sickle cell anaemia (or HbS condition), is a disease related to Haemoglobinopathies. Its inability to synthesize Oxygen ($O_2$) to its full capacity by an individual who is suffering from the disease or the trait, which results a risk to survival liability. This is identified as due to a point mutation or single mutation at the $6^{th}$ position of the â-globlin chain of the haemoglobin gene. The single aminoacid substitution ($â_6$ Glu to Val) changes the haemoglobin structure, which is phenotypically identified as sickle cell shaped form (or half moon shaped form) of the RBC.

Mutation is an important factor or ingredient leading to the appearance of new characters in the population. The fate of the mutation as a new character in a population depends on its advantage or disadvantage that it can impinge to the

survival fitness of the population. For example, mutation is a significant evolutionary force which can change allele frequency variation in a population under a favourable environment. How we can know the relation between the mutation and allele frequency change from HWE.

a) **Change in gene frequency due to mutation (μ):**

Random changes that happen at the DNA sequence, especially at the coding region of the gene can create an allele which can alter the gene frequency in the population in successive generations. This can be investigated theoretically given the mutation rate per generation in the population. This has been shown in Box 2.7 for bi allelic loci.

The theoretical results suggest that the change in gene frequency of a mutant allele, after 't' generations, depends on the initial allele (gene) frequency before mutation and the mutation rate of the allele per generation in the population.

This is an important result and can help to calculate change in gene frequency after 't' generations given the mutation rate (μ) per generation and the initial gene frequency in the population.

b) **Rate of mutation (μ):**

Though the mutation is random, but the rate of mutation varies. It is site specific – there are 'hot-spots' where mutation rate is more frequent than in other parts of the genome. In general, the coding part of the gene does not support mutation to occur, as a result of proof reading process and functional importance of the codons. However, mutations occur at higher rate in the intronic region, and in the repeat sequences than in exons or codons. Also the mitochondrial non-coding parts, *viz*., hyper variable regions HV1, HV2 in the D-loop has higher rate of mutation than in nuclear genome.

**BOX 2.7**

**Change in gene frequency due to mutation (μ)**

If there are two alleles 'A' and 'a' with its frequencies '$p_0$' and '$q_0$' at the initial stage (say at time '$t_0$') in a population and 'μ' is the mutation rate that changes allele 'A' to 'a' per generation, then gene frequency (g.f.) of 'A' will decrease by an amount '$\mu p_0$' in the first generation. Therefore the g.f. of 'A' allele in the first generation after mutation will be:

$$P_1 = p_0 - \mu p_0 = (1 - \mu) P_0$$

In the (next) second generation the gene frequency is expected to be:

$$P_2 = P_1 - \mu P_1 = (1 - \mu) P_0 - \mu(1 - \mu) P_0$$
$$= (1 - \mu) P_0 \quad [(1 - \mu)]$$
$$= (1 - \mu)^2 P_0$$

After 't' generations the g.f. of 'A' is expected to be

$$P_t = (1- \mu) P_{t-1} = (1- \mu)^2 P_{t-2} .. = (1-\mu)^t P_0$$

When μ is very small $(1-\mu)^t$ can be approximately equated to $= e^{-ut}$, (where e is natural logarithm to base e), therefore, gene frequency after 't' generations will be

$$P_t \sim P_0 e^{-ut}$$

Therefore, the mutations that occur at HV1 and HV2 regions of mitochondrial genome help us to investigate the short-term evolution or micro-evolutionary

trends in sub-populations. This has helped us to address some of the questions of human origins or to verify the Darwin's hypothesis that the Africa is the origin of Man. This also helps to enquire the antiquity and past genetic history of diverse populations and their diversity and relationship with other human populations.

### 2.4.1.2 Genetic Drift

Genetic drift is an important non-systematic evolutionary force. To understand the concept of genetic drift, let us know what the word 'drift' conveys, in general. One of the descriptions for the word 'drift' in the English Dictionary is: "move aimlessly from one place or activity to another' – this is more with reference to things or events that we experience with practical world e.g., drifting by air, wind and water or ocean. Similar phenomena can also happen with respect to gene frequency in a small population. In small populations, as a result of population-events such as pandemic diseases, earthquakes etc., the population size is drastically reduced which can have significant effect on the genetic diversity and gene frequency: for example, the gene frequency can drift from one generation to generation randomly leading to either loss or fixation of alleles over generations (in the absence of other interfering factors). In small populations or due to demographic and ecological effects the population size drastically reduced to a fraction (or a random sample) of the original population with allelic representation different from the original population. In these cases, there will be random changes in gene frequency, which appear to drift at varying frequencies in successive generations in an erratic manner. For example, the studies on the origins of Man, suggest that decreasing heterozygosity and linkage disequilibrium levels away from Africa are supportive of the role of genetic drift among human populations.

To understand how the genetic drift can happen or possible, one can investigate and/or understand by attempting some simple examples or simulation exercises. These are available on the online resources. One such example is illustrated in Box 2.8.

a) **Bottle-neck effect**

Genetic Drift can happen in a variety of ways due to different events that populations experiences in empirical situation. These have been referred as part of ecological factors that disturb the population size (see 2.4.1). Historically the world has experienced several pandemic diseases in the past: e.g. Syphilis, Plague, leprosy, malaria, HIV infection, etc. which has killed or wiped out bulk of the population. The natural geographical events like earthquakes, tsunami etc. had killed vast majority of the populations. Even the political and man interfering events like explosion of atomic bombs, world wars etc. have affected the demographic size of the populations. Each such event is followed by a drastic reduction in population sizes. In genetic terms it means reduction in genetic diversity (at the time of the event), and those survived will have different allelic profile or gene frequency and the stability of a particular allele over generations depends on the demographic structure of the population.

'Breeding individuals' part of the demographic structure of a population is of particular genetic importance. They are capable of mating and producing children. They will be a fraction (of the total population) who contribute to the next generation or gene pool and is referred as 'effective size' ('Ne').

**BOX 2.8**

**Simple exercises to understand the genetic drift**

There are different ways to replicate to illustrate the random drift phenomena. One such simple example could be the following:

- Start with a jar that contain with N number of blue, red, yellow balls.
- At the first step blindly or randomly take out (sat e.g., by hand) some balls and put them in the second bottle.
- Then from the second bottle, take some balls (e.g., by hand) and put in the third bottle.

If you have started with large sample (N) of mixed coloured balls you can repeat the same. Otherwise, at the third/fourth bottle you can count how many or red, blue and yellow balls. Compare the outcome with the original number of red, blue and yellow balls at the start. They will differ from the original number at the start. You may also find the absence of a particular colour at the fourth (or nth) bottle.

In case of Genetic Drift, similar such random sampling of gene frequency changes happen over successive generations in a small population. One can search several such simple examples on the online resources on genetic drift – bottleneck effect, founder effect etc.

Genetic drift can alter the 'effective size' of a population and change the genetic diversity. After successive generations, the gene frequency in the population will be significantly different from the gene frequency before genetic drift. This is similar to the bottle neck, where the narrow neck of the bottle restricts the flow and this event is referred as 'bottle neck-effect' in population genetics. Such bottle neck effect resulting to sudden population size reduction had been experienced by several human populations in the past historical times affecting the genetic structure: genetic diversity, gene frequency changes.

b) **Founder effect**

The word 'founders' refers to the ancestors or the earliest settlers who colonised or founded the new population in alien territories. It could be an historical adventure of ware fare, or exploration to a new island or new area or it could also be due to chance factors like surviving from a sudden calamities like ship wreck, etc. or it could be serial migration of people at different timing to other places: in all the cases, a few founders start living and establishing a new subpopulation.

In genetic scenario, the few founders represent a random sample of the genes from the original population or gene pool from which they got separated. It is possible that, some of the rare alleles that are in the large population, by chance, may not be present in the founder individuals. It could be that, among the founders, especially if the founders are related, by chance, some of alleles may be of a higher frequency than the original population. Therefore, in the new colony after generations the gene pool will have either absence of the allele or higher frequency of the rare allele than when compared to the original population.

c) **Serial founder effect**

It is possible that people or organisms migrate repeatedly over time or waves of migration from a region to found new colonies. Such repeated waves of migration at different time periods produce successive subpopulations or gene pools whose genetic profile will be different. There appears to be waves of out of Africa migration to other continents that had happened at different time periods in the past, whose genetic signature can now be traced among the extant populations in South Asia, Europe, and America etc. The mitochondrial, X and Y chromosomal haplogroup distribution of continental populations can be explained as a result of founder effect of out of Africa hypothesis of human origins.

d) **Empirical studies of founder effect in Man**

The importance of 'Founder effect' as significant evolutionary factor has been outlined by German evolutionary biologist Ernst Mayr (1942). Founder effect is the "*The establishment of a new population by a few original founders (in an extreme case, by a single fertilized female) which carry only a small fraction of the total genetic variation of the parental population*.» This is sampling effect especially the genetic composition and evolution of the successive generations entirely depends upon the few founders. A few examples illustrating the role of genetic drift in the gene frequency changes are shown in Box 2.9

**BOX 2.9**

**Studies on Genetic Drift**

- **Tristan da Cunha is an island**; the few hundred individuals (<300) living on the island are mostly the (15) descendants (8 males and 7 females) who had founded the island in 1816-1908. Three of the founders were Asthma sufferers and there is high incidence of Asthma in the population. In a study of the 9 Y-chromosome haplotypes of the island, seven of them are traced to its 7 male founders.

- **Amish population, USA:** All most all the Amish population (~249K) descended from about 200 founders from German during 18$^{th}$ century. The population is endogamous, they show high frequency of genetic disorders as a result of founder effect that include dwarfism, metabolic disorders, unusual distribution of blood types, metabolic disorders etc.

- **'Blue Fugates' of Appalachian, Kentucky, USA:** In 1800, Martin Fugate and his wife settled in trouble some creek in Kentucky. They carried recessive gene methemoglobinemia (met-H). Due to deficiency of an enzyme diaphorase (NADH methemoglobin reductase) met-H levels rise and this gives rise to reduced oxygen-carrying capacity. This gives a tinge of blue skin of the homozygous condition. Isolation and inbreeding has caused to increase of blue people which are traced to the founders Fugates.

- **India:** In the northeast populations, some of them live in geographical isolation, practice endogamy show unusual frequency of a few genetic

traits which are expected to be due to genetic drift and founder effect. Some of them include:

- Complete lack of A2, cde, K, pc, and AK2 genes, lack of isozyme ALDH-1 (Roychoudhury and Nei 1997), a high prevalence (about 50%) of lactase malabsorption (Flatz 1987).
- Low frequency of AIBG*2 allele ( Juneja et al. 1989), high frequency of G6PD deficiency in Naga (Seth and Seth 1971), absence of 'Gd_' variant in Adi and Hmar and high frequency of this variant in Bodos (Saha et al. 1990).
- Continuing from classical genetic observations, unique and rare allele frequency of microsatellite loci among the Adi subpopultions (Krithika et al. 2005). High frequency of susceptibility of tuberculosis in some clans of tribes, stomach cancer, high incidence of cardio deaths etc.
- Absence of attached ear lobe among the Nandiwalas in Maharashtra.
- Population size reduction and allele frequency changes among Ahmedias of Kashmir population.

### 2.4.1.3 Natural Selection

Charles Darwin (and Wallace) has described natural selection as one of the important factor (key mechanism) of evolution. Natural selection happens where there is differential rate of reproductive success among different genotypes (underlying the phenotype, or trait or observed character). How selection operates at the molecular (genome) level for example, especially change in gene frequency considered, theoretically, in population genetics.

Due to differential reproductive success involving these variant of the trait, there will be more offspring with the variant than those individuals with other variant of the trait. In Darwinian sense '*fitness*' ('*Darwinian Fitness*') refers to ability to contribute successfully to the next generation. This is also referred as 'adaptive value' or 'selective value'. Therefore, if the differences of fitness are in a way associated with the presence or absence of a particular allele (or gene) in the individual's genotype then selection operates at the genetic level.

When a gene is subjected to selection (or under selective pressure), its frequency in the offspring is not the same as in the parents (or in the previous generation) as parents with different genotypes pass on their genes *unequally* to the next generation. This leads to change in gene frequency and consequently also of genotype frequency, as a result of selection (of a particular gene). The theoretical investigation of change in gene frequency of an allele under selection pressure is more complex, than factors like mutation, migration. There could be different situations under which selection can operate in a population and different situations need to be incorporated in theoretical models. Here we will consider a few of those situations (types of selection) in a more descriptive way, rather than theoretically, which is beyond the scope of the present purpose.

Theoretically, selection is measured by '*fitness*' ('*W*') or by selection coefficient ('*s*'). *Fitness* refers to '*relative rate of survival*'. The selection coefficient ('*s*') is defined as ( *1-W* ) and the value varies between 0 and 1. Once the fitness is quantified and defined the different types of dominance can be taken as degrees of dominance with respect to fitness (this is different from the dominance effect of the gene). In general, most mutant genes are completely recessive compared to the wild type as can be observed from phenotypic form of the trait. This does not imply that the heterozygotes are equally fit when compared to homozygote.

Before we get to know the effect of selection on gene frequency, it is required to know different types of selection and its fitness values. Some of the known selection types are: no dominance, partial dominance, complete dominance, over dominance. The fitness values for the four types are shown below (See Box 2.10). The change in gene frequency with respect the four types of selection (with fitness values) are given in Box 2.11.

**BOX 2.10**

**Types of dominance or degree of dominance and fitness**

**a. No dominance:**

| $A_2A_2$ | $A_1A_2$ | $A_1A_1$ |
|---|---|---|
| I | I | I |
| 1 – s | 1 – 1/2s | 1 |

**b. Partial dominance:**

| $A_2A_2$ | $A_1A_2$ | $A_1A_1$ |
|---|---|---|
| I | I | I |
| 1 – s | 1 – hs | 1 |

**c. Complete dominance:**

| $A_2A_2$ | $A_1A_2$,$A_1A_1$ |
|---|---|
| I | I |
| 1 – s | 1 |

**d. Over dominance:**

| $A_2A_2$ | $A_1A_1$ | $A_1A_2$ |
|---|---|---|
| I | ! | I |
| $1 – s_2$ | $1 – s_1$ | 1 |

a) **Types of selection**

Selection is a systematic force and operates in different ways. Selection takes place when there is differential fitness of a heritable trait. Based on the effect on the allele frequencies, the selection can be seen operating into three types.

***Directional selection***: occurs one extreme value or allele is selected. In case if one of the allele of a variety of the trait has greater fitness and producing more offspring of that allele or a variety, then the selection is said to be directional. The effect of directional selection is fixation of allele with greater fitness and the loss of the allele with least fitness. For example: well known cases come from the parasitic world, especially resistance to antibiotics in case of some of the vector-borne diseases. Initially as a result of antibiotic the parasite growth comes down to zero, but the parasites develops some mutant or new variant which gets resistance against the antibiotics or better fitness in the presence of antibiotics, in due course, the less fit variant is replaced by new variant which can survive against antibiotics. This can be illustrated as a shift in the mean of the character of a distribution (See box 2.12).

## BOX 2.11: Change in gene frequency under selection

First we will consider the basic formulae for the change in gene frequency that is achieved in one generation of selection. Under the similar notation that has been used above for other factors (p = gene freq. of $A_1$ and q = gene freq. of $A_2$), the below table shows the genotype frequencies under HWE before selection to the allele for the three genotypes (first line).

| | Genotypes | | | |
|---|---|---|---|---|
| | $A_1A_1$ | $A_1A_2$ | $A_2A_2$ | Total |
| Initial frequency | $p^2$ | 2pq | $q^2$ | 1 |
| Coefficient of selection | 0 | 0 | | s |
| Fitness | 1 | 1 | 1 – s | |
| Genetic contribution | $p^2$ | 2pq | $q^2$ (1—s) | $(1 - sq^2)$ |

Here we consider selection acting on the recessive genotype $A_2A_2$ with a selection coefficient: 's' acting against it. This will have a differential fitness to the genotypes that will be as given in the second line. By multiplying the initial frequency by the fitness values gives the frequency of each genotype after selection. This is the third line – the genetic contribution to allow to selection to operate over life cycle. Therefore, after selection, there will be a loss of fitness that is proportional to an amount $(1 - sq^2)$. From this we can calculate the frequency of $A_2$ gametes produced (frequency of $A_2$ genes in the progeny). The new g.f. is (where p = (1 – q)

$$q_1 = [q^2 (1 - s ) + pq] / (1 - sq^2)$$
$$= [q - sq^2] / (1 - sq^2)$$

The change in gene frequency $\Delta q$, resulting from one generation of selection is

$$\Delta q = q_1 - q = sq^2 (1 — q) / (1 - sq^2)$$

This tells us that the effect of selection on gene frequency depends not only on the intensity of selection s, but also on the initial gene frequency (of the recessive allele).

*Different type of selection*

What we have considered above is in general selection with respect to recessive allele q under selective pressure. But there are variety (or types) of selection that can act on the allele frequency. Depending upon the type of selection the change in gene frequency will consequently change. These are shown in the following table.

| | **Initial freq. & fitness of Genotypes** | | | **New gene frequency due to selection at q** | **Change in gene frequency** |
|---|---|---|---|---|---|
| | $A_1A_1$ $p^2$ | $A_1A_2$ 2pq | $A_2A_2$ $q^2$ | $q_1$ | $\Delta q = q_1 - q$ |
| 1. | 1 | 1– ½ s | 1– s (q – ½ sq – ½ $sq^2$) / (1 – sq) | – ½ sq (1—q) / ( 1– sq) |
| 2. | 1 | 1 – hs | 1– s (q – hspq – $sq^2$) / (1–2hspq–$sq^2$) | –spq[q + h(p-q)]/ (1–2hspq–$sq^2$) |
| 3. | 1 | 1 | 1 – s (q – $sq^2$) / (1 – $sq^2$) | – [$sq^2$ (1—sq)]/ (1 – $sq^2$) |
| 4. | 1–s | 1–s | 1(1–sq + $sq^2$) / (1– s(1-$q^2$) | + [$sq^2$ (1 - sq)]/ [1 – s(1 – $q^2$)] |
| 5. | 1–$s_1$ | 1 | 1– $s_2$ (q – $s_2q^2$) / (1 – $s_1p^2$ – $s_2q^2$) | + [pq($s_1$p – $s_2$q)] / (1–$s_1p^2$ – $s_2q^2$) |

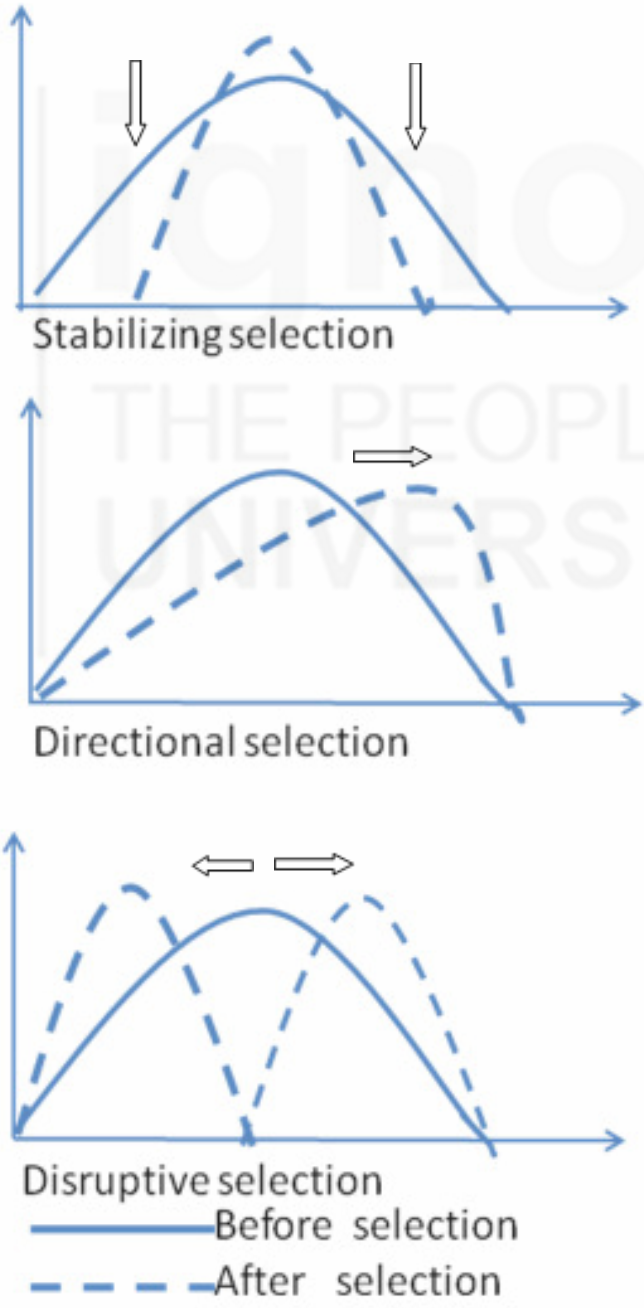
Above the different types of dominance are:

1. No dominance, selection against $A_2$ 2. Partial dominance of $A_1$: selection against $A_2$
3. Complete dominance of $A_1$: selection against $A_2$
4. Complete dominance of $A_1$: selection against $A_1$
5. Over dominance: Selection against $A_1A_1$ and $A_2A_2$ (Applicable to any degree of dominance with fitnesses expressed relative to $A_1A_2$)

***Stabilizing selection***: The extremes are selected in favour of the middle. In case of stabilizing selection, the two extreme values of a trait or alleles will have lower fitness than the intermediate value or the heterozygote alleles of a trait. One of the well known examples includes birth weight. The average birth weight of offspring ranges between 2500g (5.5pounds) to 4500g (10 pounds). Offspring with weight less than 2500g are low birth weight and greater than 4500g are the heavy babies and both have less chance of survival. As a result the selection favours the offspring with the average birth weight. Stabilizing selection is also the reason in case of height distribution in a population. This can be illustrated as a change in mean values of the distribution (See Box 2.12)

**BOX 2.12**

**Different types of selection. Change in gene frequency in case of Stabilizing, Directional and disruptive (balancing) selection**



***Balanced Selection***: In case of balanced selection, the heterozygotes have higher fitness than either of the homozygotes. This is also called heterozygous advantage or over-dominance. The best example is the sickle cell anaemia. In non-malarial environment the homozygote state of the sickle cell anaemia will have low fitness and as a result the allele gets lost in the population in due course of time. However, in malarial environment, Homozygote sickle cell anaemic individuals have the better fitness as equal to the normal homozygote individuals; as such both the alleles will be maintained in the population. (See Box 2.12).

***Disruptive selection***: both the extreme value (alleles) of a trait gets selected. It is one form of balanced selection. In case of disruptive selection, the extreme values or the alleles (low and high) of a trait will have a higher fitness when compared to the average value. As a result of disruptive selection the extreme values will increase as against the average values of the trait. This can be explained as leading to bimodal distribution (See Box 2.12).

b) **Opportunity for natural selection**

In general, to investigate natural selection in human populations is complex. Since natural selection operates on fertility and mortality, it can help us to get an overall idea of operation of natural selection. Indeed, Crow (1958) has formulated an index (Crow's Index) to examine the maximum intensity of (natural) selection that is more applicable for human populations; the index is based on the demographic components of fertility and mortality rates. According to Crow, "*there can be selection only if, through differential survival and fertility, individuals of one generation are differentially represented by progeny in succeeding generations.The extent to which this occurs is a measure of* ***'total selection intensity'***. *It sets an upper limit on the amount of genetically effective selection.*"

The total selection intensity (as defined by Crow) has two components: A fertility component ($I_f$) and mortality ($I_m$). The fertility and mortality patterns depend on several factors that vary across populations such as age at marriage, menarche, and survival to reach to fertility age, variation in fertility and age of death etc. Likelihood of these occurring needs to be calculated based on age-sex structure.

The fertility and mortality also include embryonic development and birth; these have been incorporated to make it more rigorous and efficient estimate by Johnston and Kensinger (1971). More details of the Crow Index and the relationship are given in Box 2.13.

The estimates of 'total intensity of selection' have been studied in wide diverse populations. In Indian scenario, tribal populations show larger 'Index of mortality than fertility components. There is also an overall declines in $I_m$ and $I_t$ among urban communities as a result of socio-economic and public health facilities. More details of the trends of the Crow's Index in Indian populations are described by Gautam (2009).

**BOX 2.13**

**Index of opportunity for selection**

**Crow (1958) and Johnston & Kensinger (1971)**

The total selection intensity ($I_t$), is computed based on

*I*m = index of opportunity for natural selection due to pre-reproductive mortality (mortality from birth to reproductive age, i.e. below 15 years).

*I*f = index of opportunity for natural selection due to fertility.

*X* = average number of live births per women who have completed their reproductive life span (aged 45 years and above).

*V*f = variance (average deviation from mean) of number of live births.

*P*d = proportion of pre-reproductive deaths.

*P*s = proportion of survivors from birth to reproductive ages.

The proportion of pre-reproductive deaths (*P*d) is calculated from children ever-born to mothers aged 45 years and above (who have completed their fertility) and pre-reproductive deaths.

The proportions of survivors were calculated by subtracting *P*d from 1:

$$I_t = I_m + I_f/P_s$$

$$I_m = P_d/P_s \;\&\; P_s = 1/P_d$$

$$I_f = V_f/X^2$$

The crow's Index of opportunity for selection was modified by Johnston and Kensinger (1971) to account for the survival and mortality component during conception, before the birth of an infant.

This include Ime = the selection due to prenatal mortality, Ped = the probability to die before birth,

Pb = the probability to survive till birth, Imc the index of total selection due to postnatal mortality,

Pd = the probability to die before reaching reproductive age,

Ps = the likelihood to survive til reproductive age, If = selection due to fertility,

V = varience due to fertility among women who had completed their fertility,

X is the mean number of births, Pd and Ps are proportion of deaths and survivors.

The modified total intensity index $I_t$ is:

$$I_t = I_{me} + (I_{mc}/P_b) + (I_f/P_b)\, P_s$$

$$I_{me} = P_{ed}/P_b,\; P_b = 1 - P_{ed}$$

$$I_{mc} = P_d/P_s$$

$$P_s = (1 - P_d)$$

$$I_f = V/X^2$$

## 2.4.1.4 Gene Flow

### a) Migration

Migration or gene flow is an important factor that can change the gene frequency. Emigration or immigration of individuals between populations can alter or change in the gene frequency. In genetic terms it is either loss of genetic diversity due to emigration or increase of genetic diversity due to immigration of individuals. There is loss of gene flow from a gene pool or gain of gene flow into a subpopulation from other gene pool. The quantitative estimate of the effect of migration in case of an allele at a single locus has been estimated by Bernstein and it has been shown (Box 2.14).

**BOX 2.14**

**Change in gene frequency due to migration (m) / gene flow or genetic admixture**

Suppose if migration is unidirectional from mainland to a nearby island and is random, then suppose

'm' is the rate of migration per generation from mainland to island

a) Pi be the frequency of gene A in immigrating individuals

'$p_0$' is the frequency of gene A in the island

b) The gene freq of A in the ***island after migration*** is

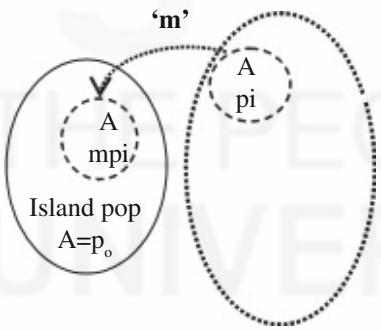
$$p_{am} = mpi + ( 1 - m ) p_0$$

The change in gene freq in one generation is

$$p_{am} - p_0 = [ mpi + (1-m) p_0] - p_0$$

$$= m (pi - p_0) + p_0 - p_0$$

$$m = (p_{am} - p_0) / (pi - p_0)$$




**NB:** This is based on Bernstein's formula for an allele at a single locus

The effect of migration rate (m) on allele frequency in a population is the proportion of differences of allele frequency in the island population ($p_i$) before and after migration ($p_{am}$) to the difference between allele frequency in the migrant population ($p_0$) and the island population ($p_i$). The above formula can be extended for a multiple loci by using least square or maximum likelihood estimate procedures. It can also be worked out based on gene identity method.

### b) Genetic admixture

Gene flow can happen between two subpopulations through random mating or admixture or marriages. The American Blacks, Anglo Indians, are examples of genetic admixed populations. The Latin American countries are populated by admixed populations contributed by native tribes, African, European and other settlers. The estimates of admixture proportion can be estimated for a gene located at a specific locus of interest or for a set of genetic markers located at different loci. The above formula (Box 2.14) can be used to estimate the 'm' the admixture

in a hybrid population. It is also possible to estimate the admixture proportions based on genetic distances and from principal component analysis (for multilocus allele frequencies).

### c) Barriers to gene flow

Human populations live over wide geographical regions forming local subpopulations; these subpopulations are formed as a result of endogamy which is promoted by geographical, cultural, linguistic, political and other factors. The same factors form barriers for gene flow and restrict the admixture, intermarriages etc. between the local populations. In India caste, geographical isolation, cultural, linguistic, political factors play a major role in restricting the gene flow or admixture or intermarriages between groups.

### d) Theoretical Models of gene flow

These factors are important to consider estimating or modelling the gene flow between populations. In population genetic point of view, there is a decrease in genetic diversity with the increasing distance or geographical location of the populations. This gives spatial pattern of gene frequency clines, which help us to understand the geographic variation of genetic markers across populations and regions.

Since gene flow can occur in different scenarios, there are a variety of theoretical models to account for different situations of spatial gene exchange or flow. For example, Sewall Wright has proposed 'island', 'neighbourhood' and 'isolation by distance' models and 'steppingstone' model by Kimura and Weiss.

**Island model*:*** It is the simple situation similar to island population. Suppose the population is distributed among a few close (equi distance) islands, each of population size N. The people tend to marry within each of the islands and gene flow is restricted, in the sense that there is equal immigration between islands, hence 'island model'. Suppose the mating takes place at random in each of such island or insular populations. The gene frequency in each of the island will differ with respect to total population (of all the islands). The theoretical results show that the deviation in such island model is exactly the variance in allele frequency among the islands. The number of homozygotes in the total population is always larger than expected from HW proportions in that population. The result is known as '*Wahlund principle'*. For a two-allele polymorphism, the genotypic proportions in the total population are:

$$AA : p^2_0 + V, \quad Aa : 2p_0q_0 - 2V \quad \text{and} \quad aa: q^2_0 + V.$$

These proportions are similar to those population practicing inbreeding with inbreeding coefficient 'F' ($F = V/p_0q_0$). Where $P_0$ is the gene frequency of allele A and V is the variance of the gene frequency among the islands. "*The change in heterozygote frequency is twice the covariance among populations in the frequency of the allele in the heterozygote, and this may be positive or negative*." (Christiasen and Feldman, 1986). One other model proposed by Sewall Wright is Neighbourhood model**.**

***Steppingstone model:*** The island model is too realistic to realise, therefore other models have been proposed which is more close to geographically structured populations. Kimura and Weiss (1964) proposed the '*stepping stone model'*. In

'one-step-linear (one dimensional) stepping stone model, the populations are arranged, rather in a linear fashion, on a long chain. The migration occurs between the neighbouring populations. This situation allows the distant populations with least migration between them are expected to behave differently than the neighbouring populations that are expected to change the gene frequency of the extreme populations as against the neighbouring populations. Kimura and Weiss (1964) have shown that the correlation in gene frequencies ( $r$ ) between demes decreases approximately exponentially as a function of the number of steps ( $x$ ) between deems.

This is expected to lead to clines in the gene frequency or geographical clines of the allele frequency.

***Isolation by distance model:*** This was proposed by Sewall Wright, which is in a similar to the stepping stone model in a continuously distributed population.

### 2.4.1.5 Genetic Equilibrium

The evolutionary forces of mutation, selection, and drift may oppose each other to create a dynamic equilibrium in which allele frequencies no longer change.

In a randomly mating population without selection or drift to change allele frequencies, and without migration or mutation to introduce new alleles, the Hardy-Weinberg genotype frequencies persist indefinitely. Such an idealized population is in a state of genetic equilibrium. In reality, the situation is much more complicated; selection and drift, migration and mutation are almost at work changing the population's genetic composition. However, these evolutionary forces may act in contrary ways to create a dynamic equilibrium in which there is no net change in allele frequencies. This type of equilibrium differs fundamentally from the equilibrium of the ideal Hardy-Weinberg population. In a dynamic equilibrium, the population simultaneously tends to change in opposite directions, but these opposing tendencies cancel each other and bring the population to a point of balance. In the ideal Hardy-Weinberg equilibrium, the population does not change because there are no evolutionary forces at work. However, opposing evolutionary forces can create a dynamic equilibrium within a population.

**Box 2.15**

**Calculating Equilibrium Allele Frequencies with Balancing Selection**

| | | | |
|---|---|---|---|
| Genotypes: | *AA* | *Aa* | *aa* |
| Relative fitnesses: | $1 - s$ | 1 | $1 - t$ |
| Frequencies: | $P^2$ | $2pq$ | $q^2$ |
| Average-relative fitness: | $\bar{w} = P^2 x (1–s) + 2pq x 1 + q^2 x (1–t)$ | | |
| Frequency of A in the next generation after selection: | $P^1 = [P^2 (1 – s) + (1/2) 2pq] / \bar{w} = p(1–sp) / \bar{w}$ | | |
| Change in frequency of A due to selection: | $\Delta p = P^1 - p = pq(tq-sp) / \bar{w}$ | | |
| At equilibrium, $p\Delta = 0$ | $P = t / (s + t)$ and $q = s / (s + t)$ | | |

## Balancing Selection

One type of dynamic equilibrium arises when selection favors the heterozygotes at the expense of each type of homozygote in the population. In this situation, called *balancing selection* or *heterozygote advantage*, one can assign the relative fitness of the heterozygotes to be 1 and the relative fitness of the two types of homozygotes to be less than 1:

| Genotype: | *AA* | *Aa* | *Aa* |
|---|---|---|---|
| Relative fitness | 1– *s* | 1 | 1– *t* |

In this formulation, the terms 1 – *s* and 1-*t* contain selection coefficients that are assumed to lie between 0 and 1. Thus, each of the homozygotes has a lower fitness than the heterozygotes. The superiority of the heterozygotes is sometimes referred to as '*overdominance'*.

In cases of heterozygote advantage, selection tends to eliminate both the *A* and '*a'* alleles through its effects on the homozygotes, but it also preserves these alleles through its effects on the heterozygotes. At some point these opposing tendencies balance each other, and a dynamic equilibrium is established. To determine the frequencies of the two alleles at the point of equilibrium, one must derive an equation that describes the process of selection, and then solve this equation for the allele frequencies when the opposing selective forces are in balance that is, when the allele frequencies are no longer changing (Box 2.15).

At the balance point, the frequency of *A* is $p = t/(s + t)$,

and the frequency of *a* is $q = s/(s + t)$

As an example, let's suppose that the *AA* homozygotes are lethal ($s = 1$) and that the aa homozygotes are 50 percent as fit as the heterozygotes $(t = 0.5)$. Under these assumptions, the population will establish a dynamic equilibrium when $p = 0.5/(0.5 + 1) = 1/3$ and $q = 1/(0.5 + 1) = 2/3$.

Both alleles will be maintained at appreciable frequencies by selection in favour of the heterozygotes – a condition known as a balanced polymorphism.

In humans, the disease sickle-cell anaemia is associated with a balanced polymorphism. Individuals with this disease are homozygous for a mutant allele of the â-globin gene, denoted $Hb^s$, and they suffer from a severe form of anaemia in which the haemoglobin molecules crystallize in the blood. This crystallization causes the red blood cells to assume a characteristic sickle shape. Because sickle-cell anaemia is usually fatal without medical treatment, the fitness of $Hb^s$ $Hb^s$ homozygotes has historically been 0. However, in some parts of the world, particularly in tropical Africa, the frequency of the H*b*$^s$ allele is as high as 0.2. With such harmful effects, why does the H*b*$^s$ allele remain in the population at all?

The answer is that there is moderate selection against homozygotes that carry the wild-type allele H*b*$^A$. These homozygotes that carry the wild-type allele H*b*$^A$. These homozygotes are less fit than the $Hb^s$ $Hb^A$ heterozygotes because they are more susceptible to infection by the parasites that cause malaria, a fitness-reducing disease that is widespread in regions where the frequency of the $Hb^S$ allele is high.

We can schematize this situation by assigning relative fitness to each of the genotype of the â-globin gene:

| Genotype: | H$b^S$ H$b^S$ | H$b^S$ H$b^A$ | H$b^A$ H$b^A$ |
|---|---|---|---|
| Relative fitness: | 1 – s | 1 | 1 – t |

If one assumes that the equilibrium frequency of H$b^S$ is p = 0.1 – a typical value in West Africa – and if one notes that s = 1 because the HbS HbS homozygotes die, one can estimate the intensity of selection against the HbA HbA homozygotes because of their greater susceptibility to malaria:

$$P = t/(s + t)$$

$$0.1 = t/(1+t)$$

$$t = (0.1)/(0.9) = 0.11$$

This result tells us that the H$b^A$ H$b^A$ homozygotes are about 11 percent less fit than the H$b^S$ H$b^A$ heterozygotes. Thus, the selective inferiority of the HbS HbS and HbA HbA homozygotes compared to the heterozygotes creates a balanced polymorphism in which both alleles of the â-globin gene are maintained in the population.

Various other mutant Hb alleles are found at appreciable frequencies in tropical and subtropical regions of the world in which falciparum malaria is – or was – endemic. It is plausible that these alleles have also been maintained in human populations by balancing selection.

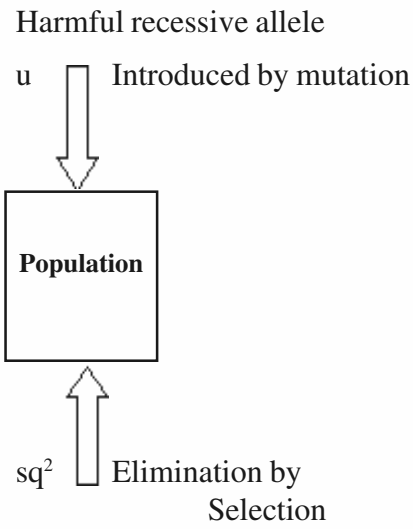
**Mutation-Selection Balance**

Another type of dynamic equilibrium is created when selection eliminates deleterious alleles that are produced by recurrent mutation. For example, let's consider the case of a deleterious recessive allele a that is produced by mutation of the wild-type allele *A* at rate *u*. A typical value for *u* is $3 \times 10^{-6}$ mutations per generation. Even though this rate is very low, over time, the mutant allele will accumulate in the population, and, because it is recessive, it can be carried in heterozygous condition without having any harmful effects. At some point, however, the mutant allele will become frequent enough for aa homozygotes to appear in the population, and these will be subject to the force of selection in proportion to their frequency and the value of the selection coefficient *s*. Selection against these homozygotes will counteract the force of mutation, which introduces the mutant allele into the population.

If one assumes that the population mates randomly, and if one denotes the frequency of *A* as *p* and that of *a* as *q*, then one can summarize the situation as follows:

| Mutation: | | | Selection: | |
|---|---|---|---|---|
| Produces a | | | eliminates a | |
| A→ a | Genotype: | AA | A*a* | *aa* |
| rate = u | Relative fitness: | 1 | 1 | 1-*s* |
| | Frequency: | $P^2$ | 2*pq* | $q^2$ |

Mutation introduces mutant alleles into the population at rate *u*, and selection eliminates them at rate $sq^2$




Mutation-selection balance for a deleterious recessive allele with frequency q. Genetic equilibrium is reached when the introduction of the allele into the population by mutation at rate u is balanced by the elimination of the allele by selection with intensity s against the recessive homozygotes.

When these two processes are in balance, a dynamic equilibrium will be established. We can calculate the frequency of the mutant allele at the equilibrium created by mutation – selection balance by equating the rate of mutation to the rate of elimination by selection:

$$u = sq^2$$

Thus, after solving for q, we obtain $q = \sqrt{u / s}$

For a mutant allele that is lethal in homozygous condition, s = 1, and the equilibrium frequency of the mutant allele is simply the square root of the mutation rate. If one uses the value for *u* that was given above, then for a recessive lethal allele the equilibrium frequency is $q = 0.0017$. If the mutant allele is not completely lethal in homozygous condition, then the equilibrium frequency will be higher than 0.0017 by a factor that depends on $1/\sqrt{s}$ . For example, if s is 0.1, then at equilibrium the frequency of this slightly deleterious allele will be q=0.0055, or 3.2 times greater than the equilibrium frequency of a recessive lethal allele.

Studies with natural population of *Drosopbila* have indicated that lethal alleles are less frequent that the preceding calculations predict. The discrepancy between the observed and predicted frequencies has been attributed to partial dominance of the mutant alleles-that is, these alleles are not completely recessive. Natural selection appears to act against deleterious alleles in heterozygous condition as well as in homozygous condition. Thus, the equilibrium frequencies of these alleles are lower than one would otherwise predict. Selection that acts against mutant alleles in homozygous or heterozygous condition are sometimes called purifying selection.

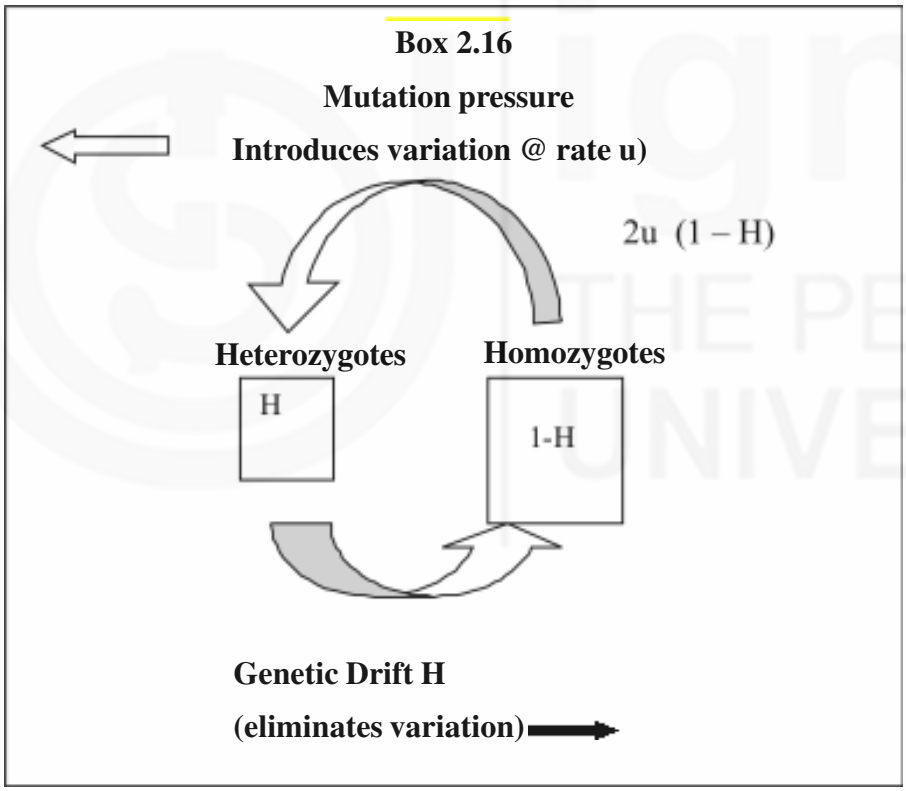
**Mutation-Drift Balance**

The random genetic drift eliminates variability from a population. Without any counteracting force, this process would eventually make all populations

completely homogeneous. However, mutation replenishes the variability that is lost by drift. At some point, the opposing forces of mutation and genetic drift come into balance and a dynamic equilibrium is established. The genetic variability can be quantified by calculating the frequency of heterozygotes in a population- a statistic called the heterozygosity, which is symbolized by the letter H. The frequency of homozygotes in a population- often called the *homozygosity*- is equal to 1-H. Over time, genetic drift decreases H and increases 1-H, and mutation does just the opposite as shown in the figure below (Box 2.16).

Let's assume that each new mutation is selectively neutral. In a randomly mating population of size N, the rate at which drift decreases H is $\frac{1}{2N}$ )H. The rate at which mutation increases H is proportional to the frequency of the homozygotes in the population (1-H) and the probability that one of the two alleles in a particular homozygote mutates to a different allele, thereby converting that homozygote into a heterozygote. This probability is simply the mutation rate μ for each of the two alleles in the homozygote; thus, the total probability of mutation converting a particular homozygote into a heterozygote is 2μ. The rate at which mutation increases H in a population is therefore equal to 2μ(1 - H).

**Box 2.16**




When the opposing forces of mutation and drift come into balance, the population will achieve an equilibrium level of variability denoted by H. This equilibrium value of H can be estimated, by equating the rate at which mutation increases H to the rate at which drift decreases it:

$$2\mu(1 - H) = \quad H$$

By solving for H, the equilibrium heterozygosity at the point of mutation-drift balance is obtained as :

$$\hat{H} = 4N\mu / (4N\mu + 1)$$

Thus, the equilibrium level of variability(as measured by the heterozygosity) is a function of the population size and the mutation rate.

If one assumes that the mutation rate is $\mu = 1\times10^{-6}$, one can plot $\hat{H}$ for different values of N. For N < 10,000, the equilibrium frequency of heterozygotes in the population will be quite low; thus, drift dominates over mutation in small populations. For N equal to 1/μ, the reciprocal of the mutation rate, the equilibrium frequency of heterozygotes would be 0.8, and for even greater values of N, the frequency of heterozygotes increases asymptotically towards 1. Thus, in large populations, mutations dominate over drift; every mutational event creates a new allele, and each new allele contributes to the heterozygosity because the large size of the population protects the allele from being lost by random genetic drift.

Values of $\hat{H}$ in natural populations vary among species. In the African cheetah, for example, $\hat{H}$ is 1 percent or less among a sample of loci, suggesting that over evolutionary time, population size in this species has been small. In humans, $\hat{H}$ is estimated to be about 12 percent, suggesting that evolutionary time population size has averaged about 30,000to 40,000 individuals. Estimates of population size that are derived from heterozygosity data are typically much smaller than estimates obtained from census data. The reason for this discrepancy is that the estimates based on heterozygosity data are *genetically effective* population sizes-sizes that take into account restrictions on mating and reproduction, as well as temporal fluctuations in the number of mating individuals. The genetically effective size of a population is almost less than the census size of a population.

(***Source:*** *Principles of Genetics (2006) by D. Peter Snustad and Michael J. Simmons. John Wiley & Sons (Asia Edition) PP. 750-754.*

## 2.5 SUMMARY

1) Understanding of Population genetics principles, requires the basic concepts of Mendelian genetics: the result of segregation, the concept 'gene', 'phenotype', 'genotype', 'dominant' , 'recessive' traits, 'allele' etc. Parental mating types and expected distribution of genotypes among the offspring.

2) Hardy-Weinberg equilibrium is the solution to an intriguing question: what happens to gene frequency of a dominant character over generations in a population. With three times more frequent than normal does this will increase over generations?

3) HWE law states that under the absence of intervening factors, especially in a large population, given random mating, no selection of any sort, no mutation and absence of demographic factors like migration, differential fertility and mortality etc., the allele frequency remain constant over generations. This can be proved theoretically, easily, for a 'biallelic locus and it can be extended to multilocus as well.

4) The importance of HWE: it gives a methodology to estimate the allele frequency in a population based on phenotypic/genotypic information of the parental mating types. It helps us to investigate the relationship between change in gene frequency with respect to mutation, migration, selection,

genetic drift etc. The entire investigation is the kernel of a branch of biomathematics or the new field: 'population genetics' and 'quantitative genetics'.

7) HWE is the bench mark of qualitative test to check whether a trait, an allele, SNP, is in equilibrium. It tells how to distinguish between the effects of evolutionary forces from the demographic factors.

8) Mutation is a non-systematic and random, but rate of mutation is site specific. Mutations are more frequent at hot-spots and are rare at the 'conserved region'. The mitochondrial non-coding genome has a higher frequency of mutations than the nuclear genome.

9) Genetic drift is a non-systematic force which can lead to significant changes in gene frequency in a small population. If an allele is rare in a small population, it can get lost or get fixed in the population over generations.

10) Founder effect is one form of genetic drift. The founders are a sample (represent a fraction of the genetic diversity) of original populations. The descendents of a few founders have the gene frequency that is dependent on the genetic composition and genetic structure of the founders. It can also happen as bottleneck effect, especially as a result of sudden population size reduction in a population, due to reasons such as natural causes or man-made causes or socio-cultural regulations. There could be serial founder effect as a result of waves of migration at different times. The mitochondrial investigation of human origins suggests that the human origins and migration to other continents appears as a result of serial founder effect from Africa.

11) Natural selection is one of the complex systematic forces that can influence significant changes in gene frequency. Selection can operate in multitude ways and it is a slow process than to the effect of migration or admixture etc.

12) Selection basically operates at differential fertility and mortality levels. It is measured as 'fitness' the ability to leave offspring and refers to 'relative rate of survival'. It is measured by 'selection coefficient' ('s') which is a function of fitness (W). The fitness or selection coefficient differs with respect to the type of dominance: complete, partial, over etc.

13) The effect of 'directional selection' to shift the mean allele frequency towards its extremes. Or it could be stabilizing selection that shifts the allele frequency of extreme alleles as a result the heterozygote frequency will increase. Or it could be disruptive selection where the extreme allele frequency increases as against the heterozygote frequency.

14) Selection can also be measured based on demographic factors of fertility and mortality trends. Crow's Index of opportunity for selection measures total selection intensity that a population can experience which depend on two components, fertility and mortality.

15) Gene flow (migration/admixture) is a systematic factor which can bring rapid changes in gene frequency within a short period. In general, human populations follow a variety of restrictions or regulations that restrict gene

flow between and within populations. The barriers for gene flow could be because of culture or due to geographical, political, religious and linguistic etc.

16) There are theoretical models to investigate the effect of spatial gene flow or population structure between populations. Island model, stepping stone model, neighbourhood model help us to investigate the spatial gene flow in different situations of population structure.

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**Sample Questions**

1) A total of 120 individuals were tested for M, N blood group and the observed genotype frequencies of MM, MN and NN are 34, 62 and 24 respectively. Calculate the gene (allele) frequencies?

2) If 'ì' is the mutation rate ('ì' = $10^{-5}$) per generation for a gene frequency of A then how many generations are required to reduce the gene frequency by a factor of ½.

3) What is Hardy-Weinberg equilibrium? Explain why HWE is important in genetic of populations?

4) In case in a population the observed gene frequencies of a particular bi-allelic locus are in HW equilibrium for the locus, does this imply the population satisfies the assumptions of the HW equilibrium? Explain?

5) What is genetic drift and how it operates in populations? Explain with Examples.

# UNIT 3 GENETIC POLYMORPHISM

**Contents**



**Learning Objectives**

After reading this unit, you will be able to:

- define the concept of genetic polymorphism;
- explain genetic polymorphism with respect to serological, biochemical and molecular markers;
- explain the genetic markers in disease association; and
- discuss the use of polymorphic markers in population and forensic studies.

## 3.1 INTRODUCTION

Genetic polymorphism can be defined as the occurrence together in the same population two or more than two alleles such that the frequency of rare allele is always >1%, and is maintained in the population not merely by the recurrent mutation. Polymorphism can be in a coding region (coding region means the portion of DNA which code for a gene, it may be synonymous or non-synonymous) or more commonly, in the noncoding regions (which does not code for functional region), often vary by ethnicity. Basic information about the types, frequencies and distribution of common polymorphisms are essential not only for the understanding of pathological entities, but also to know our evolutionary past and provide guidance about our biological future. The most common polymorphism in our genome are single base pair sequence variation i.e. SNP

but other types like copy number changes, insertions, deletions, duplications and rearrangements also occur. The methods to asses this diversity is variable. Few examples of polymorphic markers are listed in table 3.1.

**Table 3.1: Example of Genetic polymorphisms**

| Type of marker | Year | No. of loci | Properties |
|---|---|---|---|
| Blood groups | 1910-1960 | ~20 | May need fresh blood, rare antisera. Genotype cannot always be inferred from phenotype because of dominance. No easy physical localization. |
| Electrophoretic mobility variants of serum proteins | 1960-1975 | ~30 | May need fresh serum, specialized assays, no easy physical localization often limited polymorphisms |
| Human Leucocyte Antigens (HLA) | 1970 | 1 (multi locus haplotype) | One linked set highly informative. Can only test for linkage to 6p21.3 |
| DNA RFLPs | 1975 | >105 (potentially) | Two allele markers, maximum heterozygosity 0.5,initially required Southern blotting, now PCR. Easy physical localization |
| DNA VNTRs (minisatellites) | 1985- | >104 (potentially) | Many alleles, highly informative can be typed by southern blotting easy physical localization. Tend to cluster near ends of chromosomes. |
| DNA VNTRs (microsatellites) (di-,tri-, and tetranucleotide repeats) | 1989- | 105 (potentially) | Many alleles, highly informative Can be typed by automated multiplex PCR, easy physical localization. Distributed throughout genome |
| DNA SNPs | 1998- | 106 (potentially) | Less informative than microsatellites. Can be typed on a very large scale by automated equipment, without gel electrophoresis, etc. |

## 3.2 BALANCED POLYMORPHISM

When natural selection favors heterozygotes over both homozygotes, the result is balanced polymorphism. It accounts for the persistence of an allele even though it is deleterious when homozygous. Some of the examples are given below:

### Sickle Cell Disease

It is an autosomal recessive disorder that causes anemia, joint pain, a swollen spleen, and frequent, severe infections. It illustrates balanced polymorphism because carriers are resistant to malaria, an infection by the parasite *Plasmodium falciparum* that causes cycles of chills and fever. The parasite spends the first stage of its life cycle in the salivary glands of the mosquito *Anopheles gambiae.* When an infected mosquito bites a human, the malaria parasite enters the red

blood cells, which transport it to the liver. The red blood cells burst, releasing the parasite throughout the body.

It is known since long that malaria is a quite common in the tropical regions of Africa. Sickle shape red blood cells provide selective advantage as malarial parasite cannot grow in these cells. Therefore, along with malaria the sickle cell anemia also increased in these parts of Africa. The sickle cell disease is less common in Caucasians due to the less frequency of malaria. This shows the heterozygous advantage of sickle cell as it provides protective effect.

The rise of sickle cell disease goes hand in hand with the cultural development with the advent of cultivation of crops gave a breeding ground to *Anopheles* mosquitoes as the malaria rose the selective pressures gave rise to the change in the shape of the RBCs from elliptical to sickle shaped and when it occurred in homozygous condition the disease was caused otherwise it had selective advantage. The spread of sickle cell disease is associated to the migratory events. Africa by people migrating from Southern Arabia and India, or it may have arisen by mutation directly in East Africa.

Settlements with large numbers of sickle cell carriers escaped devastating malaria. They were therefore strong enough to clear even more land to grow food-and support the disease-bearing mosquitoes. Even today, sickle cell disease is more prevalent in agricultural societies than among people who hunt and gather their food.

**G6PD Deficiency**

It is a sex-linked enzyme deficiency. It affects 400 million people throughout the world. It results into hemolytic anemia which is life-threatening. It is under the influence of certain environmental conditions like eating fava beans, inhaling certain types of pollen, taking certain drugs, or catching certain infections. It has been seen in Africa that hemizygous males and heterozygous males for this enzyme deficiency are at less risk for malaria again revealing a selective advantage for heterozygotes. Therefore, natural selection acts in two directions hence it could be one of the example of balanced polymorphism.

## 3.3 TRANSIENT AND BALANCED POLYMORPHISMS

Polymorphism occurs when two or more clearly different phenotypes exist in the same population of a species-in other words, the occurrence of *more than one form* or *morph*.

A transient polymorphism is one that is changing in frequency over time. In transient polymorphism, one form is gradually being replaced by another. As the name implies, it represents a temporary situation as a by-product of directional natural selection.

The phenomenon of industrial melanism occurs in a number of moth species in Europe and the United States. The British ecological geneticist, E. B.Ford, first called attention to this phenomenon as a way of demonstrating the effect of natural selection in nature (as opposed to artificial selection experiments which have long enjoyed success in the lab). Ford noted that a light colored moth species,

*Biston betularia*, occasionally undergoes mutation at a single locus to produce a dark or melanic individual. Since the mutant allele is dominant, any gamete containing this mutant will produce a melanic individual upon syngamy. The first melanic specimen in this species was found in a collection from Manchester, England dated 1848, but by 1895 about 95% of all collected specimens were dark morphs, referred to as the form *carbonaria.* In a series of 12 observations and mark-recapture experiments during the 1950s, H. B. D. Kettlewell demonstrated that the two forms (light and dark) were differentially preyed upon by birds. He found that the birds selectively caught and ate more individuals of the form that did not match its background as compared to the one that was masked. In industrialized areas of England where the substrate (walls and tree trunks) upon which the moths rested were darkened by pollutants in the smoke poured out by factories, the *carbonaria* form possessed a selective advantage. Rural areas, unaffected by pollutants, afforded the light form an adaptive advantage. The environmental change brought on by the industrial revolution did not produce the *carbonaria* form (which presumably appeared from time to time due to recurrent mutation); it only protected the dark moths from bird predation (the agent of natural selection). The fact that the light form still exists in rare numbers in industrialized areas testifies to the amount of time selection requires to eliminate a recessive allele.

**Mendilian Population**

A population is a group of individuals who share a common gene pool where the characters are transmitted in a Mendelian fashion from one generation to the next generation. A group of individuals within which marriages are performed is called a Mendelian population. In a given Mendelian population, which is under Hardy-Weinberg equilibrium, the resultant genotype and phenotype frequencies are more or less permanently established.

## 3.4 SEROLOGICAL MARKERS

Blood groups are the best cited examples of serological markers. Both ABO and Rh are quite important serological markers as they can be used to study population diversity. These blood groups cause newborn hemolytic diseases. They also have a role in blood transfusion and also solid organ transplantation. They follow mendalian inheritance. ABO blood groups were discovered by Landsteiner in 1900 and are cited as a best example of triallelic inheritance. Blood groups can be tested by using antisera and red blood cells using simple agglutination techniques. Presently more advanced molecular techniques are also used.

The ranges of phenotypes in humans are a direct result of genetic variations which act together with environmental and behavioral factors to produce diversity. The identification of gene polymorphisms, which control the blood group antigen expression, contributes to the understanding of the biological significance of blood group systems. In addition to assisting in the characterization of allelic variations, the identification of gene polymorphisms allows us to estimate the processes involved in the formation of different populations (the founder effect, genetic drift, migration, etc.). Thus, blood group gene polymorphisms are valuable predictors of genomic ethnic ancestry.

## 3.5 BIOCHEMICAL POLYMORPHISMS

There is marked difference between individuals on the basis of biochemical markers like G6PD, human enzymes and proteins etc. This has been explained in the above section. However, here we would like to throw some light on the molecular basis of G6PD variants.

### Molecular Basis of G6PD Variants

The G6PD gene, located on chromosome Xq28 region, is 18 Kb long consisting of 13 exons transcribed to a 2.269 Kb messenger RNA with 1.545 Kb of coding regions. The commonest variant in South China, G6PD Canton, has been sequenced and was found to be due to a mutation at nucleotide (nt) position 1376 of cDNA, G to T, resulting in a missense mutation in amino acid position 459, Arg to Leu. With improved DNA technology, the whole cDNA sequence can be amplified and screened for mutation directly. PCR technique and restriction analysis has been used.

### World Incidence and Distribution of G6PD Deficiency

G6PD deficiency in male subjects can be detected easily by a number of screening tests. The simplest one is the fluorescent spot test developed by Beutler and Mitchell which relied on the fluorescence of NADPH, generated by an adequate amount of G6PD enzyme. This test can also be done on blood sample dried on filter paper similar to the Guthrie cards. In Hong Kong, the routine screening of newborns have included test for G6PD deficiency.

## 3.6 MOLECULAR MARKERS

Although ~99% DNA is known to be similar between individuals but still sequence differences exist between individuals in non-coding regions of the genome and such polymorphic regions are useful for various kinds of analyses in population genetic studies. A genetic marker can be a nucleotide sequence of variable length, varying from a single base pair to several hundred base pairs. Selection of markers for any study is dictated by the nature and purpose of the study. The more commonly used markers in population genetics studies can broadly be grouped as follows:

### 3.6.1 Repetitive DNA Sequence Variants

#### Tandem Repeats

Besides the interspersed repeats (SINEs and LINES), Tandem repeats are the other kind of repeated elements found in the genome. These are highly variable tandemly repeated arrays of 2 or more base pair core units in the non-coding regions of the genome and are located adjacent to each other. On the basis of size of the core unit, they are categorised into minisatellites (10-60 bp[1] ), Short Tandem Repeats (STRs) or microsatellites (<10 bp). When the number of nucleotides in the core unit is not known or is variable then it is called Variable Number Tandem Repeats (VNTRs).

#### Insertion/ Deletion Polymorphisms

An InDel or Insertion-Deletion polymorphism refers to insertion or deletion of a DNA sequence of variable length in the genome. The concerned DNA sequence

may vary in length from a single nucleotide to several hundred nucleotides. They are widely spread across the genome and constitute around 1.5 million of more than 10 million polymorphisms known in humans.

***Alu* InDels –** *Alu* Insertion/ Deletion polymorphisms (*Alu* InDels) involve *Alu* sequences that are characterized by the cleavage action of *Alu*I restriction endonuclease.

Properties of Alu sequences such as their known ancestral state, identity by descent, wide occurrence and stability make them ideal markers for human evolutionary and diversity studies.

## 3.6.2 Non- Repetitive DNA Sequence Variants

### Single Nucleotide Polymorphisms (SNPs or *Snips*)

SNP or Single Nucleotide Polymorphism is a single nucleotide (base pair) change in a DNA sequence. As with all polymorphisms, for an alteration to be considered a snip it must be present in ≥ 1% of the population being considered. They make up about 90% of all the human gene sequence variation. SNPs may be present in coding regions (exons) or non-coding regions (introns) or intergenic regions.

**Restriction Fragment Length Polymorphisms (RFLPs)** are the characteristic pattern of fragments of DNA produced when a DNA sequence is cleaved by specific enzymes belonging to endonuclease class of enzymes. The property of these enzymes that enables them to cleave DNA segment only at specific locations known as restriction sites have led to their use in detecting genetic differences on the basis of absence or presence of restriction sites.

## 3.6.3 Lineage Markers

### Mitochondrial Markers

Maternally inherited mitochondrial genome consists of multiple copies of circular mitochondrial DNA or mtDNA. Markers present on this haploid genome are primarily used for tracing maternal ancestral lineage(s) in populations because of their uniparental inheritance.

### Y-chromosomal Markers

Like mtDNA, Y- chromosome has a uniparental inheritance but in the male line and can thus be used for tracing paternal ancestral lineages. In absence of recombination, Y-chromosome is more or less transmitted unchanged from one generation to next and the few changes that may occur usually do not have any effect as around 98% of the DNA is in non-coding region.

### Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium predicts that in the absence of evolutionary forces, both allelic and genotypic frequencies remain constant in a population and that if the equilibrium is disturbed a new equilibrium will be reached within one generation based on the allelic frequencies of the remaining population. The conditions that must be met for the predictions of the Hardy-Weinberg equilibrium to be valid are described below:

1) Random mating: Mating patterns must randomly reflect the entire breeding population, with no dependence on genotype or closeness of relationship (either positive or negative).

2) No sex bias in allelic frequencies: The distribution of alleles must be the same in both sexes.

3) All genotypes equally viable and fertile: There must not be any selective advantages or disadvantages. This is seldom true in a real population, and often must be taken into account in terms of evolutionary pressures.

4) Mutation rate too low to alter ratios: The basic assumption is that alleles are stable through many generations and are not altered or degraded significantly by mutation. In practice this is generally not a serious problem.

5) Closed population (no in or out migration): The "population" that is being considered must be a constant one. Introduction of new genes into the breeding pool or loss of genes from the breeding pool by migration between "populations" can distort trends.

6) Population must be large: The population must be large enough so that there are no confounding effects due to genetic drift (random events altering allelic frequencies by pure chance) or due to "founder" effects, where a recessive gene becomes fixed in a population because too many of its members are descendants of a single individual.

The Hardy-Weinberg law can also be applied to multiple alleles and X-linked alleles. The genotypic frequencies expected under Hardy-Weinberg equilibrium will differ according to the situation.

## 3.7 TOOLS FOR STUDYING POLYMORPHISMS

Both conventional and advanced techniques are used to study polymorphisms. Conventional techniques are blood groups by carrying out simple agglutination techniques or protein electrophoresis for studying the protein polymorphisms etc. Under advanced techniques are the tools for studying molecular markers, and the foremost requirement for carrying out molecular analysis of any kind is the availability of the genetic material. As mentioned earlier, DNA is the focal point of human diversity and disease-association studies by virtue of the fact that it is the blueprint of our existence. There are several techniques for isolating DNA such as manual methods (like Phenol Chloroform, Salting-out) and kits. The technique of DNA isolation or extraction varies depending on the starting material, but, it is the technique of PCR which is the most useful for DNA analysis.

**Polymerase Chain Reaction (PCR)**: It involves cycling of DNA sample through a series of heating and cooling cycles with the required raw materials and enzymes to achieve its exponential amplification. The technique has come a long way since its invention. Instead of having to manually maintain the heating and cooling cycles, automated thermal cyclers are now available; and instead of having to add fresh polymerase (earlier derived from *E. coli*) after every cycle because of its denaturation due to heating, thermally stable DNA polymerases such as *Taq* DNA Polymerase are now made use of.

Amplification of DNA by PCR has found applications in a variety of fields ranging from forensics to archaeology; study of variation and evolution to mutation detection; gene mapping and cloning and DNA sequencing to epidemiology among several others.

**Restriction Digestion:** It is the method of cutting DNA sequences into fragments using restriction endonucleases or enzymes that cut at specific recognition sites. This generates DNA fragments of varying lengths producing a variation pattern known as Restriction Fragment Length Polymorphisms (RFLPs). The variation may be produced in response to absence or presence of particular SNP(s) or an insertion or deletion event in that region and is recognised in the form of banding pattern. Resulting fragments are separated according to molecular size using gel electrophoresis. There are several classes of endonucleases- Type I, Type II, Type III and Type IV but the most commonly used restriction enzymes are of type II and they cleave DNA fragment at specific sites within or close to the recognition sequence. Most of these enzymes cut palindromic sequences.

The technique is useful in detection of mutations/ SNPs. It is also used to detect VNTRs. The technique has been widely used for constructing physical maps of the genome, genetic linkage maps; in forensic testing; and in epidemiological and evolutionary studies.

**Electrophoresis:** It is one of the few techniques that has been in use since the beginning of study of classical genetic markers and is still in use for molecular markers. It is the method of separating macromolecules (both proteins and nucleic acids) on the basis of size, electric charge or other physical properties under the influence of electric field.

**Sequencing**: DNA sequencing refers to establishing the exact sequential arrangement of bases in a stretch of DNA. Knowledge of exact sequence of bases in a gene is crucial especially in ascertaining the function of genes. This is also important as the disease-causing alterations in the genes can then be identified.

The selection of technique and markers depends upon the purpose of study. In the following section we have discussed the uses of polymorphic markers.

Uses of polymorphisms: All the markers listed in table 3.1 can be used for population diversity studies. Now a days most extensively studied markers are Single nucleotide polymorphisms. Genomics and specially SNP research can be used to improve health care through gene therapy, to yield new targets for drug discovery, to renew the process of drug development and to discover new diagnostics.

## 3.8 GENETIC MARKERS AND DISEASE

Understanding the genetic basis of complex human diseases (like hypertension, cardiovascular disease, diabetes etc.) has been increasingly emphasized as a means of achieving insight into disease pathogenesis, with the ultimate goal of improving preventive strategies, diagnostic tools, and therapies. Genetic approaches to complex disorders thus offer great potential to improve our understanding of their pathophysiology, but they also offer significant challenges. These can be studied either using linkage analysis. In linkage analysis we use families and try to find out which polymorphic marker is near to the disease gene and then try to map the gene on the human genome. The other approach is where we study populations of both types of individuals. One would be those suffering with a disease and the other would be who are not suffering with the disease. We take different polymorphic markers and study in these two sets of samples. Then we

compare both the groups and if both the groups differ significantly at these markers we propose that these markers may be associated with the disease.

Association studies can be a very powerful approach for finding genetic determinants of a complex disorder. It has been suggested that if hundreds of thousands of single nucleotide polymorphisms (SNPs) were identified across the genome, then it would be possible to perform genome-wide association studies to identify the regions of linkage disequilibrium around disease susceptibility genes. In addition, they noted that much smaller sample sizes would be required to detect association than to detect linkage. The SNP Consortium is rapidly identifying single nucleotide polymorphisms, and within next several years, genome-wide association studies may become a reality.

These association studies can result into positive association or negative association. Some time they result into false positive or false negative results. The following general guidelines, summarized in Table 3.2, may be useful for genetic association studies. First, are the candidate gene(s) under study should be biologically reasonable. Several factors can determine the appropriateness of a candidate gene. If human genetic linkage studies have identified a chromosomal region linked to a disease, or if an animal model for a disease is influenced by a particular gene or syntenic chromosomal region, positional candidate genes in such genomic regions warrant strong consideration. In addition, the biologic plausibility of a candidate gene for involvement in disease pathogenesis is important. However, obvious limitations of this candidate approach are the large number of potential candidate genes for complex diseases and the reality that only known genes can be investigated. Although candidate genes can be selected for study on this basis, they should not be ruled out on the basis of our current understanding of disease pathophysiology- important new insights may be missed if potential candidate genes must fit into current pathophysiologic models.

**Table 3.2: Evaluation of candidate gene case-control association studies**

| Issue | Key Questions | Possible Solutions |
|---|---|---|
| Selection of candidate | Is candidate gene biologically reasonable? | Demonstration of biologically functional effect |
| Gene polymorphism | Is the candidate gene a positional candidate? | Within linked region in man or systemic from animal model |
| Population stratification | Are cases and controls matched? | Matching on ethnicity<br>Family-based association designs<br>Negative results with multiple unlinked markers |
| Hardy-Weinberg (H-W) equilibrium | Is control group in H-W equilibrium? | Calculation of H-W equilibrium with goodness-of-fit test (2 alleles) or simulation (multiple alleles) |
| Multiple comparisons | How many alleles were tested?<br>How many genetic loci were tested? | Bonferroni correction<br>Estimation of empirical P values |

A second criterion in evaluation of case-control association studies is the careful selection of cases and control subjects. Do the case subjects meet appropriate criteria for disease affection? Are control subjects free from symptoms of disease, associated intermediate phenotypes, and potential confounders? Have control subjects been exposed to relevant environmental influences involved in disease pathogenesis while remaining clearly unaffected? Were the cases and controls matched on demographic and environmental factors? Was consideration of population stratification included, either by attempting to match ethnicity or by typing unlinked markers.

A third criterion in the evaluation of case-control studies is assessment of Hardy-Weinberg equilibrium in the markers studied within the control group. Hardy-Weinberg equilibrium indicates that the genotype frequencies can be determined directly from the allele frequencies; failure to demonstrate Hardy-Weinberg equilibrium could result from genotyping errors, inbreeding, genetic drift, mutation, or population substructure. Hardy-Weinberg equilibrium can be readily assessed with a goodness-of-fit chi square test for biallelic markers; for markers with multiple alleles (such as short-tandem repeat markers), more accurate determination of Hardy-Weinberg equilibrium can be obtained with Markov Chain Monte Carlo methods. Significant deviations from the expected proportions of homozygote and heterozygote classes in a population of case subjects may be caused by association with the disease allele. Lack of consistency with Hardy-Weinberg equilibrium among control subjects should prompt investigation for potential complications, including genotyping errors and population stratification. A final criterion for evaluation of a case-control study is correction for multiple comparisons. This remains a problematic topic requiring additional statistical genetic research. However, an effort to correct for spurious associations, which can result from testing a large number of alleles, is warranted. The multiple comparison issue is especially problematic with markers that have multiple alleles like short-tandem repeat polymorphisms; the conservative Bonferroni approach to use a corrected significance value calculated by multiplication of the observed *P* value by the number of alleles tested. Bonferroni corrections for the total number of alleles at all loci are probably too conservative because the alleles at one locus are not independent of each other and closely linked loci are probably not independent either. A less conservative but more computationally intensive approach is to estimate empirical significance values using simulation approaches.

### Genome Wide Studies

Unlike the direct approach of case-control association with candidate genes, genome scanning (screening) is an indirect strategy that does not rely on conjecture. Basically, either affected individuals, usually siblings, from a number of families or families with two or more affected individuals are genotyped with polymorphic DNA markers that cover the entire chromosome complement. A set of about 400 short tandem repeat polymorphic markers that are spaced at about every 10cM is used for most genome scans. This level of resolution has been enhanced with the assembly of about 3000 simple sequence repeat polymorphic markers that are about 1.5cM apart. Single –nucleotide polymorphic sites (SNPs) are preferred for genome scans because they are uniformly distributed about every 300 bases throughout the genome and easily identified with automated equipment. Eventually, sets of SNPs will supersedes short tandem repeat polymorphic sequence marker systems.

Furthermore, major landmark attempts that have also been made to study various aspects of human genome, and few are listed below.

***Human Genome Project*** (HGP): A National Institute of Health (NIH, US) initiative started in 1990, HGP was a multinational collaborative project aimed at identifying all the genes in the human DNA and determining the sequence of about 3 billion nucleotide pairs that constitute the human DNA to understand the species' genetic makeup.

First draft was released in 2001 followed by the complete draft in 2003. Some of the main findings from the draft sequence are as follows:

- Total number of genes was estimated at 30, 000.
- The average gene was found to consist of 3000 bp but sizes vary greatly.
- Repeated sequences that do not code for proteins ("junk DNA") make up at least 50% of the human genome.
- About 1.4 million locations with SNPs were identified.

Findings from HGP are already having profound impact on diverse areas of research including molecular medicine (improved diagnosis of disease, earlier detection of genetic predispositions to disease, rational drug design etc.), bioarchaeology, anthropology, evolution and human migration, DNA forensics (identification), agriculture, livestock breeding etc.

***Human Genome Diversity Project*** (HGDP): HGDP was formally organised in 1993 under Stanford University's Morrison Institute, and was aimed at understanding the diversity patterns worldwide, the contributing factors and the implications of the observed diversity patterns. Findings from the project could also shed light on the origins and migration patterns of the entire human species. HGDP could also aid in understanding the role played by environmental factors in complex human diseases.

***HapMap Project***: The International HapMap Consortium is an international collaborative venture between Japan, the United Kingdom, Canada, China, Nigeria, and the United States aimed at developing haplotype map of the human genome in a bid to identify genetic determinants of complex diseases. The information made available through the HapMap project is helping researchers find genes that affect health, disease, and individual responses to medications and environmental factors.

***Indian Genome Variation*** (IGV): IGV was the first large scale effort to document and understand the genomic structure of enormously varied Indian populations. The study found high degree of genetic differentiation among the different ethnic groups.

### Genetic Testing and Counseling

Frequently the question may arise as to whether the patient has a certain disease for which there is a genetic basis. Often among the 10,000 conditions for which a genetic basis has been identified, the diagnosis can be made from evaluation of personal and family history, physical examination, and conventional laboratory tests. A useful database for identifying these conditions is available on Online Mendelian Inheritance in Man (OMIM) (www.ncbi.nlm.nih.gov/omim). This

catalog is updated regularly and can be searched using multiple terms. The entries provide information about the clinical signs as well as the genetic basis for the condition, if known, including mutations that have been found to cause the condition. To determine whether genetic testing is available for a given condition and to find a laboratory, a useful link is GeneTests, a free online service (www.genetests.org). The entries in this catalog indicate the test menus and contact information for the laboratories, as well as whether the testing is provided on a routine or research basis. A very useful adjunct in the GeneTests Website is GeneReviews, which provides succinct summaries about many genetic conditions and the ways the genetic testing can be used for diagnosing these conditions, including prediction of natural history.

The clinician is likely to encounter many situations in which a genetic test may be useful. Sometimes genetic testing is required from diagnosis when it cannot be made by clinical criteria alone. The fragile X syndrome is the most common genetic form of mental retardation. Although the diagnosis may be suggested by the presence of the characteristic signs—large ears, protruding chin, and large testes—the only way to diagnose fragile X is by genetic testing. For the various forms of spinocerebellar ataxia, there is considerable overlap. Yet, these can be readily distinguished by their specific mutations. Patients with atypical forms of certain diseases may have a negative gold standard test, but positive genetic test. For most patients with cystic fibrosis, the diagnosis can usually be made by a sweat chloride test. However, a number of individuals have been described with pulmonary disease suggestive of this condition for whom the sweat chloride test is normal. For these patients, the diagnosis has been based on observation of mutations in both copies of their CFTR genes.

For some conditions, the signs of disease may not yet have developed, yet on the basis of one's family history, one may want to know about the risk of developing disease. This is true for the person whose parent(s) may have died from Huntington's disease, a progressive neurodegenerative disease or for the person whose mother and sister may have died from breast or ovarian cancer, suggesting a heritable risk. For these individuals, a positive genetic test result will indicate an increased, although not necessarily absolute, risk for developing the disease.

Genetic testing is used for assessing reproductive risks—by testing the parents for carrier status and by testing the fetus. Individuals with a positive family history of genetic disease (usually autosomal recessive or X-linked) or who come from ethnic groups with an increased prevalence of autosomal recessive or X-linked diseases are candidates for carrier screening. Currently, carrier screening for cystic fibrosis, fragile X syndrome, and spinal muscular atrophy is recommended in the United States. For people of Mediterranean, African, or South Asian ancestry, hemoglobinopathy screening is recommended. For individuals of Ashkenazi Jewish ancestry, screening for Tay-Sachs disease, Canavan disease, cystic fibrosis, Gaucher disease, Bloom syndrome, Fanconi anemia, Niemann-Pick disease, familial dysau-tonomia, maple syrup urine disease, glycogen storage disease, and familial hyperinsulinism is available. An individual who is a carrier for a certain condition may choose not to marry another individual who is a carrier for the same condition. Alternatively, if a carrier couple is identified, they may choose to have prenatal diagnosis to determine whether their fetus is affected with this condition. This can be performed either at 10-11 weeks using the procedure of chorionic villus sampling where a bit of placenta is obtained under ultrasound

guidance. As another option, an amniocentesis can be performed at 15-18 weeks of pregnancy to obtain cells from the amniotic fluid. These couples might also choose to have pre- implantation genetic diagnosis with selection implantation of only those embryos that are deemed unaffected.

Not all genetic testing involves looking for heritable mutations. Sometimes it is used to look for genetic alterations that are confined to a specific population of cells. These alterations may cause certain cells to become cancerous, or if cancerous, to progress to a more aggressive stage. Genetic testing can be used to identify chromosomal translocations between two non-homologous chromosomal segments and in the process diagnose a specific form of leukemia. For example, the translocation between chromosomes 1 and 19 in leukemic cells is diagnostic of the acute promyelocytic form of this disease and the translocation between chromosomes 9 and 22 is diagnostic of the chronic myelogenous form. The expression patterns of RNA transcribed from many genes can be assessed to predict the natural history of the disease. This approach has been used to predict breast cancer outcome and whether more or less aggressive therapies should be used to treat patients.

Individuals might also have genetic tests of identity. These might be voluntary and selected to test specific questions, such as whether they are members of a known patrilineal lineage, such a people with a specific surname. These tests analyze a series of polymorphic genetic markers on the Y chromosome. On the basis of the general pattern of markers, or "haplogroup," they may be told of the geographic region where their Y chromosome originated. According to the number of markers that match with people who are suspected to be of the same lineage, individuals may be advised about the common ancestors or other people in that lineage. Such testing is also possible for matrilineal lineages by testing mitochondrial DNA markers.

## 3.9 GENETIC MAPPING OF DISEASE GENE ON HUMAN CHROMOSOME USING POLYMORPHIC MARKERS

In genetic mapping the diseased gene polymorphic markers play a very important role. These markers could be short tandem repeats, variable number of tandem repeats, blood groups, restriction fragment length polymorphism etc. Mapping can be done in a step wise manner.

Collect all the pedigrees where the disease is found. Analyse all the members against various polymorphisms and perform linkage analysis.

Linkage study entails collecting blood cells from members of several two – and three – generation families or from individuals of a large multiple generation family with a specific genetic disorder. The blood can be cultured and cell lines can be maintained large number of polymorphic markers (probes), representing sites from all parts of all autosomes, are used. A two –point (two – locus) LOD score is calculated for each polymorphic locus and the site of the genetic disease from all informative parent offspring combinations and finally the linkage is established. However, genotyping errors can give –ve or +ve LOD score. Hence perfect genotyping is must to get the correct results.

## 3.10 USE OF POLYMORPHIC MARKERS IN FORENSIC TESTING

Polymorphic markers have great utility in personal identification. As mentioned above no two individuals are alike. These differences are at both phenotypic and genotypic levels. The genetic differences can be identified by testing these markers. This testing is provided by commercial firms that market directly to consumers. Identity genetic analysis may also be involuntary and used for paternity testing of children or fetuses or for identification of forensic samples in murder, assault or rape cases, in which the perpetrator of the crime left a tissue sample of blood, semen, hair, or other tissue type from which DNA can be extracted and the test can be performed. However, it must be kept in mind that there are ethnic differences in the distribution of these markers. Hence every population should have its own genetic profile.

## 3.11 USE OF POLYMORPHIC MARKERS IN POPULATION STUDIES

**Population Diversity Studies**

Human genome varies from individual to individual and therefore no two individuals look alike. This was noted long back. Historically, individual variation was studied on the basis of conventional somatoscopic markers. However, with the advancement of technology various genetic markers were discovered and the gene frequency data for studying the evolution of human races was analyzed using these markers. Initially, the classical serological and biochemical markers have played important roles in various types of human population genetic studies. One of the problems that limited their practical utility results from the limited number of possible genotypes at each of such loci. The discovery of hyper variable DNA loci offers the opportunity to ameliorate this problem. It was later realized that comparison of gene frequencies for one or two loci are not reliable since each locus has a different geographical distribution, hence the differences observed may be because of chance factor. Only when a large number of loci are used, the genetic relationship among populations could be drawn successfully. Recent analysis based upon polymorphic markers reveal that inter and intragroup genetic variation may be of a lesser magnitude and may not be of significance if proper markers are not selected and more so if statistical tools used are not highly powerful. However, it is important to record population variation because it is helpful to know the various mechanisms involved in causing variation and it further enhances our knowledge about the molecular basis of disease susceptibility.

## 3.12 SUMMARY

It is difficult to attribute any functional significance to genetic polymorphisms. However, the non-coding sequences of the genes which are located far away from the functional region of the gene may affect the function of the gene. However, these sequences are otherwise useful in studying population diversity, disease gene mapping, forensic investigations etc. Recently after the advent of microarray genes for many complex disorders have been found by using genome wide association studies.

## Suggested Reading

Gardner, E.D, Simmons, M.J and Snustad, D.P. 2003. *Principles of Genetics,* 8th Edition, New York: John Wiley and Sons.

Simmons, S and Simmons, M.J. 2003. *Principles of Genetics,* 3rd Edition, New York: John Wiley and Sons.

## Sample Question

1) Define polymorphism with few examples.
2) What are the evolutionary forces that affect gene frequency of polymorphic markers?
3) Give some uses of polymorphic markers.
4) What is law of Hardy Weinberg?
5) What is genetic testing?
6) Describe the utility of studying molecular markers in anthropological genetics.

# MANE-001
# Human Genetics

Indira Gandhi
National Open University
School of Social Sciences

Block

# 2

## HUMAN POPULATION GENETICS

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## Print Production

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July, 2012



ISBN-978-81-266-6134-3



*Further information on Indira Gandhi National Open University courses may be obtained from the University's office at Maidan Garhi. New Delhi-110 068.*

Printed and published on behalf of the Indira Gandhi National Open University, New Delhi by the Director, School of Social Sciences.

Laser Typeset by : Tessa Media & Computers, C-206, A.F.E.-II, Okhla, New Delhi

Printed at :

# BLOCK 2 HUMAN POPULATION GENETICS

The science of population genetics is an integral component of biological anthropology in understanding human evolution and in pursuing the goal of human origins vis-à-vis the divergence of human groups. Population genetics provides underpinning for all evolutionary biology. The word evolution includes all changes, large or small, visible and invisible, adaptive and non-adaptive. In this context, the existence of genetic polymorphisms in human populations becomes immensely important in seeking answers to our understanding of the evolutionary significance of these variations. Stated simply, population genetics is the study of genetic variation through gene and genotype frequencies in a population, and predicting the way they would change or would be maintained over a period under the differential effects of various micro-evolutionary forces. There are four basic evolutionary forces: mutation, natural selection, genetic drift and gene flow. Mutations are copying errors during DNA replication and transcription, which introduce new alleles into the population. Natural selection is the differential transmission of alleles into the next generation due to the consequences of functional differences on an individual's survival and reproductive success. Genetic drift is the differential transmission of alleles into the next generation as a result of random sampling, and has the greatest potential impact in small populations. Gene flow spreads alleles from one population into another via migration, making them more genetically similar to each other, and countering genetic differentiation by drift. These four evolutionary forces are reflected in patterns of diversity, measured by the numbers of different alleles at a gene locus, the frequencies of each allele, and the interrelatedness of each allele to the others present at the same time. To understand why genetic diversity accumulates to a particular level, why it has its observed distribution, and how its turnover occurs, we investigate the interaction of these four evolutionary forces using population genetic models (Relethford and Harding 2001). Besides, population structure, inbreeding and the Mendel's principles of inheritance and associated factors that violate those principles, also produce differential genetic variation. It is also worth mentioning here that the concept of neutral or nearly neutral mutations along with genetic drift has been consistent with many observations of genetic variations. It is seen for example approximately 0.08% of the nucleotide base pairs (bp) in human DNA vary among individuals. Why these and not others? One explanation is that selection favors functionally different DNA alleles in different circumstances. Another is that DNA variation is tolerated when the alleles of a gene are functionally equivalent. The former explanation clearly applies to some variation, but the latter explanation, formalized as neutral theory, is invoked most often. Either way, the aim of population genetics is to model the dynamics of evolutionary change within and between populations. Moreover, anthropological genetics also deals with change in genetic diversity, population size in the context of evolutionary changes pertaining to *Homo sapiens*. Thus, assuming a simple population genetic model evolutionary population size of humans is estimated to be about 10 000. Population genetic models have also been used to estimate the time depth of typical diversity in the human nuclear genome, and it is suggested to be approximately 800 000 years. Studies on genetic diversity have revealed that population differences are mainly due to the presence of low-frequency alleles that have not diffused far from their geographic place of origin.

Further, by studying formal models of gene frequency change, population geneticists therefore hope to shed light on the evolutionary process, and to permit the consequences of different evolutionary hypotheses to be explored in a quantitatively precise way.

The field of Population Genetics was firmly established with the pioneering work of R.A. Fisher, J.B.S. Haldane and Sewall Wright in 1920's and 1930's. Their achievement was to integrate the principles of Mendelian genetics, which were rediscovered at the turn of century, with Darwinian natural selection. Though the compatibility of Darwinism with Mendelian genetics is today taken for granted, in the early years of the twentieth century it was not. Many of the early Mendelians did not accept Darwin's 'gradualist' account of evolution, believing instead that novel adaptations must arise in a single mutational step; conversely, many of the early Darwinians did not believe in Mendelian inheritance, often because of the erroneous belief that it was incompatible with the process of evolutionary modification as described by Darwin. By working out mathematically the consequences of selection acting on a population obeying the Mendelian rules of inheritance, Fisher, Haldane and Wright showed that Darwinism and Mendelism were highly compatible; which played a key part in the formation of the 'neo-Darwinian synthesis', and thus explains why population genetics came to occupy such a pivotal role in evolutionary theory.

With the advent of different laboratory techniques and various polymorphic serological and biochemical markers, the impetus in studies was gained to a large extent. Later, the power of molecular methods also gave a new dimension to the field of population genetics. Now, with the help of different techniques, and with completion of the various genome projects, the methods of "functional genomics" are scaling up to identify the roles of novel genes. Inevitably increasing attention is being paid to the significance of genetic variations in populations. Prompted by the high incidence of multifactorial diseases as a group, the medical community has become acutely aware of the need to understand the basic structure of genetic variations in populations in order to determine the aspects of the variation that can cause disease. Although, multifactorial diseases have received great attention in the recent past, but the scope of population genetics actually is much broader because the anthropological genetics not only explains the causes of human diversity and the evolutionary history but also focus on understanding adaptation to local environments.

Thus, Population genetics is the key to our understanding of human variation, and by linking medical and evolutionary themes; it enables us to understand the origins and impacts of our genomic differences. Despite current limitations in our knowledge of the locations, sizes and mutational origins of structural variants, the overall growth in this field has brought new insights into recent human adaptation, genome biology and disease association studies. Population genetics provides models for investigating the balance of evolutionary forces acting on genetic diversity. Studies that use these models have found that the evolution of contemporary human genetic diversity has occurred over the past several hundred thousand years or longer. Our species is geographically widespread, but shows low levels of differences among population groups suggesting persistent levels of gene flow as well as dispersal. It is difficult to classify humans into groups by their DNA profiles, and impossible to successfully apply a biological concept of race of diversity within living human populations.

The origin of modern human genetic diversity is still widely debated. Genetic data indicates the importance of Africa in modern human evolution, in line with the observations from the fossil record of the first appearance of modern anatomical form in Africa. Whether Africa is the only region that we can trace our ancestors to, or whether it is the primary region remains to be seen. Some genetic evidence does suggest ancient contributions in southern Asia, a region where the fossil evidence for replacement is equivocal. It may be the case that our origins are best described as 'mostly (but not exclusively) out of Africa' (Relethford and Harding 2001).

# UNIT 1 MEANING AND SCOPE

**Contents**



**Learning Objectives**

After reading this unit, you will be able to:

- define biological inheritance and describe its historical development;
- discuss breeding isolation and its implications in human population genetics;
- define and outline various mating patterns;
- explain inbreeding and types of consanguineous marriages; and
- measure inbreeding in families and in populations, its consequences of inbreeding/consanguineous marriage.

## 1.1 INTRODUCTION

Population Genetics can be defined as the study of genetic transmission in populations of interbreeding organisms. It is the study of the nature and source of the inherited differences and involves predicting the changes that may take place in relative frequencies of different genes that may be found in a population, and determining a condition under which equilibrium between forces affecting their frequencies may be obtained.

The science of population genetics deals with Mendel's laws and other genetic principles as they affect entire populations of organisms. The organisms may be

human beings, animals, plants or microbes. The populations may be natural, agricultural or experimental. The environment may be city, farm, field or forest. The habitat may be soil, water or air. Because of its wide ranging purview, population genetics cuts across many fields of modern biology. A working knowledge has become essential in genetics, evolutionary biology, systematic, plant breeding, animal breeding, ecology, natural history, forestry, horticulture, conservation and wildlife management. A basic understanding of population genetics is also useful in medicine, law, biotechnology, molecular biology, cell biology, sociology and anthropology.

Population genetics also includes the study of the various forces that result in evolutionary changes in species through time. By defining the framework within which evolution takes place, the principles of population genetics are basic to broad evolutionary perspectives on biology. Many oddities in biology become comprehensible in the light of evolution: they result from shared ancestry among organisms, and they attest to the unity of life on earth. One of the purposes of population genetics is to study the mechanism of origin and maintenance of genetic variability. The genetic variability is studied in terms of polymorphism of various genetic markers, as polymorphism usually refers to alternative hereditary forms that can easily be distinguished from each other and whose inheritance is clearly understood. Human populations are polymorphic for a large number of genetic loci. These polymorphic loci are useful for constructing linkage maps, genetic counselling, paternity testing and studying the evolutionary relationships of human populations. The association between gene frequencies and environmental factors has been made for number of polymorphic loci. It seems that this type of association study is an effective way to make an inference about selective mechanisms, which could be done by having the data on the distribution of gene frequencies among various populations, provided that the population is endogamous. If there are gene frequencies for a number of loci in a population, the heterozygosity for individual locus can be calculated and average heterozygosity per locus of a population is obtained. The average heterozygosity indicates the magnitude of genetic variation that exists within a population.

### 1.1.1 Inheritance – Historical Development

Probably no area of biology arouses more interest or creates more controversy than evolution, which deals with the origin of living organisms, the genetic diversity of populations, the mechanisms of speciation, and the biological history of our planet. Evolution is the branch of biology that attempts to explain how to tens or hundreds of millions of different species of plants, animals and micro organisms arouse in the course of several billion years of earth's history. Evolutionary science analyzes the various forces that cause species to adapt, to change and to eventually become extinct.

Evolution and genetics are inextricably connected because the biological changes that occur in organisms through time are due to changes in their hereditary information changes in their genes. To a geneticist, evolution refers to changes in gene and genotype frequencies that arise and accumulate through time in populations and organisms (it is populations, not individual organisms, which evolve). These cumulative changes in gene and genotype frequencies are subject to natural selection, a process originally proposed by Charles Darwin. Charles Darwin, in his landmark work 'On the Origin of Species by Means of Natural

Selection' altered the course of intellectual history in the Western World. Like Newton, Copernicus, Galileo and others, Darwin proposed a mechanistic explanation for natural phenomena. Darwin argued that the diversity of life on earth could be accounted for by the operation of simple and observable processes that are part of our everyday experience. Darwin made the assumptions that phenotypic variation is partly determined by hereditary differences between individuals. It is remarkable that the mechanisms of heredity transmission were not then known because as Darwin himself realised, the theory of evolution by natural selection depended critically on the way in which hereditary information is passed from generation to generation in interbreeding populations, which he himself was unable to account for. Darwin realised that for his theory of natural selection to be plausible and for the adaptive modifications, observed as phenotypes, to be passed on from one generation to the next, a mechanism of hereditary transmission was required otherwise the offspring will not resemble their parents and fitness-enhancing traits will not spread through the population but he was not able to shed light on this. The fact that he could not explain the mechanism of inheritance was the main objection to his theory of evolution. He accepted a weak form of Lamarck's use and disuse theory to explain origin of variation and attempted to work out a way for explaining mechanism of inheritance by assuming that in response to environmental stimuli, somatic cells of body would release entities called 'gemmules'. According to him, these 'gemmules' carried information on the traits of the organism and would accumulate in germ cells and get passed on to the next generation. But he could not prove this. He knew that for selection to operate in a population and gradually alter it, continuous supply of variation was required. There were scholars who believed in importance of selection for creating new adaptive changes. Others doubted that selection acting on continuous variations was sufficient to transform one species into another. Still others advocated Lamarckism. It took the discovery of genes and mutations in the 20$^{th}$ century to render natural selection feasible and unavoidable as an explanation for evolution.

Historically the first and still the most conclusive evidence for the existence of genes come from the phenomenon of segregation of traits observed in the offspring of hybrids between individuals or strains that differ in some recognizable aspect. The principle behind the transmission was for the first time demonstrated by Austrian monk Gregor J. Mendel (July 20, 1822 – January 6, 1884). His publications on the results obtained from the *very carefully selected* breeding experiments on '*hybridisation*' conducted on *Pisum sativum* in monastery gardens of Austria for eight years (1856-1863) indicated that the transmission of these (seven) characters (height, size and shape of seed, colour of flowers etc. among pea plants) are particulate in nature. The hereditary transmission of the above traits follows specific pattern what has termed as law of independent assortment and law of segregation. According to Mendel's laws, given the parental phenotype/ genotype combination, it is possible to predict the likelihood genotype/phenotypes of the offspring. Another important aspect of the discovery of Mendel's laws is that the transmission of hereditary traits from parents to offspring follows simple binomial expectation with stochastic perturbation, which is more likely to be true in a large sample size.

The Mendelian's concept of hereditary unit is contradictory to the erstwhile popular Greek school of thought '*blending theory of inheritance'* that involves mixing nature of inheritance of characters from parents to offspring. In this regard,

it is important to mention that Darwin has proposed '*pangenesis'* to explain the mechanism of inheritance and in doing so; he postulated '*gemmules*' particle nature of inheritance. According to Darwin, The '*gemmules'* is produced by the cells and could diffuse to other parts of the organism and produce new cells etc. However, this did not stand the scrutiny of the developments of empirical experiments of hereditary by others like Francis Galton etc. As against these backdrops, independently and at the same time (1860s), the Mendel's hereditary experiments have demonstrated the existence of particular nature of hereditary units, and rightly, he located its existence in germ cells.

The principle of segregation and dominance and principle of independent assortment was formulated by Gregor Mendel in 1866 under such peculiar circumstances that the scientific world failed to recognize or appreciate it until after a lapse of 34 years. Mendel was not primarily a biologist but a monk in the Augustinian Monastery at Brunn, Austria (now Brno, Czechoslovakia). After seven years of experimental work in the monastery gardens, he presented the results of his experiments, together with the generalizations we now know as 'Mendel's Law', at two meetings of the Natural History Society of Brunn in 1865. The results and the theory were printed in the annual proceedings of the society, which appeared and distributed to libraries in Europe and America in 1866.

Possibly, people expected that the results obtained by Mendel is more related to hybridisation and are of importance to breeding animals and the implications that Mendel's discovery of hereditary units, the secret behind the heredity and its implications to overall biology, was possibly realised by a few, until after three decades later by the rediscovery of his laws in 1900 simultaneously by Hugo De Vries in Holland, Carl Correns in Germany and Erich Von Tschermak in Austria, who found Mendel's forgotten paper and proclaimed its importance. Immediately his conclusions began to be confirmed and extended by experiments carried on in various parts of the world on many kinds of plants and animals.

It was from the Mendel's rediscovery of laws of hereditary that describes how the characters that he selected – e.g., height, seed colour – among plants follows simple principles of transmission, it became clear that the *cause for the hereditary transmission of a particular (Mendelian) character* is governed by what Mendel hypothesised as '*hereditary unit*'. This hereditary unit was later described as 'gene' by William Bateson and the subject that deals with the heredity and its transmission rules and regulations is the discipline of 'Genetics'. The hereditary transmission of traits, its variation or extent of diversity among regional populations, how its changes over time, what are the factors and causes that influence these changes all are important for us and the study that deals with it in brief is the 'human population genetics'. It also deals with theoretical and empirical studies to understand the how different traits change over time and factors governed its mechanism at the population level, at amino acid, enzyme, molecular level etc.

## 1.2 UNIT OF STUDY

One of the major intellectual challenges of early twentieth century genetics was the integration of Mendelian genetics and the theory of evolution by natural selection. The resulting synthesis combined the postulates of evolution by natural

selection presented. Darwin with the statistical rules of genetic transmission in the population to construct mathematical theory of evolution. This synthetic theory of evolution, based on population genetics, is sufficient to account for the evolution of the great variety of life forms on earth. Thus, Population Genetics, as we know it today, arose from the need to reconcile Mendel with Darwin.

It may not be less worth mentioning here that genotype frequencies are determined in part by pattern of mating. To deduce the genotype frequencies from the estimated allele frequencies, British mathematician, G.H.Hardy and German Physician, W.Weinberg independently formulated a model in 1908 describing the relationship between the gene and genotype frequencies, and this relationship is now popularly known as Hardy-Weinberg principle or Hardy-Weinberg equilibrium (H.W.E), which eventually laid down the foundation of genetic analysis of population.

Population genetics deals with the changes in gene frequency over generations in a population. In the context of evolution, according to Darwin, it is the species living in a given eco-niche evolves over time and this is brought by what he called as 'natural selection', the driving force for the evolutionary change among species. The unit of study in the biological realm of Darwinian evolution is the species. Apart from the phenotypic characters that is/are characteristic (identity) of given species — what we can understand from the common English usage that is quite opt to the topic, for example, "birds of the same feather flock together", — interbreeding within a species is prime distinguishing character that defines a species, the study unit. This is in a way identified by characteristic species specific phenotypic traits. In general, in nature, mating is restricted to within the species; as such the phenotypic and genetic characters are transmitted through generations and become restricted to a given species. Therefore, mating within a species is compatible and matings between species are unlikely and are supposed to be incompatible. However, Man was successful in bringing out interspecies matings between related species e.g. Lion and Tiger, Horse and Donkey, etc. in artificial circumstances (cross-breeding) creating some unusual types such as 'tigon' and 'mule' (was supposed to be infertile). The mating within a species or a group restricts the transmission of characters through generations within the group or a species. This is similar to water pond or pool/lake where the water is contained and restricted to its geographical space.

In general, in Man, human populations live in groups over a neighbourhood area in a wide geographical or eco-regions. In general, the marriages are expected to be within a particular community, group or clan or caste etc. Marriages within a community or group or clan, is said to be endogamous. Similar to water tight compartments, the endogamy within a community restricts transmission of genetic characters within its community, over generations. In population genetics, the endogamous populations, where marriages are restricted within a community are construed as 'gene pools' where genes/alleles or consisting of a variety of traits with different frequencies confined to the population or the gene pool.

### 1.2.1 Gene Pool

Gene pool refers to the endogamous population where the marriages are restricted within the group, restricting the genes within its group or community. This is also called as Mendelian population (A reproductive community of individuals

which share in a common gene pool). How do we investigate whether a population is endogamous? or how do we investigate the extent of a Mendelian population or a gene pool among human populations?

The boundaries of a gene pool in human populations can be investigated by the extent of endogamy that is practiced in a local/regional population and by studying the marital alliances or the pattern of mating. It is not always easy to estimate the extent of endogamy, except in small isolated populations with known or defined barriers that restricts marriages within the population. These barriers could be geographical and/or cultural. Examples where geographical factors play major barriers to marriages within in the community include island populations or a few tribal populations who live in hunting and gathering sustainable livelihood in interior jungles or high mountain ranges of difficult terrain or inhospitable environmental conditions. Even when the population is large, they might subdivide to form several endogamous populations. In this case, the cultural factors that regulate marriages lead to endogamy within in the community. Identification of these cultural isolates helps us to investigate or define the 'gene pool. At empirical level one gets information about marriages within the community and estimates "endogamy index". It is defined as per cent occurrence of number of marriages within the community to the total number of marriages. The index ranges from 0 to 100 and a value of above 95% can serve as indication to suggest the population is an endogamous and can be considered as a gene pool. The index can be investigated for each generation to get an idea to track changes in endogamy levels in the recent past. In case one would like to investigate the status of endogamy beyond 5 or 6 generations, one can choose other informative data. One such data is marriages among surnames or clans. This can help us to get to know the relative levels of endogamy in the recent past beyond several generations.

### 1.2.2 Breeding Isolation

In human populations, breeding isolation can give an estimate the spatial distribution of gene pool. The breeding isolation can be investigated by marital distance between the birth places of the spouses in a population. In general there is a tendency to marry within the communities over a neighbourhood region. The distance between the birth places of the spouses gives an extent of spatial distribution of gene pool. In general, in traditional rural, tribal societies and in among some urban societies a positively skewed marital distribution is expected. A low mean marital distance (MMD) is supposed to be characteristic of traditional societies. In urban and metropolitan societies is expected to show higher mean and higher variance of MMD. In a study conducted among the regional populations of *Yanadi* (a tribal population from Andhra Pradesh) who differ in their subsistence pattern in islands, coastal and plateau and hill forest areas shows the typical positively skewed MMD. The mean also varies from hunting gathering to agricultural societies, lower among hunting gathering and higher the mean among agricultural societies of the same tribe. It also shows the spatial distribution of extent of gene pool among the Yanadi regional populations (Figure. 1.1).

The urban population tend to have large and higher values of MMD and their gene pool extend over a large spatial distance. Among these populations they are characteristic of lower endogamy rates, which become difficult to define them as breeding isolates or gene pools. Another difficulty is in urban communities

are tend to show higher interbreeding with other communities, which poses problems for considering them as breeding isolates or gene pools for population genetic studies. Though there are very few studies about the interbreeding or admixture in various populations, but in general such rates are about 1-2 per cent among the rural endogamous groups and among urban communities it can vary as high as 10%. Due to socio-economic reasons such admixture or interbreeding is increasing among urban societies.

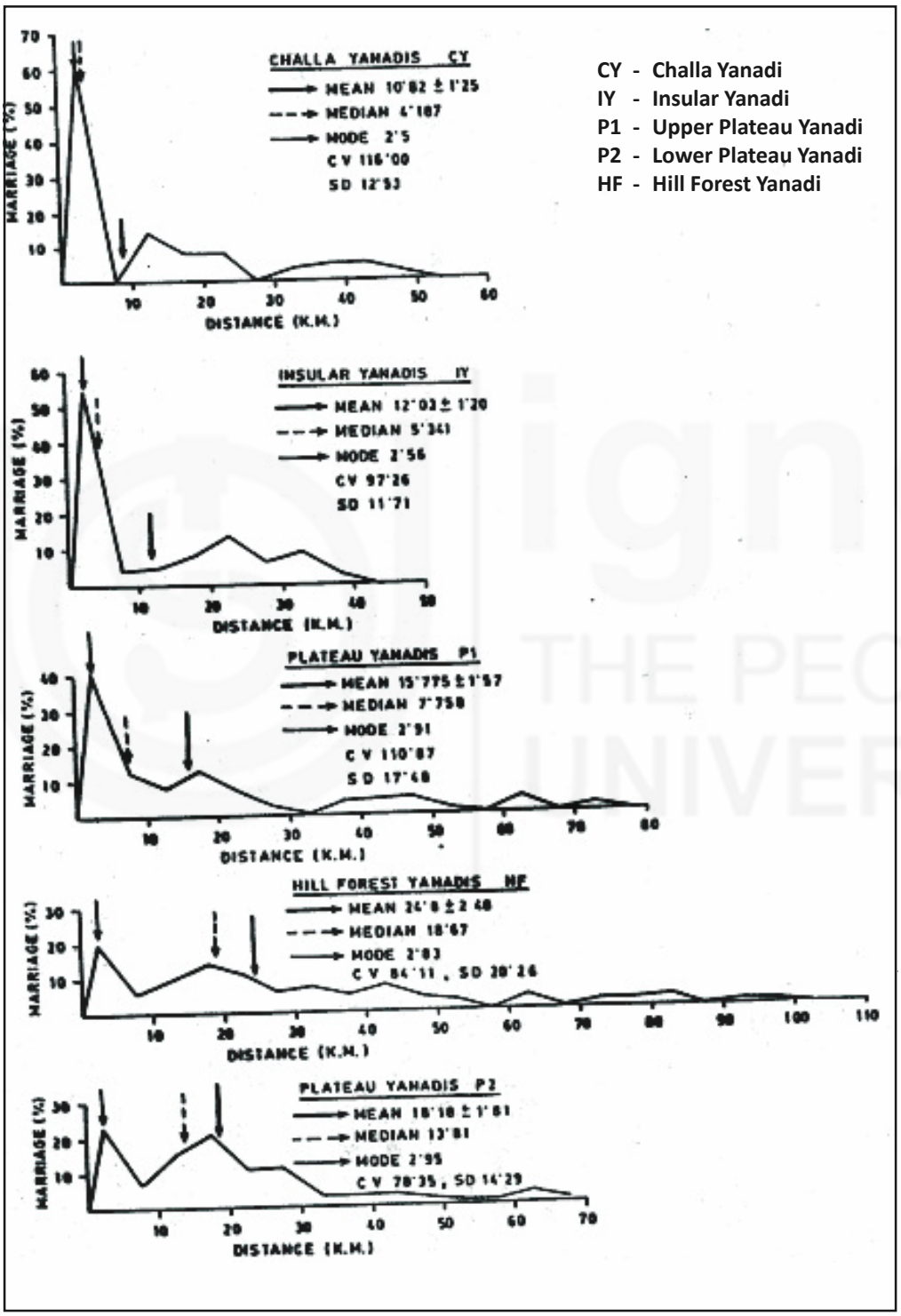



**Fig.1.1: Distribution of matrimonial distance in regional breeding populations of the Yanadi tribe**

## 1.3 MATING PATTERNS

Hereditary transmission of characters from generation to generation depends on type and selection of males and females that they participate in sexual selection leading to reproduction. Both in animals and human populations mating behaviour is very complex and it is non-random. The selection of male and female is characterised by a variety of patterns and certain pattern is characteristic of some groups. Charles Darwin while elucidating his hypothesis of origin of species by natural selection, considered sexual selection is an important criterion that plays a role in the successive reproduction that might lead to proliferation of desired qualities in the offspring. Those who are not so successful in selecting a mate will have least possibility of successful reproduction of their abilities being transmitted in the next generation. These abilities or characters stands eliminated in successive generations. In human populations, there is a wide variety of mating patterns and marriage norms, rules and regulations. These vary in different societies and communities.

Human populations are organized conglomerate entities. It generally consists of demographic, cultural, linguistic, political sub structuring; each with its own defined functional domain. Of these several entities, culture is one important component of human populations, which can have a profound biological significance. For example, populations have varying rules and regulations in choosing a mate or marriage partner. Charles Darwin in 1871 in his book 'Descent of Man and selection in relation to sex', recognized sexual or mate selection as important causative factor as that of natural selection in Evolution.

The non-random nature of choice of mate or mate selection can lead to significant genetic changes. There are different types of mating. The genetic significance of each type of mating can be investigated theoretical and its theoretical expectation of the possible genetic changes over generations can be worked out or marriages in diverse societies. These are described below.

### 1.3.1 Random Mating

Random mating is also referred as 'panmixia'. Panmixia or Random mating refers to the situation in a population where there is equal opportunity for both the sexes to mate and reproduce irrespective of any choice or selection. Random mating refers to a situation where absence of cultural rules and regulations that involve preferences or avoidances of choice or selection of marriage partners. If an individual mate or marry without any criteria of selection or choice what so ever, it is random mating. Random mating can also refer to those mating with respect to individual genotype or phenotype, though they may be marrying individuals within the community. Therefore, the random mating also refers to the characters which are not involved, either directly or indirectly, in the choice or selection by the respective mates or marriage partners.

Random mating is one important criterion in Hardy-Weinberg equilibrium which ensues, in a large population, stability of gene frequency over generations. Otherwise deviations from random mating, a common phenomenon among human populations, is an important factor which changes the gene frequency.

### 1.3.2 Assortative Mating

In all human populations, from simple societies of tribes and to complex societies of urban in metropolis, people usually like to select their marriage partners, mates or spouse, i.e., non-randomly, with desirable characters of their liking. This is referred as 'assortative mating', an important factor of non-random selection of mates that exists in all human societies. Assortative mating is one of the deviations from random mating assumption of Hardy-Weinberg equilibrium. According to H W Law the gene frequency in a population is expected change as a result of non-random mating due to assortative mating**.**

Choice of mate selection is more concerned directly with respect to some specific phenotypic characters (rarely genotypes are involved) and it might involve, indirectly, other characters which are associated or related to selected traits or characters among spouses or mates. For example, choice of marriage partner could be based on such desired or preferred traits e.g., skin colour, age preferences, intelligence, social status etc. In case one selects intelligence as the criterion it might be associated with high social status, which may not be desired trait.

In general, the institution of marriage, in human populations, prescribes rules and regulations that allow members of the community choice to select his/her spouse or marriage partner. Assortative mating refers to the preferential marriages (or mating) between individuals that involve either similar traits such as intelligence or phenotypic characters such as height, skin colour etc. or it might involve different traits of phenotypic characters. Assortative mating is one type of non-random mating that commonly occurs among human populations.

Assortative mating could be of two types: positive and negative mating. In positive assortative mating (homogamy) type both the spouses choose or select their mates on the basis of common phenotype or of similar characteristics. Conversely, the negative assortative (heterogamy) mating types are those where the partners select their mates based on different or dissimilar phenotypic traits.

For example, if tall and fair people choose to marry those with similar traits of equally tall and fare peoples, then it is assortative mating involving phenotypic characters of tall and fair skin colour. Similarly the opposite can also happen where people choose their mates unlike themselves or with dissimilar or opposite traits. In general, in some societies, in mate selection, there is preference (or selective advantage value) for fair skin colour people to get married. Conversely, people with dark skin colour are avoided or least preferred as ones' spouse. In a patrilineal society, it is observed that males with dark skin colour prefer to marry fair skin brides. In these communities, there is negative assortative mating involving selection of dissimilar characters (dark and fair skin colour) between the males and females. In simple positive assortative mating people marry their spouse who are similar to them and in negative assortative mating spouse avoid similar characters or marry their spouse with dissimilar or diverse phenotypic traits.

## 1.4 INBREEDING

In many societies marriages takes place between relatives. In general, breeding among close individuals within a group is referred as 'inbreeding', and it is

applicable in case of plants and animals. The extent of inbreeding depends on the amount of common ancestry shared by the parents of the 'inbred' child (the progeny of a consanguineous marriage or inbreeding) or in genetic terms it is the proportion of genes that the parents have in common. It is opposite to outbreeding referring to breeding with the individuals from outside the group or unrelated individuals.

In human societies the marriages contracted among relatives and are referred as 'consanguineous marriages' or 'inbreeding'. Consanguineous is more with reference to cultural context and inbreeding is more with reference to general breeding that include Man and other organisms.

### 1.4.1 Consanguineous Marriages

In general relatives are referred as blood relatives (see introduction above for details) as per the belief that blood is responsible for hereditary transmission. In Greek the word 'Consanguin' refers to blood and relatives are referred as 'consanguines'. The other word for relatives is kin. Hence, marriages contracted between relatives (consanguines) or among kin are referred as consanguineous marriages.

In several human societies there is a preference and practice of marriages among close relatives. Possibly owing reasons due to social, cultural and economic reasons, several societies practice consanguineous marriages.

### 1.4.2 Types of Inbreeding/Consanguineous Marriages

There are different types of inbreeding and/or consanguineous marriages that are practiced that differ among communities or societies. Self fertilization is one of the closest form of inbreeding, which is observed among some flowering-plants, some hermaphroditic animals. The types of close inbreeding that are disapproved in most of the human societies but rarely observed in Man include sib-mating, parent-offspring. In early history of mankind, e.g., in ancient Egypt, the royal families had followed brother-sister marriages for some generations. This was based on the belief that royal blood is pure and to maintain the purity of blood they followed sib-sib mating. The Egyptian queen Cleopatra is one such example of generations of sib-sib mating in the royal family.

The cousin mating or marriages among near-relatives are practiced in several human populations. These different types of close marriages can be investigated by drawing a pedigree with all the information of relationship between husband and wife in a family that can extend to several generations. Aunt-nephew and Uncle-niece marriages are the marriages that occur across two different generations. In Indian populations Uncle-niece marriages are more common to Aunt-nephew marriages. Depending on the type of consanguineous relationship between husband and wife, they are categorized as first degree, second degree (and etc.) consanguineous marriages. The first cousin marriages are categorized as first degree consanguinity. This involves individuals who belong to same generation but come from different families whose parents share a common ancestor (grand parental level). These are referred as first cousin marriages, marriage among cousins who share a common grand parent. An individual if he

or she marries his/her paternal or maternal daughter or son (uncle's daughter or son respectively) the cousin marriages is the first cousin marriage. Similarly in case of second cousin marriages, the second cousins share a common ancestor at great grand parent level. In between there could be marriages between individuals that belong to two generations e.g., one-and-half cousin marriages etc.

The cousin marriages could be of different types: for example, parallel, cross and double cousin marriages etc. The marriage between the cousins who were descendents of two brothers or sisters (or whose (either) parents are either brothers or sisters) is referred as parallel cousin marriages. An individual marries his or her paternal uncle's daughter or son is referred as parallel cousin marriage. The word 'parallel' refers to same sex (either brothers or sisters) at the parental level. In case of cross cousin marriages, it is the marriage between the offspring (cousins) who's (either) parents are brother and sisters. In cross cousin marriage an individual marries his or her maternal uncle's daughter or son is the cross cousin marriage type. Apart from the above two there could be one more type of cousin marriages, it is called as double cross cousin marriages. In double cross cousin marriage, the marriages between a brother and a sister of a family marry their cousins a sister and a brother of their uncle or aunts respectively. In cross cousin marriage either a boy or a girl marries his or her uncle's daughter or son (cousin) respectively. In double cross cousin marriage type both a boy and a girl marry their uncle's or aunts' girl or son (cousins) respectively.

The prevalence of consanguineous marriages varies across different populations across regions and through time (generations). At least in the early history of Man such close inbreeding should have been very common as a result of population structure that is conducive of small population size, isolation and subsistence pattern of hunting and gathering and early agricultural life. It should have been a common practice in the history of mankind till 19-20$^{th}$ century or two centuries ago before the era of breeding and principles of inheritance and its consequences were investigated. Charles Darwin, who married her cousin Emma Wedgewood, had of the opinion: it is likely that we are all descendents of cousin marriages.

### 1.4.3 Consequences of Inbreeding

One of the important aspects of inbreeding is that it increases the chance of some of the recessive alleles with deleterious effects to get expressed in the inbred (the offspring of consanguineous marriages) which get transmitted from one or more common ancestors carrying (harbour) the rare recessive alleles.

Theoretically, inbreeding in a population, in general, increases the number of homozygotes at any autosomal locus. In case of recessive Mendelian diseases this can lead to increased risk of genetic diseases among the inbred children. In the same logic, in case the alleles are not deleterious in nature, but are advantageous, increase of such genes through inbreeding in a population is expected to be beneficial. The consequence of inbreeding whether it is deleterious or advantageous depends on the status of the allele that is involved in the expression among the inbred and transmission from the common ancestors.

Such cases pose health and survival problems of deleterious or debilitating diseases. This is health and socio-economic burden to the society and to the country. Increase of deleterious characters as a result of inbreeding is referred as 'inbreeding load'.

Inbreeding in a small population lead to decrease in the genetic diversity, and it may lead to decreased Darwinian fitness of an organism. In case if these results to less capability to survive in changing environment, or eco-niche, with inbreeding such characters will eventually allow the species to extinction. Such characters are supposed to have less Darwanian fitness. Such decline in reproductive performance and fitness with inbreeding in a population is referred as 'inbreeding depression'.

In case the population is large enough, some of the undesirable characters which are deleterious, over long inbreeding in a population, suppose to get eliminated by selection and thus attributes to better fitness and increased survival advantage to the population.

The above theoretical expectations differ from the empirical studies conducted on the consequences of inbreeding and its effects in human populations. These results are not uniform across populations and there has been a debate on the deleterious or harmful effects of inbreeding in human populations. This could be partly due to problems of study design, the methodology used and the history of inbreeding in the population. Some studies show significant differences with respect to some characters considered among the inbreeding and non-inbred population, while others have not found such differences in other populations studied. These studies on inbreeding effects are concerned with characters such as fertility, offspring mortality, morbidity, diseases, and rare genetic disorders, etc.

### 1.4.4 Measures of Inbreeding

The consanguineous marriages or mating in a population increases the chance of inheriting two identical copies of a homologous gene in the offspring from one or more common ancestors. The possibility that an inbred-individual gets two identical copies (homozygote) of such homologous genes due to inbreeding is described as 'identical by descent' (or 'ibd'). And the two alleles are called 'autozygous'. Apart from the case of close kin marriages that can lead to increased likelihood of both the identical copies to get expressed in the offspring by descent, it can also happen by chance alone. In cases of chance occurrence of homozygosity of two identical copies being expressed in an individual independent of inheritance from a common ancestry or absence of inbreeding, then such homozygous alleles are said to be described as 'identical by state' (or 'ibs') and the two alleles are called as 'allozygous'. The extent of autozygosity depends on the type of consanguinity followed in a family or in a population. The degree or extent of consanguinity or inbreeding in a family or in a population can be quantified by suitable measures.

There are at least two types of inbreeding measures. These measures can be calculated or estimated at different levels or type of data: pedigree data, population data and based on variety of traits: phenotypic, genetic and genomic information. The two measures are:

1) A genetic similarity measure to find out how or to what extent two related individuals are genetically similar; and

2) The effect of inbreeding on the fitness of inbred offspring, described as 'inbreeding depression'

a) **Inbreeding depression:** Inbreeding is expected to reduce the fitness of an individual when compared to non-inbred population. With inbreeding within variation will reduce and between variations is expected to increase. The extent of Inbreeding depression can be estimated in a pedigree among the inbred and outbred individuals and in a population. This can be investigated in case of single trait or multiple loci and phenotypic characters. The measures to estimate the extent of inbreeding depression include deviation from the mean of phenotypic characters, or deviation or differences between frequencies of a trait between the outbred and inbred individuals in a population and taking care of the variance of the trait concerned.

## 1.4.5 Inbreeding Coefficient 'F'

It is the measure of extent of genetic similarity among inbred individuals as a result of inbreeding. Sewall Wright (1922,1923) defined inbreeding coefficient as the 'correlation between uniting gametes' however such correlation can also occur because of other types of mating e.g., assortative mating.

Malecot (1948) defined inbreeding coefficient 'F' based on the probability that the homologous genes of uniting gametes are 'identical by descent (ibd)'.

Therefore, F is the probability of autozygosity

Inbreeding coefficient == coefficient of consanguinity

== coefficient of ancestry

An inbred individual has genes that are likely to be same with the ancestor.

The inbreeding coefficient can be calculated (estimated) by different methods for pedigrees and populations and for a variety of data as well.

### 1.4.5.1 Inbreeding in Families (Pedigree)

In case of individual inbreeding coefficient ($F_x$) from a detailed pedigree where individual x is an inbred offspring as a result of consanguineous marriage of the parents and/or grand parents etc. The inbreeding coefficient of the individuals can be calculated as follows:

The inbreeding coefficient of an individual, according to Sewall Wright (1922) is

$$F_X = \Sigma [(1/2)^{n_1 + n_2 + 1} (1 + F_A)]$$

Where x is the inbred individual

$n_1$ is the number of generations from the inbred (x) to the male common ancestor

$n_2$ are the number of generations from the inbred (x) to the females common ancestor

$F_A$ is the inbreeding coefficient of the ancestor

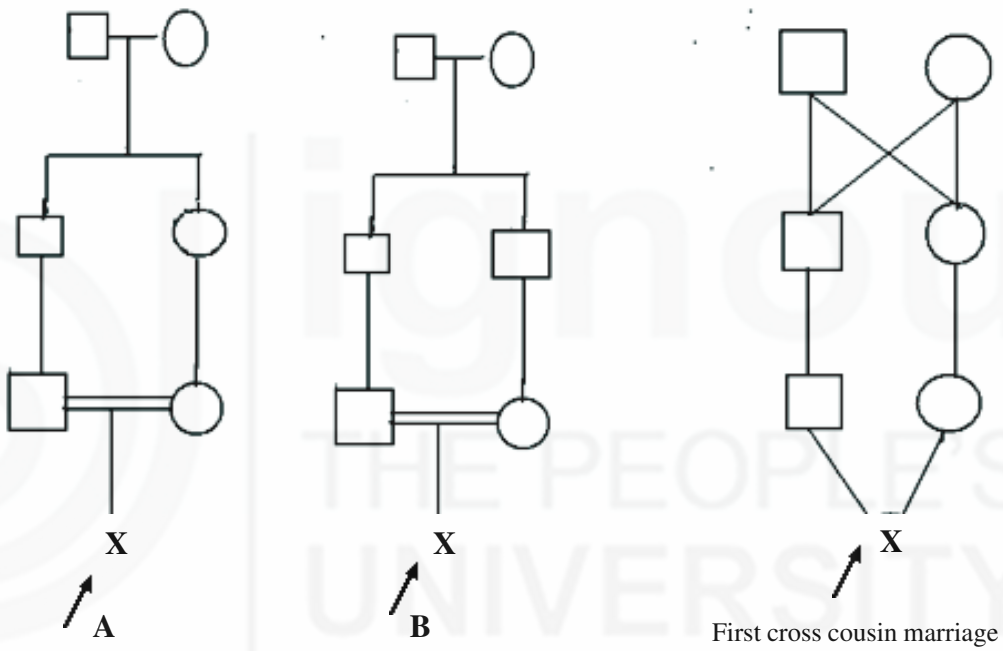
The inbreeding coefficient (F) can also be calculated from path analysis:

$$F_X = \Sigma [(1/2)^{n_p - 1} (1 + F_A)] \text{ and}$$

$$F_X = \Sigma [(1/2)^{n_a} (1 + F_A)]$$

Where, $n_p$ is the number of paths that connects x to the common ancestor, and $n_a$ is the number of ancestors from the inbred to the ancestor in the earliest generation. $F_A$ is the inbreeding coefficient of the common ancestor. If the common ancestor is non-inbred, then $F_A$ is zero (in both the methods).

The value of F ranges from 0 to 1. If F is zero then there is no inbreeding in the population and higher the value higher the inbreeding coefficient. This states proportion of common genome shared by the inbred as a result of inbreeding. The offspring of first cousin marriage is 0.0625 or 6.25% of the genome is supposed to be same with that of the common ancestor.




First cousin marriage
**A:** cross cousin
**B:** parallel cousin

In the above diagram first cross cousin and parallel cousin marriages are shown. X is the inbred (↗), his or her parents are cousins and an example of consanguineous marriage ( = ).

The right hand side of the diagram is short form of representing the inbreeding in the pedigrees. Here the ancestors are not themselves are inbred, so that the term $(1 + F_A)$ is zero for the above pedigree.

The inbreeding coefficient of the x in the above can be calculated as per the above formulae: The pedigree shows two loops. Here $F_A$ is zero as the ancestors are unrelated and not inbred.

There are 5 ancestors (a) in the first loop starting from x.

Another 5 ancestor (a) in the other loop starting from x.

If one wants to count number of paths, then there are six paths (lines) (p) that connect x through one common ancestor and another six paths (lines) (p) through the other common ancestor.

By using any one of the formulae it is easy to calculate $F_X$ for example:

$$F_X = \Sigma [(1/2)^{n_a}(1 + F_A)] = (1/2)^5 + (1/2)^5 (1) = (1/16)$$

$$F_X = \Sigma [(1/2)^{n_p - 1}(1 + F_A)] = (1/2)^{6-1} + (1/2)^{6-1}(1) = (1/16)$$

The inbreeding coefficient of a first cross/parallel cousin marriage offspring is 0.0625. Similarly it is possible to calculate the inbreeding coefficient of different type of related marriages practiced in the population through pedigree method. The same can be extended to complex pedigree structures with multiple loops extending to several generations as well.

The above inbreeding coefficient refers to autosomal loci. But the same can be extended to estimate the inbreeding coefficient for sex-linked chromosomes (Fs) as well. In that case there is no inbreeding coefficient for a male offspring and the path if there are two consecutive males Fs will be zero.

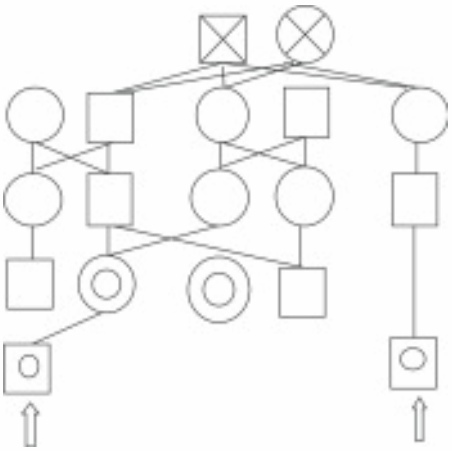
Inbreeding coefficient for different type of consanguineous marriages in human populations is shown below. More close the relationship the higher the inbreeding coefficient. In general the above is very simple example of individual cases of consanguineous or related marriages to illustrate the inbreeding by pedigree method. The different inbreeding coefficients of known related marriages are shown in Table 1.1. However, in empirical study, the pedigree information is a tedious exercise to draw large family data and from them to identify the complex inbreeding that might run several generations.

**Table 1.1: Type of consanguineous marriage and inbreeding coefficient**

| Type of consanguineous or related marriage | Inbreeding coefficient | |
|---|---|---|
| Uncle – Niece or Aunt – Nephew (UN/AN) | 1/8 | 0.025 |
| First cousin marriage (cross, parallel) IC | 1/16 | 0.0625 |
| First cousin once removed IC1 | 1/32 | 0.03125 |
| Second cousin 2C | 1/64 | 0.015625 |
| Double cross first cousin marriage D1C | 1/16 | 0.0625 |
| Double cross second cousin marriage D2C | 1/32 | 0.03125 |

Here for example, we have a one such complex inbreeding drawn from field work studies among tribal villages which shows one such large complex inbreeding network involving several families (see box 1). Indeed to calculate the inbreeding coefficient for such complex pedigree will be difficult. But now a days there are several methods of drawing the related marriages and luckily there are softwares on the internet services they can provide quick way of drawing the pedigree of complex nature and to calculate the inbreeding coefficient as well.

**Box 1**



In some populations the inbreeding marriages may run in several generations. Here is one such short pedigree of 5 generations showing only those individuals leading to inbreeding. This is drawn from large pedigree involving 20 families from a tribal village. The pedigree shows four inbred individuals with varied inbreeding coefficient. I expect you will be interesting to calculate the inbreeding coefficient of the four individuals

### 1.4.5.2 Inbreeding in Populations ($F_p$)

By knowing different types of inbreeding coefficient in a population it is possible to calculate the inbreeding coefficient for a particular population where inbreeding is preferred by tradition. This will be the average inbreeding coefficient practiced in the population.

Suppose in a survey one has observed different types of consanguineous types of marriages with each occurring at some frequency or proportion.

If '$c_i$' is the proportion of 'ith' types of marriages ('ith' e.g., = Uncle-Niece marriages, First Cross Cousin, Second Cross Cousin once removed, Double cross cousin etc. marriages observed in a population)

The inbreeding coefficient in the population is:

$$F_p == \Sigma (c_i . F_i)$$

Where, $c_i$ is the proportion of type (ith) of consanguineous marriage and $F_i$ is the inbreeding coefficient of **ith** type of consanguineous marriage.

## 1.4.6 Extent of Consanguineous Marriages in India

In India several communities practice consanguineous marriages and the extent of consanguineous marriages vary. For example a review work indicates that the extent of average inbreeding coefficient among Muslim population in different states of the country vary from 0.007 in West Bengal to 0.26 in Madhya Pradesh (Table 1.2). In case of castes and tribes, such marriages in different regions show an average of 0.0325. The extent of consanguineous marriages and average inbreeding levels practiced in the country is shown in Fig. 1.2. (Malhotra and Vasulu, 1999).

**Table 1.2: Extent of consanguineous marriages and inbreeding among muslims in different states of India**

| State/ Region | No. of Marriages | Type of Marriage | | | Inbreeding Coefficient |
|---|---|---|---|---|---|
| | | UN | I.C | Total | |
| Andhra Pradesh | 356 | 3.37 | 32.87 | 36.2 | 0.025 |
| Tamil Nadu | 6116 | 0.88 | 16.29 | 19.5 | 0.012 |
| Kerala | 215 | — | 16.74 | 22.3 | 0.012 |
| Maharashtra | 2014 | — | 14.00 | 20.71 | 0.010 |
| Madhya Pradesh | 351 | 7.41 | 19.09 | 59.3 | 0.026 |
| Rajasthan | 412 | — | 31.55 | 41.3 | 0.022 |
| Uttar Pradesh and | 1000 | — | 27.7 | 49.4 | 0.020 |
| Delhi | 1483 | — | 11.4 | 27.6 | 0.010 |
| West Bengal | 835 | — | 20.36 | 22.2 | 0.013 |
| | 471 | — | 8.28 | 19.3 | 0.007 |

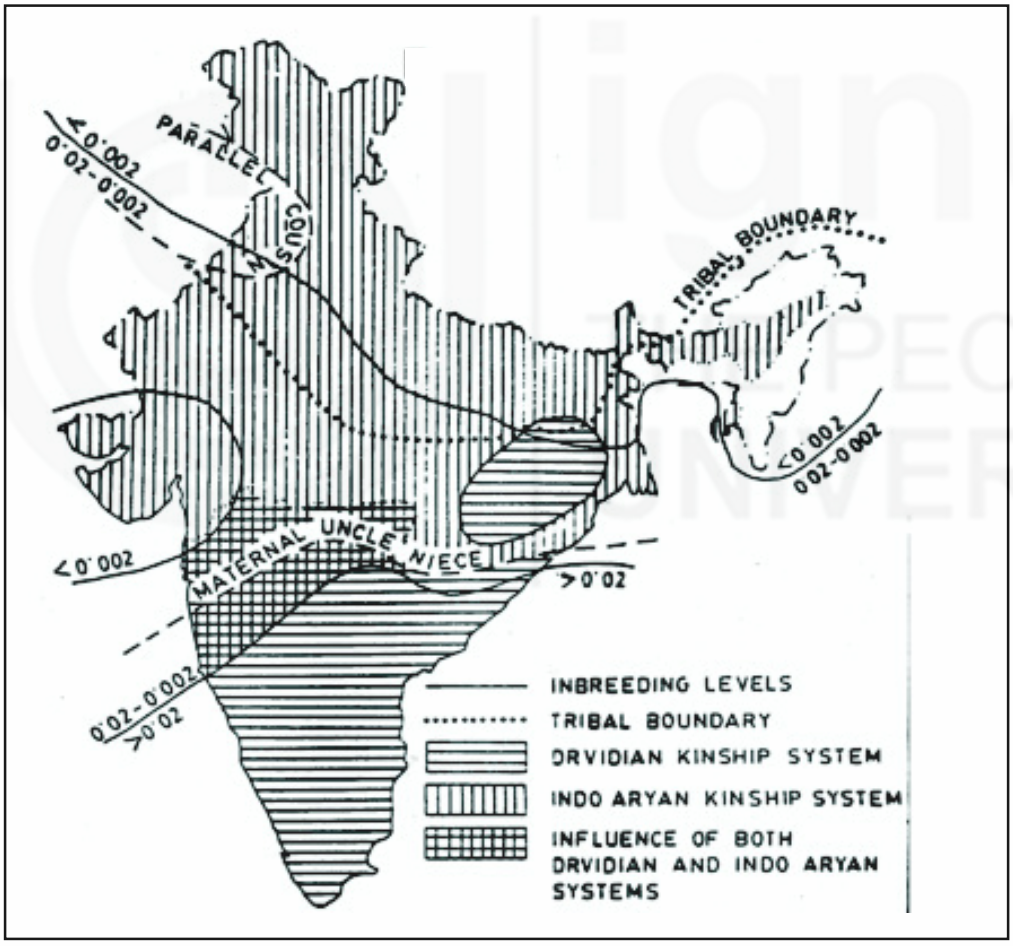



**Fig.1.2: Extent of consanguineous marriages and inbreeding levels in India**

## 1.5 SUMMARY

1) An understanding of the human population genetics starts with an idea of the concept of heredity, historical development of the concept and to the present knowledge of the concept of Mandelian hereditary unit and genetics and its developments.

2) In human populations the study of population genetics is the gene pool, the study unit. To identify and investigate the extent of gene pool requires understanding of the breeding isolation, especially who marries whom and where. This demographic information helps us to understand the extent of endogamy.

3) Sexual selection is one of the important mechanism that can significantly influence the pattern of gene transmission in a population. In case of random mating, where everybody can marry anybody as per HW equilibrium the genetic pattern is expected to be influenced by selection, drift and mutational pressures.

4) In human populations mating is non-random. Each population follows a variety of rules and regulations in selecting their marriage or mating partners. There are preferences or criterion of mate selection, one such criterion is based on e.g., morphological characters, intelligence, socio-economic status and is referred as assortative mating. One can choose to marry a partner with desirable similar characters as ones mate, where it is called as positive assortative mating or dissimilar or opposite characters where it is called as negative assortative mating.

5) Several human populations prefer to marry their own relatives. Such related mating or marriages are called inbreeding. Consagnuineous marriages in Man are one of the common type of marriage. There are a variety of types of related marriages and all result in inbreeding. Inbreeding leads to a greater chance of inheriting two copies of the homologous genes from one or more common ancestors. This is referred as identity by descent (ibd). In case of inbreeding there is decrease in variability of a trait. There is decrease in heterozygosity of several characters. This decrease in survival and fertility due to inbreeding is called inbreeding depression.

6) Theoretically it is possible to investigate the extent of genetic similarity between related individuals. One such measure is the inbreeding coefficient. By knowing types of consanguineous type or inbreeding practiced, one can calculate the inbreeding coefficient for different types of related marriages. One can also calculate the inbreeding coefficient in a population and examine the trends of levels of inbreeding practiced over time and region among diverse populations.

Overall this unit gives an idea of developments of the concept of inheritance how it is developed from the earlier time to the current understanding of genetics. This gives an idea of what is the unit of study in human population genetics, and how we investigate the breeding isolation in Man. It gives an idea of what is random mating, non-random mating, deviations from random mating in Man. Inbreeding and consanguineous marriages and how to measure them, the pattern and levels of inbreeding in Indian populations.

**Suggested Reading**

Charles Darwin 1859. *Origin of Species by Natural Selection.*

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**Sample Questions**

1) What is gene pool? How do we investigate the extent of gene pool in human populations.

2) What is Assortative mating and how it is different from random mating and inbreeding.

3) What are different measures of inbreeding?

# UNIT 2 HARDY-WEINBERG EQUILIBRIUM

**Contents**



**Learning Objectives**

After reading this unit, you will be able to:

- define what is Hardy-Weinberg Equilibrium or Law;
- depict the importance of HW Equilibrium and the field of population genetics;
- explain the method how to estimate the genotype and phenotype frequencies from HW theorem and to calculate in empirical situation; and
- evaluate the theory behind the deviations from H-WE, especially the gene frequency changes with respect to Mutation, Genetic drift, Selection, Gene flow and how to investigate them in empirical situations in human populations.

## 2.1 INTRODUCTION

Living organisms are endowed with unique abilities, traits that allow them to survive in a given environment. These traits or abilities may show or exhibit enormous variations within species and across species. Some of these traits are unique to that species; some traits are common within and across species with little variation, these are adaptive characters and gives survival advantage.

These traits are the '*phenotypic*' forms that can be observed as a quantitative trait (or measurable) or classified as types or categories. These traits are hereditary and transmitted across generations: either in the same form or in slight variable form. At times some new traits or variations of the trait appear among the offspring. Some of these traits are governed by '*genes'* or located in the '*genome'* of an organism. The nature of heredity of some of these traits could be complex and/or it could follow some simple principles of transmission.

Human population genetics deals with how these traits or variation change in a population over space and time (generations)? What are the factors that influence the variation of these traits in the population? To what extent these traits are hereditary and are influenced by environment? Can we understand them by simple theoretical models? Can we study how different forces operate differentially in different populations to give a characteristics distribution of gene and genotype frequencies?

Population genetics is the study of gene and genotype frequencies in populations of interbreeding organisms (small or large, natural or artificial) and predicting the way these frequencies are maintained or changed under the combined influence of various factors. It is concerned with applying models of gene frequency change involving different factors in the context of Mendelian genetics to examine evolution in a quantitative manner. In order to understand the pattern of allele frequencies we need to have a defined population, in this case a '*Mendelian population*'. Dobzhansky (1951) defined it as the reproductive community of individuals which share a common gene pool. Evolutionary studies involve reconstructing past demographic events that have led to the present day diversity patterns. Use of various models allows one to examine interplay of various factors and make inferences about the past based on present day data. But one has to be careful about interpreting results obtained from any model considering that all models have some assumptions inherent to them.

## 2.2 HARDY-WEINBERG EQUILIBRIUM

During the early 1900s people were interested to validate the Mendelian laws of genetics to other organisms, including Man. Are there Mendelian traits in Man?

**Mendelian Genetics in Man**

**BOX 2.1**

Brachydactyly **in a population:** All the mating types are:

The mating types include individuals Normal and Brachydactyly (a Dominant Mendelian trait)

N – Normal and B –Brachydactyly

a. Both parents are normal — All the offspring are N

b. One parent is N and the other B (heterozygous)

c. One parent is N the other B

d. Both parents are brachydactyly B (heterozygous)

e. Both parents are brachydactyly B (homozygous)

a NN – NN = All the offspring N
b NB – NN = ½ : ½ = N : B
c BB – NN = all are B
d NB – NB = 1 N : 1 B: 2 B
e BB – BB = All the offspring B

Of the 5 possible combinations of parental mating types 4 types of matings results brachydactyly offspring.

Therefore, B are more frequent than N in a population as per Mendelian expectation. However Normal individuals are more frequent than Brachydactyly in a population.

# there is apparent contradiction between what is observed and what is expected (Mendelian)!

At Cambridge one research scholar was studying 'brachydactyly' – a trait characteristic of small or short digital length ('*brachy*' and '*dactyl*' in Greek

means 'short' and 'digit' respectively) than the normal type. The trait runs in some families. Does 'brachydactyly' follow Mendelian principles? The results of the study showed that '*brachydactyly*' is dominant Mendelian trait and the pedigrees showed 3:1 ratio of brachydactyly to normal offspring. This has invoked an important and interesting question? If it is a dominant trait, there will be more and more brachydactyly individuals in the population, but normal individuals are more frequent than brachydactyly individuals (See Box 2.1)

GH Hardy has solved the puzzle theoretically and published the theorem in Science (Hardy, 1908). GH Hardy's proof illustrates that the gene (or allele) frequency, — here in this case, frequency of brachydactyly individuals in a population, — will not increase over generations, but remain the same, under equilibrium conditions or in the absence of confounding variables. In 1908, Dr. W. Weinberg independently also published similar results (Weinberg, 1908) and is called as HWEqulibirium**.** (See Box 2.2)

**BOX 2.2: Historical anecdotes: HWEqulibrium/Law**

In 1908, a German physicist Dr. Weinberg published similar results on Mendelian genetics in a German journal. It was discovered by Dr. Curt Stern (publication in Science 1943), and the Hardy theorem was rightfully referred as HW Law or HWEquilibrium. However, in 1903, at least five years earlier, two scientists have considered similar such possibility of change in gene frequency. They are: WE Castle 1903 in America and Karl Pearson 1903 in England. These two papers considers the question of equilibrium state of gene frequency and change in gene frequency partially with respect to some factors.

a) **What is H-W EQUILIBRIUM/LAW?**

HWE states that in a randomly mating population of sufficiently large size, and in the absence of the influencing factors such as; mutation, migration, selection, genetic drift and inbreeding, the gene and genotype frequencies will remain constant from generation to generation.

The mathematical proof of invariance of gene frequency under given assumptions, require:

a) simple knowledge of school algebra and

b) basic concepts of Mendelian genetics (See Box 2.3).

The proof in case of autosomal '*biallelic*' trait is given in Box 2.4. (for further reading see references)

**BOX 2.3: Basic concepts**

**Phenotype :** A trait or a character that is observed as types or measurable and is transmitted from parents to offspring. Some phenotypes are complex with unknown genotypes, and some are directly governed by hereditary units (genes).

**Gene :** The causative factor of hereditary transmission of traits (phenotypes) and are located in the chromosomes (the hereditary materials in cell nucleus and in mitochondria).

**Allele** : Genes, the causative factor of hereditary transmission can exist or express in different forms and are referred as 'alleles'.

**Codominant:** Where both the alleles are equally expressive in the offspring.

**Recessive** : The alleles whose expression is suppressed at phenotypic level. The heterozygote offspring of a recessive allele will express the phenotype of the dominant allele.

**Haploid** : Organisms which carry one set of chromosomes.

**Haplotype** : It is short form of 'Haploid genotype'**.** Refers to genetic markers located on one chromosome. A haplotype can be identified by SNP (single nucleotide polymorphism).

**Diploid** : Organisms which carry two sets of chromosomes, each set derived from either of the parent. Man is diploid and carries two sets of chromosome (2N).

A diploid individual can carry two copies (alleles) of the gene in each of the chromosome that he or she gets from his or her parents. The two copies could be of the same type (form/status) or of different type (form/status).

**Homozygous:** The two alleles that an individual carries are of the same or identical types.

**Heterozygous:** The two alleles that an individual carries are of different type.

**Genotype** : Is the combination of alleles that a diploid individual can carry in each of the chromosomes.

For example, in case of a 'biallelic' gene say A, B two forms (alleles) of the gene that occur in each of the two sets of the chromosomes. There could be three different genotypes: AA, AB, BB.

AA and BB : two different homozygotes (genotype).

AB = BA : heterozygote (genotype).

The box shows the "Punnet's square" – method of scoring different combination of genotypes based on the male and female gametes or mating types. This can be extended to multiple alleles.

**Polymorphism:** If a gene exists in more than one form or morph (alleles) and that occurs in stable frequency in a population.

**Punnet's square**

| Female gamete | Male Gamete A | Male Gamete B |
|---|---|---|
| A | AA | BA |
| B | AB | BB |

**Genotype**

**BOX 2.4: Hardy-Weinberg theorem or principle: Proof**

In case of genetic trait that has positive family history in a population, let us assume that the gene is biallelic and therefore the two alleles are: B1 and B2 and let

'p' is the frequency of 'B1 allele' and

'q' is the frequency of B2 allele,

N is the total individuals and

So that (p + q = 1) or p = (1 – q) or q = (1 – p)

An individual in the population can have three types of genotypes: B1B1, B1B2, and B2B2. And let the frequency of the above three genotypes in the parental population are: P, H and Q respectively.

| | Gene (alleles) | | Genotypes | | |
|---|---|---|---|---|---|
| | B1 | B2 | B1B1 | B1B2 | B2B2 |
| Frequencies | p | q | P | H | Q |

If there are total N individuals in the sample, there will be

P individuals with genotype B1B1 type,

H individuals with genotype B1B2 type and

Q individuals with genotype B2B2 type

So that sum of (P +H + Q) = N,

Assuming all the individuals of the three genotypes are equally fertile, then given the genotypes, one can calculate the frequencies 'p' and 'q' in the population, by gene counting method:

The gene (allele) frequency 'p' = [P + ½ (H)]1/N = (B1B1)/N + ½ (B1B2/N), and

The gene (allele) frequency 'q'= [Q + ½ (H)]1/N = (B2B2)/N + ½ (B1B2/N)

This is the gene (allele) frequencies of 'p' and 'q', which are also the gametes produced in the population.

Only some of the gametes form zygotes that will eventually become individuals in the next generation. The allele (gene) frequency in the zygote is unchanged provided there is no reproductive advantage of either of the allele and the zygotes formed represent a large sample of the parental gametes.

Random mating between individuals is equivalent to random union among their gametes. Therefore, in the next generation, the genotype frequencies among the zygotes (fertilized eggs) are the result of random union of two types of gametes. The genotype frequencies among the progeny are therefore can be worked out by Punnet's square. Or it is the multiplication of the frequencies of the gametic types produced by the parents. Viz.,

| | Genotype | | |
|---|---|---|---|
| | **B1B1** | **B1B2** | **B2B2** |
| **Frequency** | **$p^2$** | **2pq** | **$q^2$** |

| Allele | B1 | B2 | |
|---|---|---|---|
| Genotype | B1B1 | 2B1B2 | B2B2 |
| Frequency | $p^2$ | 2pq | $q^2$ |
| Absolute freq. | P | H | Q |

## BOX 2.4 (Contd.)

### Hardy-Weinberg theorem or principle: Proof

In a population there will be three different types of genotypes among males and females, who will mate randomly and they will give rise to their offspring who will represent the same genotypes in the next generation. We will have to work out the frequencies of offspring genotypes given the three genotypes among the male and female parents. This is worked out easily by Punnet's square: the frequencies of different mating types among the male and female genotypes in the population and different possible genotypes among the offspring are.

**Frequency of different mating types and the offspring genotypes**

| | Male Parent — Genotype | | | |
|---|---|---|---|---|
| Female Parent | | B1B1 | B1B2 | B2B2 |
| | Freq. | $p^2$ | $2pq$ | $q^2$ |
| B1B1 | $p^2$ | $p^4$ | $2p^3q$ | $p^2q^2$ |
| B1B2 | $2pq$ | $2p^3q$ | $2p^2q^2$ | $2pq^3$ |
| B2B2 | $q^2$ | $p^2q^2$ | $2pq^3$ | $q^4$ |

Once we know the possible offspring genotypes as a result of random mating among the three parental genotypes we can calculate the expected frequencies among the offspring genotypes for different combination of mating types in the population. Given the three genotypes six possible mating types are possible in the population and each mating type will give rise to offspring of different possible combination of genotypes. These are worked out in the following table and this gives the allele frequencies in the offspring population in the next generation:

**Frequency of different mating types and the offspring genotypes**

| Parent Female X Male (Genotype) | Freq. | Offspring — Genotype B1B1 | B1B2 | B2B2 |
|---|---|---|---|---|
| B1B1 X B1B1 | $p^2$ | $p^4$ | — | — |
| B1B1 X B1B2<br>B1B2 X B1B1 | $4p^3q$ | $2p^3q$ | $2p^3q$ | — |
| B1B1 X B2B2<br>B2B2 X B1B1 | $2p^2q^2$ | — | $2p^2q^2$ | — |
| B1B2 X B1B2 | $4p^2q^2$ | $p^2q^2$ | $2p^2q^2$ | $p^2q^2$ |
| B1B2 X B2B2<br>B2B2 X B1B2 | $4pq^3$ | — | $2pq^3$ | $2pq^3$ |
| B2B2 X B2B2 | $q^4$ | — | — | $q^4$ |
| | 1 | $p^2 (p^2 + 2pq + q^2)$ | $2pq (p^2 + 2pq + q^2)$ | $q^2 (p^2 + 2pq + q^2)$ |
| | | $p^2$ | $2pq$ | $q^2$ |

Thus the genotypic frequencies in the offspring remain the same in two successive generations, assuming that allele frequencies are not influenced by selection, mutation and mating is random and there is no differential fertility and mortality and the population is large.

The above is true for *autosomal loci* and can be extended for multiple loci. It is also true for sex-linked trait. Here the gene frequencies will oscillate (by ½) between two sexes in successive generation and will soon reach to equilibrium.

### 2.2.1 Importance and Implications of HWE

What are the implications and why it is so important? In brief, it is the fundamental theorem of population genetics.

- **Methodology:** Tells us how to calculate (or estimate) the allele frequency or genotype frequency from observed phenotypes in an empirical situation. It can help us to investigate how many alleles are governed by a phenotypic trait.

- **Evolution:** It is quantitative way of understanding the mechanism of evolutionary factors and its influences. Evolution is a dynamic and complex phenomenon and it is hardly possible to study evolution in the laboratory conditions. It gives insights into the inter-relationship between the forces and how to study the effects of each of these forces and the gene frequency. (See the box 2.5 for the relationship between gene frequencies and genotype frequencies).

- It is the benchmark criterion to test whether a new trait is in equilibrium or if not how to test the reasons for the deviations.

- It helps us in genetic counselling to expect the likelihood of a child being homozygous for a recessive deleterious trait given the parental genotype. It helps in forensic science in cases like identification of suspects, parent-offspring disputes etc.

- **Quantitative Genetics**: HWE helps us to investigate complex genetic traits, to estimate the role of environment and genetic components, spatial distribution of gene frequency etc.

Further implications of this principle are as under:

- In case in a population a particular trait or character is in HWE, (the converse) it does not mean that the assumptions are satisfied. (The theoretical proof is complicated and it is available).

- The allele frequencies remain constant from generation to generation. This means that hereditary mechanism itself does not change allele frequencies. It is possible for one or more assumptions of the equilibrium to be violated and still not produce deviations from the expected frequencies that are large enough to be detected by the goodness of fit test.

- When an allele is rare, there are many more heterozygotes than homozygotes for it. Thus, rare alleles will be impossible to eliminate even if there is selection against homozygosity for them.

- For populations in HWE, the proportion of heterozygotes is maximal when allele frequencies are equal ($p = q = 0.50$), and when this happens the heterozygote frequency will be 0.50 (2x0.50x0.50). Unless HWE is violated (as in selective loss of homozygotes), heterozygosity can never be more than 0.50 at any biallelic locus. The relationship between gene frequency and genotype frequency is illustrated in Box 2.5.

**BOX 2.5**

**The relationship between gene frequency and genotype frequency**

It is interesting to know the relation between the gene and genotype frequency for a biallelic loci which is under H-W equilibrium. The graph shows the changes in the three genotype frequencies as against the change in allele frequencies A1 and A2 from 0 to 1 in Cartesian coordinates (drawn on x and y axis).

A1A1/A2A2 – homozygotes, A1A2 – heterozygotes

**Relationship between genotype frequency and gene frequency - biallelic GENE**

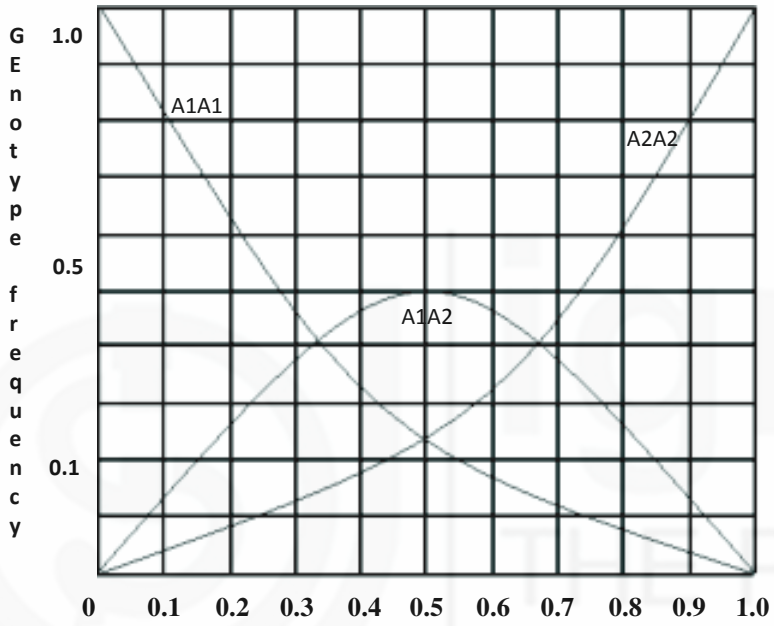



Fig: Left top curve – A1A1, Right top curve – A2A2, Lower curve – A1A2

The graph shows two interesting properties of the HWE:

- ✓ The frequency of the heterozygotes can reach to a maximum of 50%
- ✓ And this can occur when the gene frequency of '*p*' = '*q*' = 50%
- ✓ When one of the gene frequencies of an allele is low, the rare allele predominantly occurs as heterozygotes and there will be few heterozygotes.

- An application of HWE is that when the frequency of an autosomal recessive disease (e.g., sickle cell disease, hereditary hemochromatosis, congenital adrenal hyperplasia) is known in a population and unless there is reason to believe HWE does not hold in that population, the gene frequency of the disease gene can be calculated. Likewise, the carrier rate may be calculated for autosomal recessive disorders if the disease gene frequency is known. For example, phenylketonuria (PKU) occurs in 1/11,000 ($q^2$), which gives a heterozygote carrier frequency of approximately 1/50 [ 2xq(1-q) ]. If the diseased individuals ($q^2$) are deducted from the whole population, the carrier rate in normal individuals approximates to [ 2q/1+q ].

- It has to be remembered that when HWE is tested, mathematical thinking is necessary. When the population is found in equilibrium, it does not necessarily mean that all assumptions are valid since there may be counterbalancing forces. Similarly, a significant deviance may be due to sampling errors (including Wahlund effect), misclassification of genotypes, measuring two or more systems as a single system, population substructure, failure to detect rare alleles and the inclusion of non-existent alleles. The Hardy-Weinberg laws rarely holds true in nature (otherwise evolution would not occur). Organisms are subject to mutations, selective forces and they move about, or the allele frequencies may be different in males and females. The gene frequencies are constantly changing in a population, but the effects of these processes can be assessed by using the Hardy-Weinberg law as the starting point.

- The direction of departure of observed from expected frequency cannot be used to infer the type of selection acting on the locus even if it is known that selection is acting. If selection is operating, the frequency of each genotype in the next generation will be determined by its relative fitness (W). Relative fitness is a measure of the relative contribution that a genotype makes to the next generation. It can be measured in terms of the intensity of selection (s), where $W = 1 - s$ $[0 < s < 1]$. The frequencies of each genotype after selection will be $p^2 W_{AA}$, $2pq W_{Aa}$, and $q^2 W_{aa}$. The highest fitness is always 1 and the others are estimated proportional to this. For example, in the case of heterozygote advantage (or overdominance), the fitness of the heterozygous genotype (Aa) is 1, and the fitnesses of the homozygous genotypes negatively selected are $W_{AA} = 1 - s_{AA}$ and $W_{aa} = 1 - s_{aa}$. It can be shown mathematically that only in this case a stable polymorphism is possible. Other selection forms, underdominance and directional selection, result in unstable polymorphisms. The weighted average of the fitnesses of all genotypes is the mean fitness. It is important that genetic fitness is determined by both fertility and viability. This means that diseases that are fatal to the bearer but do not reduce the number of progeny are not genetic lethal and do not have reduced fitness (like the adult onset genetic diseases: Huntington's chorea, hereditary hemochromatosis). The detection of selection is not easy because the impact on changes in allele frequency occurs very slowly and selective forces are not static (may even vary in one generation as in antagonistic pleiotropy).

- All discussions presented so far concerns a simple biallelic locus. In real life, however, there are many loci which are multiallelic, and interacting with each other as well as with the environmental factors. The Hardy-Weinberg principle is equally applicable to multiallelic loci but the mathematics is slightly more complicated. For multigenic and multifactorial traits, which are mathematically continuous as opposed to discrete, more complex techniques of quantitative genetics are required.

## 2.3 APPLICATION IN HUMAN POPULATION GENETICS

The behaviour of HW principle under different assumptions is the discipline of 'population genetics', which describes, primarily, the changes in gene frequency that are influenced by demographic factors, population structure variables,

historical, random events, sampling fluctuations and evolutionary factors of selection and mutation. In simple the four main factors that influence the gene frequency in a population are: mutation and genetic drift (non-systematic factors), migration and selection (systematic factors). The genetic drift is effective, more specifically, in populations whose size is small or limited e.g., an isolate or an island population or a small endogamous population. These are described in detail below:

- For example, HWE has helped us to find out to investigate the number of alleles of ABO locus and how to calculate the gene frequency of ABO locus (e.g., Bernstein has given the method of correction ) (see Box 2.6)
- We were able to understand how HbS despite its deleterious effect it maintains its equilibrium in the population.
- HWE helps to understand the some of the health problems in some isolated populations, whose propagation is the result of genetic drift, and selection or inbreeding etc.
- HWE has forensic applications in solving problems related to disputed paternity, to provide evidence in case of crime to detect the culprit, property or biological inheritance cases.
- It helps in understanding the complex genetic disorders, to be able to estimate the contribution of genetic versus environmental effects.
- HWE helps to understand to investigate the human origins, the role of selection versus demographic effects on the genetic diversity in a population.

**BOX 2.6**

**HWE – Gene frequency estimation: Gene counting method**

Given the information about the genotypes, HWE helps us to estimate the allele frequency by 'gene counting method' (how many alleles a genotype contains). For example,

- As each homozygote carries two alleles and each
- Heterozygote carries one allele, therefore, estimate of an allele frequency in a population of size N individuals (or 2N alleles) will be
  = (2 homozygotes + heterozygote) *(1/2N)
- In a population there will be three genotypes and their absolute frequency will be say N1, N2 and N3 (where N1 + N2 + N3 = N). If there are two alleles say 'A' and 'a' with a frequency 'p' and 'q' respectively (where p + q = 1).
- By gene counting method assuming HW law the gene (allele) frequencies of p = (1/2N) * (2N1 + N2), q = (1/2N) * (2N3 + N2) and p = (1 – q)

## 2.4 DEPARTURE FROM HWE

In general, the factors that are assumed to be non-operative under HWE are hardly realised in the living systems. The living system (populations or organisms) are structured (non-random entities) and are influenced by multiple and interactive factors that operate through space and time. With the help of HW equilibrium it is possible to investigate and estimate the effect of these individual forces that change gene frequency in human populations.

### 2.4.1 Factors Affecting Change in Gene Frequency

The four aspects of the H-W assumptions are:

i) Demographic:
   a) Size, mating, fertility and mortality, and migration

ii) Evolutionary:
   a) Mutation, selection, gene flow

iii) Population structure:
   a) Social and Cultural factors
      i) Matings and Marriage specifications that regulate the marriage or mating type in a population.
      ii) Non-random mating – Sexual selection of mates

iv) Ecological:
   a) Population bottle-neck events:
      i) Pandemic: disease, earthquake etc.
      ii) Historical: wars etc.,

Of the above factors, for the present academic purpose, we will be dealing a few factors and examine how these factors change or influence the gene frequency in a population and how to estimate them in empirical situation.

#### 2.4.1.1 Mutation

Mutation is a random change in phenotypic or genotypic forms that occur once a while in a population. The probability or likelihood of occurrence, in a population, in general, is of the order of one over several lakhs or tens of thousand of individuals.

For example, several of the Mendelian syndromes and disorders that have been discovered in human populations are the result of mutation. In general, it is observed to be a single mutation or point mutation. At molecular level, mutation primarily refers to changes in the DNA sequences (or SNPs — Single Nucleotide Polymorphism) in the genome of an individual (population) with phenotypic manifestations resulting to non-normal cases, some of them are clinically or medically identified as diseases or a syndrome. If one can search *web* resources, there is a data base created by Hopkins institute and or by NIH (America) on a list of Mendelian syndromes, which can be found by a search criterion OMIM (Online Mendelian Inheritance in Man). One can also find such data bases from a variety of sources.

Some examples will help us to get an idea of mutation and its effects. Sickle cell anaemia (or HbS condition), is a disease related to Haemoglobinopathies. Its inability to synthesize Oxygen ($O_2$) to its full capacity by an individual who is suffering from the disease or the trait, which results a risk to survival liability. This is identified as due to a point mutation or single mutation at the $6^{th}$ position of the â-globlin chain of the haemoglobin gene. The single aminoacid substitution ($â_6$ Glu to Val) changes the haemoglobin structure, which is phenotypically identified as sickle cell shaped form (or half moon shaped form) of the RBC.

Mutation is an important factor or ingredient leading to the appearance of new characters in the population. The fate of the mutation as a new character in a population depends on its advantage or disadvantage that it can impinge to the

survival fitness of the population. For example, mutation is a significant evolutionary force which can change allele frequency variation in a population under a favourable environment. How we can know the relation between the mutation and allele frequency change from HWE.

a) **Change in gene frequency due to mutation (μ):**

Random changes that happen at the DNA sequence, especially at the coding region of the gene can create an allele which can alter the gene frequency in the population in successive generations. This can be investigated theoretically given the mutation rate per generation in the population. This has been shown in Box 2.7 for bi allelic loci.

The theoretical results suggest that the change in gene frequency of a mutant allele, after 't' generations, depends on the initial allele (gene) frequency before mutation and the mutation rate of the allele per generation in the population.

This is an important result and can help to calculate change in gene frequency after 't' generations given the mutation rate (μ) per generation and the initial gene frequency in the population.

b) **Rate of mutation (μ):**

Though the mutation is random, but the rate of mutation varies. It is site specific – there are 'hot-spots' where mutation rate is more frequent than in other parts of the genome. In general, the coding part of the gene does not support mutation to occur, as a result of proof reading process and functional importance of the codons. However, mutations occur at higher rate in the intronic region, and in the repeat sequences than in exons or codons. Also the mitochondrial non-coding parts, *viz*., hyper variable regions HV1, HV2 in the D-loop has higher rate of mutation than in nuclear genome.

**BOX 2.7**

**Change in gene frequency due to mutation (μ)**

If there are two alleles 'A' and 'a' with its frequencies '$p_0$' and '$q_0$' at the initial stage (say at time '$t_0$') in a population and 'μ' is the mutation rate that changes allele 'A' to 'a' per generation, then gene frequency (g.f.) of 'A' will decrease by an amount '$\mu p_0$' in the first generation. Therefore the g.f. of 'A' allele in the first generation after mutation will be:

$$P_1 = p_0 - \mu p_0 = (1 - \mu) P_0$$

In the (next) second generation the gene frequency is expected to be:

$$P_2 = P_1 - \mu P_1 = (1 - \mu) P_0 - \mu(1 - \mu) P_0$$
$$= (1 - \mu) P_0 \quad [(1 - \mu)]$$
$$= (1 - \mu)^2 P_0$$

After 't' generations the g.f. of 'A' is expected to be

$$P_t = (1- \mu) P_{t-1} = (1- \mu)^2 P_{t-2} .. = (1-\mu)^t P_0$$

When μ is very small $(1-\mu)^t$ can be approximately equated to $= e^{-ut}$, (where e is natural logarithm to base e), therefore, gene frequency after 't' generations will be

$$P_t \sim P_0 e^{-ut}$$

Therefore, the mutations that occur at HV1 and HV2 regions of mitochondrial genome help us to investigate the short-term evolution or micro-evolutionary

trends in sub-populations. This has helped us to address some of the questions of human origins or to verify the Darwin's hypothesis that the Africa is the origin of Man. This also helps to enquire the antiquity and past genetic history of diverse populations and their diversity and relationship with other human populations.

### 2.4.1.2 Genetic Drift

Genetic drift is an important non-systematic evolutionary force. To understand the concept of genetic drift, let us know what the word 'drift' conveys, in general. One of the descriptions for the word 'drift' in the English Dictionary is: "move aimlessly from one place or activity to another' – this is more with reference to things or events that we experience with practical world e.g., drifting by air, wind and water or ocean. Similar phenomena can also happen with respect to gene frequency in a small population. In small populations, as a result of population-events such as pandemic diseases, earthquakes etc., the population size is drastically reduced which can have significant effect on the genetic diversity and gene frequency: for example, the gene frequency can drift from one generation to generation randomly leading to either loss or fixation of alleles over generations (in the absence of other interfering factors). In small populations or due to demographic and ecological effects the population size drastically reduced to a fraction (or a random sample) of the original population with allelic representation different from the original population. In these cases, there will be random changes in gene frequency, which appear to drift at varying frequencies in successive generations in an erratic manner. For example, the studies on the origins of Man, suggest that decreasing heterozygosity and linkage disequilibrium levels away from Africa are supportive of the role of genetic drift among human populations.

To understand how the genetic drift can happen or possible, one can investigate and/or understand by attempting some simple examples or simulation exercises. These are available on the online resources. One such example is illustrated in Box 2.8.

a) **Bottle-neck effect**

Genetic Drift can happen in a variety of ways due to different events that populations experiences in empirical situation. These have been referred as part of ecological factors that disturb the population size (see 2.4.1). Historically the world has experienced several pandemic diseases in the past: e.g. Syphilis, Plague, leprosy, malaria, HIV infection, etc. which has killed or wiped out bulk of the population. The natural geographical events like earthquakes, tsunami etc. had killed vast majority of the populations. Even the political and man interfering events like explosion of atomic bombs, world wars etc. have affected the demographic size of the populations. Each such event is followed by a drastic reduction in population sizes. In genetic terms it means reduction in genetic diversity (at the time of the event), and those survived will have different allelic profile or gene frequency and the stability of a particular allele over generations depends on the demographic structure of the population.

'Breeding individuals' part of the demographic structure of a population is of particular genetic importance. They are capable of mating and producing children. They will be a fraction (of the total population) who contribute to the next generation or gene pool and is referred as 'effective size' ('Ne').

**BOX 2.8**

**Simple exercises to understand the genetic drift**

There are different ways to replicate to illustrate the random drift phenomena. One such simple example could be the following:

- Start with a jar that contain with N number of blue, red, yellow balls.
- At the first step blindly or randomly take out (sat e.g., by hand) some balls and put them in the second bottle.
- Then from the second bottle, take some balls (e.g., by hand) and put in the third bottle.

If you have started with large sample (N) of mixed coloured balls you can repeat the same. Otherwise, at the third/fourth bottle you can count how many or red, blue and yellow balls. Compare the outcome with the original number of red, blue and yellow balls at the start. They will differ from the original number at the start. You may also find the absence of a particular colour at the fourth (or nth) bottle.

In case of Genetic Drift, similar such random sampling of gene frequency changes happen over successive generations in a small population. One can search several such simple examples on the online resources on genetic drift – bottleneck effect, founder effect etc.

Genetic drift can alter the 'effective size' of a population and change the genetic diversity. After successive generations, the gene frequency in the population will be significantly different from the gene frequency before genetic drift. This is similar to the bottle neck, where the narrow neck of the bottle restricts the flow and this event is referred as 'bottle neck-effect' in population genetics. Such bottle neck effect resulting to sudden population size reduction had been experienced by several human populations in the past historical times affecting the genetic structure: genetic diversity, gene frequency changes.

b) **Founder effect**

The word 'founders' refers to the ancestors or the earliest settlers who colonised or founded the new population in alien territories. It could be an historical adventure of ware fare, or exploration to a new island or new area or it could also be due to chance factors like surviving from a sudden calamities like ship wreck, etc. or it could be serial migration of people at different timing to other places: in all the cases, a few founders start living and establishing a new subpopulation.

In genetic scenario, the few founders represent a random sample of the genes from the original population or gene pool from which they got separated. It is possible that, some of the rare alleles that are in the large population, by chance, may not be present in the founder individuals. It could be that, among the founders, especially if the founders are related, by chance, some of alleles may be of a higher frequency than the original population. Therefore, in the new colony after generations the gene pool will have either absence of the allele or higher frequency of the rare allele than when compared to the original population.

c) **Serial founder effect**

It is possible that people or organisms migrate repeatedly over time or waves of migration from a region to found new colonies. Such repeated waves of migration at different time periods produce successive subpopulations or gene pools whose genetic profile will be different. There appears to be waves of out of Africa migration to other continents that had happened at different time periods in the past, whose genetic signature can now be traced among the extant populations in South Asia, Europe, and America etc. The mitochondrial, X and Y chromosomal haplogroup distribution of continental populations can be explained as a result of founder effect of out of Africa hypothesis of human origins.

d) **Empirical studies of founder effect in Man**

The importance of 'Founder effect' as significant evolutionary factor has been outlined by German evolutionary biologist Ernst Mayr (1942). Founder effect is the "*The establishment of a new population by a few original founders (in an extreme case, by a single fertilized female) which carry only a small fraction of the total genetic variation of the parental population*.» This is sampling effect especially the genetic composition and evolution of the successive generations entirely depends upon the few founders. A few examples illustrating the role of genetic drift in the gene frequency changes are shown in Box 2.9

**BOX 2.9**

**Studies on Genetic Drift**

- **Tristan da Cunha is an island**; the few hundred individuals (<300) living on the island are mostly the (15) descendants (8 males and 7 females) who had founded the island in 1816-1908. Three of the founders were Asthma sufferers and there is high incidence of Asthma in the population. In a study of the 9 Y-chromosome haplotypes of the island, seven of them are traced to its 7 male founders.

- **Amish population, USA:** All most all the Amish population (~249K) descended from about 200 founders from German during 18$^{th}$ century. The population is endogamous, they show high frequency of genetic disorders as a result of founder effect that include dwarfism, metabolic disorders, unusual distribution of blood types, metabolic disorders etc.

- **'Blue Fugates' of Appalachian, Kentucky, USA:** In 1800, Martin Fugate and his wife settled in trouble some creek in Kentucky. They carried recessive gene methemoglobinemia (met-H). Due to deficiency of an enzyme diaphorase (NADH methemoglobin reductase) met-H levels rise and this gives rise to reduced oxygen-carrying capacity. This gives a tinge of blue skin of the homozygous condition. Isolation and inbreeding has caused to increase of blue people which are traced to the founders Fugates.

- **India:** In the northeast populations, some of them live in geographical isolation, practice endogamy show unusual frequency of a few genetic

traits which are expected to be due to genetic drift and founder effect. Some of them include:

- Complete lack of A2, cde, K, pc, and AK2 genes, lack of isozyme ALDH-1 (Roychoudhury and Nei 1997), a high prevalence (about 50%) of lactase malabsorption (Flatz 1987).
- Low frequency of AIBG*2 allele ( Juneja et al. 1989), high frequency of G6PD deficiency in Naga (Seth and Seth 1971), absence of 'Gd_' variant in Adi and Hmar and high frequency of this variant in Bodos (Saha et al. 1990).
- Continuing from classical genetic observations, unique and rare allele frequency of microsatellite loci among the Adi subpopultions (Krithika et al. 2005). High frequency of susceptibility of tuberculosis in some clans of tribes, stomach cancer, high incidence of cardio deaths etc.
- Absence of attached ear lobe among the Nandiwalas in Maharashtra.
- Population size reduction and allele frequency changes among Ahmedias of Kashmir population.

### 2.4.1.3 Natural Selection

Charles Darwin (and Wallace) has described natural selection as one of the important factor (key mechanism) of evolution. Natural selection happens where there is differential rate of reproductive success among different genotypes (underlying the phenotype, or trait or observed character). How selection operates at the molecular (genome) level for example, especially change in gene frequency considered, theoretically, in population genetics.

Due to differential reproductive success involving these variant of the trait, there will be more offspring with the variant than those individuals with other variant of the trait. In Darwinian sense '*fitness*' ('*Darwinian Fitness*') refers to ability to contribute successfully to the next generation. This is also referred as 'adaptive value' or 'selective value'. Therefore, if the differences of fitness are in a way associated with the presence or absence of a particular allele (or gene) in the individual's genotype then selection operates at the genetic level.

When a gene is subjected to selection (or under selective pressure), its frequency in the offspring is not the same as in the parents (or in the previous generation) as parents with different genotypes pass on their genes *unequally* to the next generation. This leads to change in gene frequency and consequently also of genotype frequency, as a result of selection (of a particular gene). The theoretical investigation of change in gene frequency of an allele under selection pressure is more complex, than factors like mutation, migration. There could be different situations under which selection can operate in a population and different situations need to be incorporated in theoretical models. Here we will consider a few of those situations (types of selection) in a more descriptive way, rather than theoretically, which is beyond the scope of the present purpose.

Theoretically, selection is measured by '*fitness*' ('*W*') or by selection coefficient ('*s*'). *Fitness* refers to '*relative rate of survival*'. The selection coefficient ('*s*') is defined as ( *1-W* ) and the value varies between 0 and 1. Once the fitness is quantified and defined the different types of dominance can be taken as degrees of dominance with respect to fitness (this is different from the dominance effect of the gene). In general, most mutant genes are completely recessive compared to the wild type as can be observed from phenotypic form of the trait. This does not imply that the heterozygotes are equally fit when compared to homozygote.

Before we get to know the effect of selection on gene frequency, it is required to know different types of selection and its fitness values. Some of the known selection types are: no dominance, partial dominance, complete dominance, over dominance. The fitness values for the four types are shown below (See Box 2.10). The change in gene frequency with respect the four types of selection (with fitness values) are given in Box 2.11.

**BOX 2.10**

**Types of dominance or degree of dominance and fitness**

**a. No dominance:**

| $A_2A_2$ | $A_1A_2$ | $A_1A_1$ |
|---|---|---|
| I | I | I |
| 1 – s | 1 – 1/2s | 1 |

**b. Partial dominance:**

| $A_2A_2$ | $A_1A_2$ | $A_1A_1$ |
|---|---|---|
| I | I | I |
| 1 – s | 1 – hs | 1 |

**c. Complete dominance:**

| $A_2A_2$ | $A_1A_2$,$A_1A_1$ |
|---|---|
| I | I |
| 1 – s | 1 |

**d. Over dominance:**

| $A_2A_2$ | $A_1A_1$ | $A_1A_2$ |
|---|---|---|
| I | ! | I |
| $1 – s_2$ | $1 – s_1$ | 1 |

a) **Types of selection**

Selection is a systematic force and operates in different ways. Selection takes place when there is differential fitness of a heritable trait. Based on the effect on the allele frequencies, the selection can be seen operating into three types.

***Directional selection***: occurs one extreme value or allele is selected. In case if one of the allele of a variety of the trait has greater fitness and producing more offspring of that allele or a variety, then the selection is said to be directional. The effect of directional selection is fixation of allele with greater fitness and the loss of the allele with least fitness. For example: well known cases come from the parasitic world, especially resistance to antibiotics in case of some of the vector-borne diseases. Initially as a result of antibiotic the parasite growth comes down to zero, but the parasites develops some mutant or new variant which gets resistance against the antibiotics or better fitness in the presence of antibiotics, in due course, the less fit variant is replaced by new variant which can survive against antibiotics. This can be illustrated as a shift in the mean of the character of a distribution (See box 2.12).

## BOX 2.11: Change in gene frequency under selection

First we will consider the basic formulae for the change in gene frequency that is achieved in one generation of selection. Under the similar notation that has been used above for other factors (p = gene freq. of $A_1$ and q = gene freq. of $A_2$), the below table shows the genotype frequencies under HWE before selection to the allele for the three genotypes (first line).

| | Genotypes | | | |
|---|---|---|---|---|
| | $A_1A_1$ | $A_1A_2$ | $A_2A_2$ | Total |
| Initial frequency | $p^2$ | 2pq | $q^2$ | 1 |
| Coefficient of selection | 0 | 0 | | s |
| Fitness | 1 | 1 | 1 – s | |
| Genetic contribution | $p^2$ | 2pq | $q^2$ (1—s) | $(1 - sq^2)$ |

Here we consider selection acting on the recessive genotype $A_2A_2$ with a selection coefficient: 's' acting against it. This will have a differential fitness to the genotypes that will be as given in the second line. By multiplying the initial frequency by the fitness values gives the frequency of each genotype after selection. This is the third line – the genetic contribution to allow to selection to operate over life cycle. Therefore, after selection, there will be a loss of fitness that is proportional to an amount $(1 - sq^2)$. From this we can calculate the frequency of $A_2$ gametes produced (frequency of $A_2$ genes in the progeny). The new g.f. is (where p = (1 – q)

$$q_1 = [q^2 (1 - s ) + pq] / (1 - sq^2)$$
$$= [q - sq^2] / (1 - sq^2)$$

The change in gene frequency $\Delta q$, resulting from one generation of selection is

$$\Delta q = q_1 - q = sq^2 (1 — q) / (1 - sq^2)$$

This tells us that the effect of selection on gene frequency depends not only on the intensity of selection s, but also on the initial gene frequency (of the recessive allele).

*Different type of selection*

What we have considered above is in general selection with respect to recessive allele q under selective pressure. But there are variety (or types) of selection that can act on the allele frequency. Depending upon the type of selection the change in gene frequency will consequently change. These are shown in the following table.

| | **Initial freq. & fitness of Genotypes** | | | **New gene frequency due to selection at q** | **Change in gene frequency** |
|---|---|---|---|---|---|
| | $A_1A_1$ $p^2$ | $A_1A_2$ 2pq | $A_2A_2$ $q^2$ | $q_1$ | $\Delta q = q_1 - q$ |
| 1. | 1 | 1– ½ s | 1– s (q – ½ sq – ½ $sq^2$) / (1 – sq) | – ½ sq (1—q) / ( 1– sq) |
| 2. | 1 | 1 – hs | 1– s (q – hspq – $sq^2$) / (1–2hspq–$sq^2$) | –spq[q + h(p-q)]/ (1–2hspq–$sq^2$) |
| 3. | 1 | 1 | 1 – s (q – $sq^2$) / (1 – $sq^2$) | – [$sq^2$ (1—sq)]/ (1 – $sq^2$) |
| 4. | 1–s | 1–s | 1(1–sq + $sq^2$) / (1– s(1-$q^2$) | + [$sq^2$ (1 - sq)]/ [1 – s(1 – $q^2$)] |
| 5. | 1–$s_1$ | 1 | 1– $s_2$ (q – $s_2q^2$) / (1 – $s_1p^2$ – $s_2q^2$) | + [pq($s_1$p – $s_2$q)] / (1–$s_1p^2$ – $s_2q^2$) |

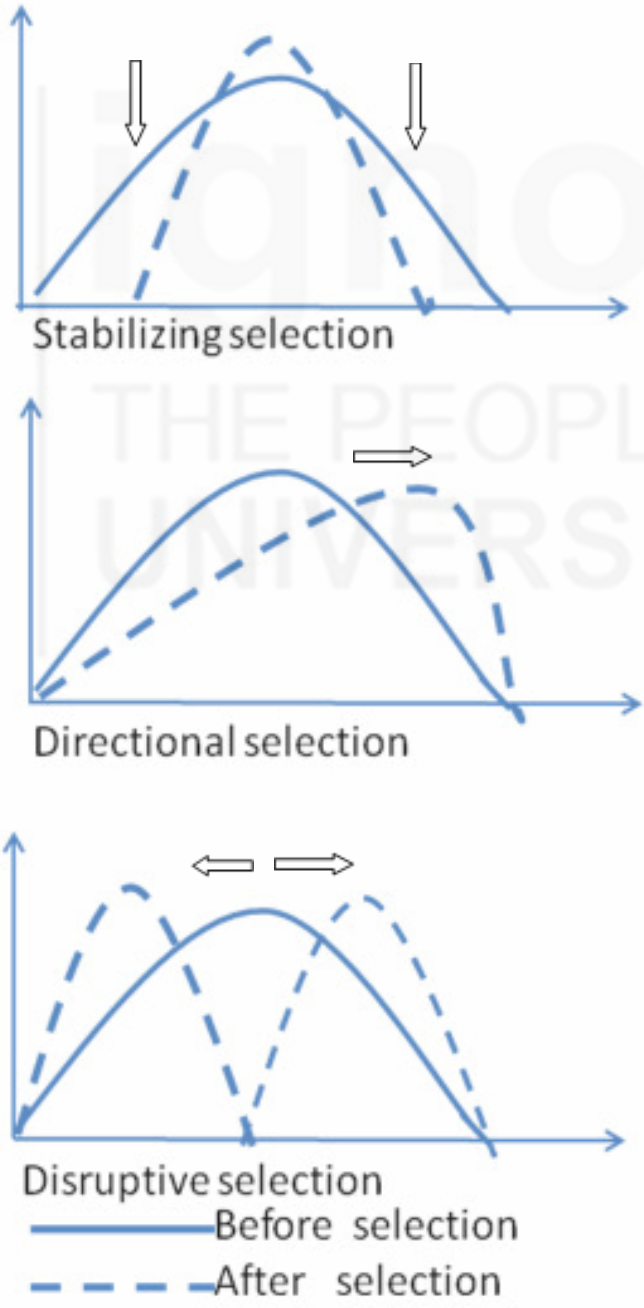
Above the different types of dominance are:

1. No dominance, selection against $A_2$ 2. Partial dominance of $A_1$: selection against $A_2$
3. Complete dominance of $A_1$: selection against $A_2$
4. Complete dominance of $A_1$: selection against $A_1$
5. Over dominance: Selection against $A_1A_1$ and $A_2A_2$ (Applicable to any degree of dominance with fitnesses expressed relative to $A_1A_2$)

***Stabilizing selection***: The extremes are selected in favour of the middle. In case of stabilizing selection, the two extreme values of a trait or alleles will have lower fitness than the intermediate value or the heterozygote alleles of a trait. One of the well known examples includes birth weight. The average birth weight of offspring ranges between 2500g (5.5pounds) to 4500g (10 pounds). Offspring with weight less than 2500g are low birth weight and greater than 4500g are the heavy babies and both have less chance of survival. As a result the selection favours the offspring with the average birth weight. Stabilizing selection is also the reason in case of height distribution in a population. This can be illustrated as a change in mean values of the distribution (See Box 2.12)

**BOX 2.12**

**Different types of selection. Change in gene frequency in case of Stabilizing, Directional and disruptive (balancing) selection**



***Balanced Selection***: In case of balanced selection, the heterozygotes have higher fitness than either of the homozygotes. This is also called heterozygous advantage or over-dominance. The best example is the sickle cell anaemia. In non-malarial environment the homozygote state of the sickle cell anaemia will have low fitness and as a result the allele gets lost in the population in due course of time. However, in malarial environment, Homozygote sickle cell anaemic individuals have the better fitness as equal to the normal homozygote individuals; as such both the alleles will be maintained in the population. (See Box 2.12).

***Disruptive selection***: both the extreme value (alleles) of a trait gets selected. It is one form of balanced selection. In case of disruptive selection, the extreme values or the alleles (low and high) of a trait will have a higher fitness when compared to the average value. As a result of disruptive selection the extreme values will increase as against the average values of the trait. This can be explained as leading to bimodal distribution (See Box 2.12).

b) **Opportunity for natural selection**

In general, to investigate natural selection in human populations is complex. Since natural selection operates on fertility and mortality, it can help us to get an overall idea of operation of natural selection. Indeed, Crow (1958) has formulated an index (Crow's Index) to examine the maximum intensity of (natural) selection that is more applicable for human populations; the index is based on the demographic components of fertility and mortality rates. According to Crow, "*there can be selection only if, through differential survival and fertility, individuals of one generation are differentially represented by progeny in succeeding generations.The extent to which this occurs is a measure of* ***'total selection intensity'***. *It sets an upper limit on the amount of genetically effective selection.*"

The total selection intensity (as defined by Crow) has two components: A fertility component ($I_f$) and mortality ($I_m$). The fertility and mortality patterns depend on several factors that vary across populations such as age at marriage, menarche, and survival to reach to fertility age, variation in fertility and age of death etc. Likelihood of these occurring needs to be calculated based on age-sex structure.

The fertility and mortality also include embryonic development and birth; these have been incorporated to make it more rigorous and efficient estimate by Johnston and Kensinger (1971). More details of the Crow Index and the relationship are given in Box 2.13.

The estimates of 'total intensity of selection' have been studied in wide diverse populations. In Indian scenario, tribal populations show larger 'Index of mortality than fertility components. There is also an overall declines in $I_m$ and $I_t$ among urban communities as a result of socio-economic and public health facilities. More details of the trends of the Crow's Index in Indian populations are described by Gautam (2009).

**BOX 2.13**

**Index of opportunity for selection**

**Crow (1958) and Johnston & Kensinger (1971)**

The total selection intensity ($I_t$), is computed based on

*I*m = index of opportunity for natural selection due to pre-reproductive mortality (mortality from birth to reproductive age, i.e. below 15 years).

*I*f = index of opportunity for natural selection due to fertility.

*X* = average number of live births per women who have completed their reproductive life span (aged 45 years and above).

*V*f = variance (average deviation from mean) of number of live births.

*P*d = proportion of pre-reproductive deaths.

*P*s = proportion of survivors from birth to reproductive ages.

The proportion of pre-reproductive deaths (*P*d) is calculated from children ever-born to mothers aged 45 years and above (who have completed their fertility) and pre-reproductive deaths.

The proportions of survivors were calculated by subtracting *P*d from 1:

$$I_t = I_m + I_f/P_s$$

$$I_m = P_d/P_s \;\&\; P_s = 1/P_d$$

$$I_f = V_f/X^2$$

The crow's Index of opportunity for selection was modified by Johnston and Kensinger (1971) to account for the survival and mortality component during conception, before the birth of an infant.

This include Ime = the selection due to prenatal mortality, Ped = the probability to die before birth,

Pb = the probability to survive till birth, Imc the index of total selection due to postnatal mortality,

Pd = the probability to die before reaching reproductive age,

Ps = the likelihood to survive til reproductive age, If = selection due to fertility,

V = varience due to fertility among women who had completed their fertility,

X is the mean number of births, Pd and Ps are proportion of deaths and survivors.

The modified total intensity index $I_t$ is:

$$I_t = I_{me} + (I_{mc}/P_b) + (I_f/P_b)\, P_s$$

$$I_{me} = P_{ed}/P_b,\; P_b = 1 - P_{ed}$$

$$I_{mc} = P_d/P_s$$

$$P_s = (1 - P_d)$$

$$I_f = V/X^2$$

## 2.4.1.4 Gene Flow

### a) Migration

Migration or gene flow is an important factor that can change the gene frequency. Emigration or immigration of individuals between populations can alter or change in the gene frequency. In genetic terms it is either loss of genetic diversity due to emigration or increase of genetic diversity due to immigration of individuals. There is loss of gene flow from a gene pool or gain of gene flow into a subpopulation from other gene pool. The quantitative estimate of the effect of migration in case of an allele at a single locus has been estimated by Bernstein and it has been shown (Box 2.14).

**BOX 2.14**

**Change in gene frequency due to migration (m) / gene flow or genetic admixture**

Suppose if migration is unidirectional from mainland to a nearby island and is random, then suppose

'm' is the rate of migration per generation from mainland to island

a) Pi be the frequency of gene A in immigrating individuals

'$p_0$' is the frequency of gene A in the island

b) The gene freq of A in the ***island after migration*** is

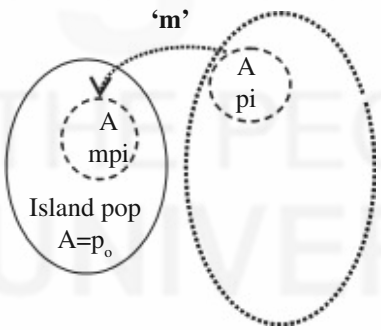
$$p_{am} = mpi + ( 1 - m ) p_0$$

The change in gene freq in one generation is

$$p_{am} - p_0 = [ mpi + (1-m) p_0] - p_0$$

$$= m (pi - p_0) + p_0 - p_0$$

$$m = (p_{am} - p_0) / (pi - p_0)$$




**NB:** This is based on Bernstein's formula for an allele at a single locus

The effect of migration rate (m) on allele frequency in a population is the proportion of differences of allele frequency in the island population ($p_i$) before and after migration ($p_{am}$) to the difference between allele frequency in the migrant population ($p_0$) and the island population ($p_i$). The above formula can be extended for a multiple loci by using least square or maximum likelihood estimate procedures. It can also be worked out based on gene identity method.

### b) Genetic admixture

Gene flow can happen between two subpopulations through random mating or admixture or marriages. The American Blacks, Anglo Indians, are examples of genetic admixed populations. The Latin American countries are populated by admixed populations contributed by native tribes, African, European and other settlers. The estimates of admixture proportion can be estimated for a gene located at a specific locus of interest or for a set of genetic markers located at different loci. The above formula (Box 2.14) can be used to estimate the 'm' the admixture

in a hybrid population. It is also possible to estimate the admixture proportions based on genetic distances and from principal component analysis (for multilocus allele frequencies).

### c) Barriers to gene flow

Human populations live over wide geographical regions forming local subpopulations; these subpopulations are formed as a result of endogamy which is promoted by geographical, cultural, linguistic, political and other factors. The same factors form barriers for gene flow and restrict the admixture, intermarriages etc. between the local populations. In India caste, geographical isolation, cultural, linguistic, political factors play a major role in restricting the gene flow or admixture or intermarriages between groups.

### d) Theoretical Models of gene flow

These factors are important to consider estimating or modelling the gene flow between populations. In population genetic point of view, there is a decrease in genetic diversity with the increasing distance or geographical location of the populations. This gives spatial pattern of gene frequency clines, which help us to understand the geographic variation of genetic markers across populations and regions.

Since gene flow can occur in different scenarios, there are a variety of theoretical models to account for different situations of spatial gene exchange or flow. For example, Sewall Wright has proposed 'island', 'neighbourhood' and 'isolation by distance' models and 'steppingstone' model by Kimura and Weiss.

**Island model*:*** It is the simple situation similar to island population. Suppose the population is distributed among a few close (equi distance) islands, each of population size N. The people tend to marry within each of the islands and gene flow is restricted, in the sense that there is equal immigration between islands, hence 'island model'. Suppose the mating takes place at random in each of such island or insular populations. The gene frequency in each of the island will differ with respect to total population (of all the islands). The theoretical results show that the deviation in such island model is exactly the variance in allele frequency among the islands. The number of homozygotes in the total population is always larger than expected from HW proportions in that population. The result is known as '*Wahlund principle'*. For a two-allele polymorphism, the genotypic proportions in the total population are:

$$AA : p^2_0 + V, \quad Aa : 2p_0q_0 - 2V \quad \text{and} \quad aa: q^2_0 + V.$$

These proportions are similar to those population practicing inbreeding with inbreeding coefficient 'F' ($F = V/p_0q_0$). Where $P_0$ is the gene frequency of allele A and V is the variance of the gene frequency among the islands. "*The change in heterozygote frequency is twice the covariance among populations in the frequency of the allele in the heterozygote, and this may be positive or negative*." (Christiasen and Feldman, 1986). One other model proposed by Sewall Wright is Neighbourhood model**.**

***Steppingstone model:*** The island model is too realistic to realise, therefore other models have been proposed which is more close to geographically structured populations. Kimura and Weiss (1964) proposed the '*stepping stone model'*. In

'one-step-linear (one dimensional) stepping stone model, the populations are arranged, rather in a linear fashion, on a long chain. The migration occurs between the neighbouring populations. This situation allows the distant populations with least migration between them are expected to behave differently than the neighbouring populations that are expected to change the gene frequency of the extreme populations as against the neighbouring populations. Kimura and Weiss (1964) have shown that the correlation in gene frequencies ( $r$ ) between demes decreases approximately exponentially as a function of the number of steps ( $x$ ) between deems.

This is expected to lead to clines in the gene frequency or geographical clines of the allele frequency.

***Isolation by distance model:*** This was proposed by Sewall Wright, which is in a similar to the stepping stone model in a continuously distributed population.

### 2.4.1.5 Genetic Equilibrium

The evolutionary forces of mutation, selection, and drift may oppose each other to create a dynamic equilibrium in which allele frequencies no longer change.

In a randomly mating population without selection or drift to change allele frequencies, and without migration or mutation to introduce new alleles, the Hardy-Weinberg genotype frequencies persist indefinitely. Such an idealized population is in a state of genetic equilibrium. In reality, the situation is much more complicated; selection and drift, migration and mutation are almost at work changing the population's genetic composition. However, these evolutionary forces may act in contrary ways to create a dynamic equilibrium in which there is no net change in allele frequencies. This type of equilibrium differs fundamentally from the equilibrium of the ideal Hardy-Weinberg population. In a dynamic equilibrium, the population simultaneously tends to change in opposite directions, but these opposing tendencies cancel each other and bring the population to a point of balance. In the ideal Hardy-Weinberg equilibrium, the population does not change because there are no evolutionary forces at work. However, opposing evolutionary forces can create a dynamic equilibrium within a population.

**Box 2.15**

**Calculating Equilibrium Allele Frequencies with Balancing Selection**

| | | | |
|---|---|---|---|
| Genotypes: | *AA* | *Aa* | *aa* |
| Relative fitnesses: | $1 - s$ | 1 | $1 - t$ |
| Frequencies: | $P^2$ | $2pq$ | $q^2$ |
| Average-relative fitness: | $\bar{w} = P^2 x (1–s) + 2pq x 1 + q^2 x (1–t)$ | | |
| Frequency of A in the next generation after selection: | $P^1 = [P^2 (1 – s) + (1/2) 2pq] / \bar{w} = p(1–sp) / \bar{w}$ | | |
| Change in frequency of A due to selection: | $\Delta p = P^1 - p = pq(tq-sp) / \bar{w}$ | | |
| At equilibrium, $p\Delta = 0$ | $P = t / (s + t)$ and $q = s / (s + t)$ | | |

## Balancing Selection

One type of dynamic equilibrium arises when selection favors the heterozygotes at the expense of each type of homozygote in the population. In this situation, called *balancing selection* or *heterozygote advantage*, one can assign the relative fitness of the heterozygotes to be 1 and the relative fitness of the two types of homozygotes to be less than 1:

| Genotype: | *AA* | *Aa* | *Aa* |
|---|---|---|---|
| Relative fitness | 1– *s* | 1 | 1– *t* |

In this formulation, the terms 1 – *s* and 1-*t* contain selection coefficients that are assumed to lie between 0 and 1. Thus, each of the homozygotes has a lower fitness than the heterozygotes. The superiority of the heterozygotes is sometimes referred to as '*overdominance'*.

In cases of heterozygote advantage, selection tends to eliminate both the *A* and '*a'* alleles through its effects on the homozygotes, but it also preserves these alleles through its effects on the heterozygotes. At some point these opposing tendencies balance each other, and a dynamic equilibrium is established. To determine the frequencies of the two alleles at the point of equilibrium, one must derive an equation that describes the process of selection, and then solve this equation for the allele frequencies when the opposing selective forces are in balance that is, when the allele frequencies are no longer changing (Box 2.15).

At the balance point, the frequency of *A* is $p = t/(s + t)$,

and the frequency of *a* is $q = s/(s + t)$

As an example, let's suppose that the *AA* homozygotes are lethal ($s = 1$) and that the aa homozygotes are 50 percent as fit as the heterozygotes $(t = 0.5)$. Under these assumptions, the population will establish a dynamic equilibrium when $p = 0.5/(0.5 + 1) = 1/3$ and $q = 1/(0.5 + 1) = 2/3$.

Both alleles will be maintained at appreciable frequencies by selection in favour of the heterozygotes – a condition known as a balanced polymorphism.

In humans, the disease sickle-cell anaemia is associated with a balanced polymorphism. Individuals with this disease are homozygous for a mutant allele of the â-globin gene, denoted $Hb^s$, and they suffer from a severe form of anaemia in which the haemoglobin molecules crystallize in the blood. This crystallization causes the red blood cells to assume a characteristic sickle shape. Because sickle-cell anaemia is usually fatal without medical treatment, the fitness of $Hb^s$ $Hb^s$ homozygotes has historically been 0. However, in some parts of the world, particularly in tropical Africa, the frequency of the H*b*$^s$ allele is as high as 0.2. With such harmful effects, why does the H*b*$^s$ allele remain in the population at all?

The answer is that there is moderate selection against homozygotes that carry the wild-type allele H*b*$^A$. These homozygotes that carry the wild-type allele H*b*$^A$. These homozygotes are less fit than the $Hb^s$ $Hb^A$ heterozygotes because they are more susceptible to infection by the parasites that cause malaria, a fitness-reducing disease that is widespread in regions where the frequency of the $Hb^S$ allele is high.

We can schematize this situation by assigning relative fitness to each of the genotype of the â-globin gene:

| Genotype: | H$b^S$ H$b^S$ | H$b^S$ H$b^A$ | H$b^A$ H$b^A$ |
|---|---|---|---|
| Relative fitness: | 1 – s | 1 | 1 – t |

If one assumes that the equilibrium frequency of H$b^S$ is p = 0.1 – a typical value in West Africa – and if one notes that s = 1 because the HbS HbS homozygotes die, one can estimate the intensity of selection against the HbA HbA homozygotes because of their greater susceptibility to malaria:

$$P = t/(s + t)$$

$$0.1 = t/(1+t)$$

$$t = (0.1)/(0.9) = 0.11$$

This result tells us that the H$b^A$ H$b^A$ homozygotes are about 11 percent less fit than the H$b^S$ H$b^A$ heterozygotes. Thus, the selective inferiority of the HbS HbS and HbA HbA homozygotes compared to the heterozygotes creates a balanced polymorphism in which both alleles of the â-globin gene are maintained in the population.

Various other mutant Hb alleles are found at appreciable frequencies in tropical and subtropical regions of the world in which falciparum malaria is – or was – endemic. It is plausible that these alleles have also been maintained in human populations by balancing selection.

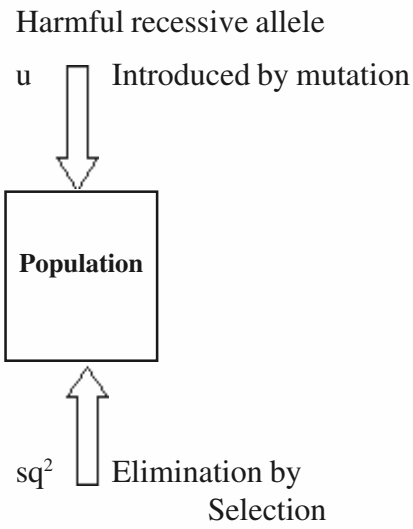
**Mutation-Selection Balance**

Another type of dynamic equilibrium is created when selection eliminates deleterious alleles that are produced by recurrent mutation. For example, let's consider the case of a deleterious recessive allele a that is produced by mutation of the wild-type allele *A* at rate *u*. A typical value for *u* is $3 \times 10^{-6}$ mutations per generation. Even though this rate is very low, over time, the mutant allele will accumulate in the population, and, because it is recessive, it can be carried in heterozygous condition without having any harmful effects. At some point, however, the mutant allele will become frequent enough for aa homozygotes to appear in the population, and these will be subject to the force of selection in proportion to their frequency and the value of the selection coefficient *s*. Selection against these homozygotes will counteract the force of mutation, which introduces the mutant allele into the population.

If one assumes that the population mates randomly, and if one denotes the frequency of *A* as *p* and that of *a* as *q*, then one can summarize the situation as follows:

| Mutation: | | | Selection: | |
|---|---|---|---|---|
| Produces a | | | eliminates a | |
| A→ a | Genotype: | AA | A*a* | *aa* |
| rate = u | Relative fitness: | 1 | 1 | 1-*s* |
| | Frequency: | $P^2$ | 2*pq* | $q^2$ |

Mutation introduces mutant alleles into the population at rate *u*, and selection eliminates them at rate $sq^2$




Mutation-selection balance for a deleterious recessive allele with frequency q. Genetic equilibrium is reached when the introduction of the allele into the population by mutation at rate u is balanced by the elimination of the allele by selection with intensity s against the recessive homozygotes.

When these two processes are in balance, a dynamic equilibrium will be established. We can calculate the frequency of the mutant allele at the equilibrium created by mutation – selection balance by equating the rate of mutation to the rate of elimination by selection:

$$u = sq^2$$

Thus, after solving for q, we obtain $q = \sqrt{u / s}$

For a mutant allele that is lethal in homozygous condition, s = 1, and the equilibrium frequency of the mutant allele is simply the square root of the mutation rate. If one uses the value for *u* that was given above, then for a recessive lethal allele the equilibrium frequency is $q = 0.0017$. If the mutant allele is not completely lethal in homozygous condition, then the equilibrium frequency will be higher than 0.0017 by a factor that depends on $1/\sqrt{s}$ . For example, if s is 0.1, then at equilibrium the frequency of this slightly deleterious allele will be q=0.0055, or 3.2 times greater than the equilibrium frequency of a recessive lethal allele.

Studies with natural population of *Drosopbila* have indicated that lethal alleles are less frequent that the preceding calculations predict. The discrepancy between the observed and predicted frequencies has been attributed to partial dominance of the mutant alleles-that is, these alleles are not completely recessive. Natural selection appears to act against deleterious alleles in heterozygous condition as well as in homozygous condition. Thus, the equilibrium frequencies of these alleles are lower than one would otherwise predict. Selection that acts against mutant alleles in homozygous or heterozygous condition are sometimes called purifying selection.

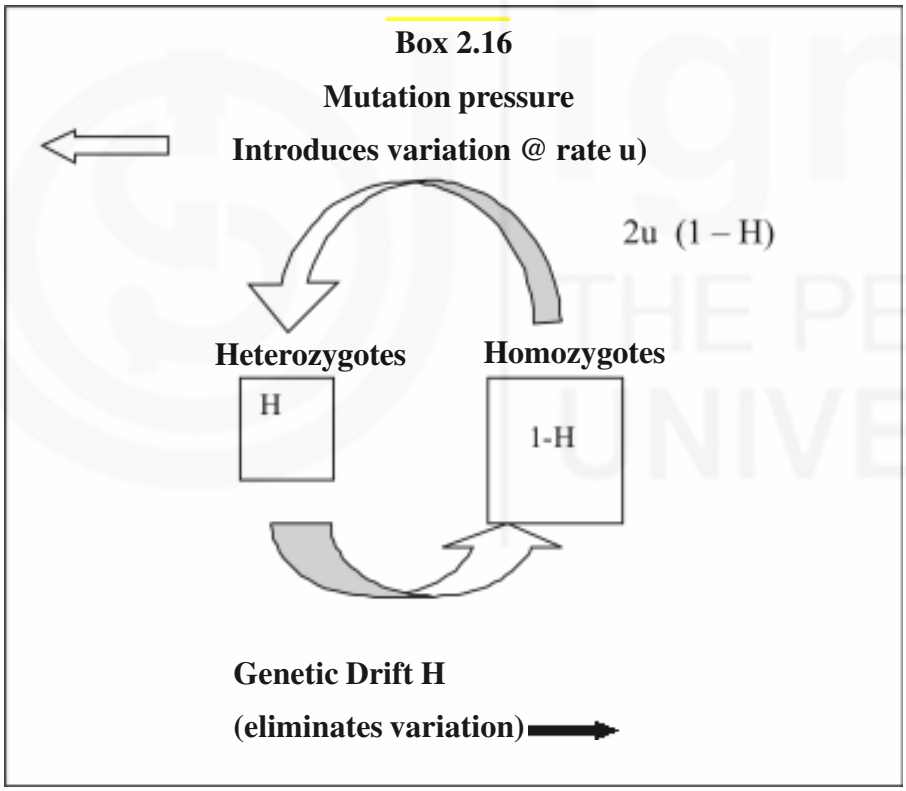
**Mutation-Drift Balance**

The random genetic drift eliminates variability from a population. Without any counteracting force, this process would eventually make all populations

completely homogeneous. However, mutation replenishes the variability that is lost by drift. At some point, the opposing forces of mutation and genetic drift come into balance and a dynamic equilibrium is established. The genetic variability can be quantified by calculating the frequency of heterozygotes in a population- a statistic called the heterozygosity, which is symbolized by the letter H. The frequency of homozygotes in a population- often called the *homozygosity*- is equal to 1-H. Over time, genetic drift decreases H and increases 1-H, and mutation does just the opposite as shown in the figure below (Box 2.16).

Let's assume that each new mutation is selectively neutral. In a randomly mating population of size N, the rate at which drift decreases H is $\frac{1}{2N}$ )H. The rate at which mutation increases H is proportional to the frequency of the homozygotes in the population (1-H) and the probability that one of the two alleles in a particular homozygote mutates to a different allele, thereby converting that homozygote into a heterozygote. This probability is simply the mutation rate μ for each of the two alleles in the homozygote; thus, the total probability of mutation converting a particular homozygote into a heterozygote is 2μ. The rate at which mutation increases H in a population is therefore equal to 2μ(1 - H).

**Box 2.16**




When the opposing forces of mutation and drift come into balance, the population will achieve an equilibrium level of variability denoted by H. This equilibrium value of H can be estimated, by equating the rate at which mutation increases H to the rate at which drift decreases it:

$$2\mu(1 - H) = \quad H$$

By solving for H, the equilibrium heterozygosity at the point of mutation-drift balance is obtained as :

$$\hat{H} = 4N\mu / (4N\mu + 1)$$

Thus, the equilibrium level of variability(as measured by the heterozygosity) is a function of the population size and the mutation rate.

If one assumes that the mutation rate is $\mu = 1\times10^{-6}$, one can plot $\hat{H}$ for different values of N. For N < 10,000, the equilibrium frequency of heterozygotes in the population will be quite low; thus, drift dominates over mutation in small populations. For N equal to 1/μ, the reciprocal of the mutation rate, the equilibrium frequency of heterozygotes would be 0.8, and for even greater values of N, the frequency of heterozygotes increases asymptotically towards 1. Thus, in large populations, mutations dominate over drift; every mutational event creates a new allele, and each new allele contributes to the heterozygosity because the large size of the population protects the allele from being lost by random genetic drift.

Values of $\hat{H}$ in natural populations vary among species. In the African cheetah, for example, $\hat{H}$ is 1 percent or less among a sample of loci, suggesting that over evolutionary time, population size in this species has been small. In humans, $\hat{H}$ is estimated to be about 12 percent, suggesting that evolutionary time population size has averaged about 30,000to 40,000 individuals. Estimates of population size that are derived from heterozygosity data are typically much smaller than estimates obtained from census data. The reason for this discrepancy is that the estimates based on heterozygosity data are *genetically effective* population sizes-sizes that take into account restrictions on mating and reproduction, as well as temporal fluctuations in the number of mating individuals. The genetically effective size of a population is almost less than the census size of a population.

(***Source:*** *Principles of Genetics (2006) by D. Peter Snustad and Michael J. Simmons. John Wiley & Sons (Asia Edition) PP. 750-754.*

## 2.5 SUMMARY

1) Understanding of Population genetics principles, requires the basic concepts of Mendelian genetics: the result of segregation, the concept 'gene', 'phenotype', 'genotype', 'dominant' , 'recessive' traits, 'allele' etc. Parental mating types and expected distribution of genotypes among the offspring.

2) Hardy-Weinberg equilibrium is the solution to an intriguing question: what happens to gene frequency of a dominant character over generations in a population. With three times more frequent than normal does this will increase over generations?

3) HWE law states that under the absence of intervening factors, especially in a large population, given random mating, no selection of any sort, no mutation and absence of demographic factors like migration, differential fertility and mortality etc., the allele frequency remain constant over generations. This can be proved theoretically, easily, for a 'biallelic locus and it can be extended to multilocus as well.

4) The importance of HWE: it gives a methodology to estimate the allele frequency in a population based on phenotypic/genotypic information of the parental mating types. It helps us to investigate the relationship between change in gene frequency with respect to mutation, migration, selection,

genetic drift etc. The entire investigation is the kernel of a branch of biomathematics or the new field: 'population genetics' and 'quantitative genetics'.

7) HWE is the bench mark of qualitative test to check whether a trait, an allele, SNP, is in equilibrium. It tells how to distinguish between the effects of evolutionary forces from the demographic factors.

8) Mutation is a non-systematic and random, but rate of mutation is site specific. Mutations are more frequent at hot-spots and are rare at the 'conserved region'. The mitochondrial non-coding genome has a higher frequency of mutations than the nuclear genome.

9) Genetic drift is a non-systematic force which can lead to significant changes in gene frequency in a small population. If an allele is rare in a small population, it can get lost or get fixed in the population over generations.

10) Founder effect is one form of genetic drift. The founders are a sample (represent a fraction of the genetic diversity) of original populations. The descendents of a few founders have the gene frequency that is dependent on the genetic composition and genetic structure of the founders. It can also happen as bottleneck effect, especially as a result of sudden population size reduction in a population, due to reasons such as natural causes or man-made causes or socio-cultural regulations. There could be serial founder effect as a result of waves of migration at different times. The mitochondrial investigation of human origins suggests that the human origins and migration to other continents appears as a result of serial founder effect from Africa.

11) Natural selection is one of the complex systematic forces that can influence significant changes in gene frequency. Selection can operate in multitude ways and it is a slow process than to the effect of migration or admixture etc.

12) Selection basically operates at differential fertility and mortality levels. It is measured as 'fitness' the ability to leave offspring and refers to 'relative rate of survival'. It is measured by 'selection coefficient' ('s') which is a function of fitness (W). The fitness or selection coefficient differs with respect to the type of dominance: complete, partial, over etc.

13) The effect of 'directional selection' to shift the mean allele frequency towards its extremes. Or it could be stabilizing selection that shifts the allele frequency of extreme alleles as a result the heterozygote frequency will increase. Or it could be disruptive selection where the extreme allele frequency increases as against the heterozygote frequency.

14) Selection can also be measured based on demographic factors of fertility and mortality trends. Crow's Index of opportunity for selection measures total selection intensity that a population can experience which depend on two components, fertility and mortality.

15) Gene flow (migration/admixture) is a systematic factor which can bring rapid changes in gene frequency within a short period. In general, human populations follow a variety of restrictions or regulations that restrict gene

flow between and within populations. The barriers for gene flow could be because of culture or due to geographical, political, religious and linguistic etc.

16) There are theoretical models to investigate the effect of spatial gene flow or population structure between populations. Island model, stepping stone model, neighbourhood model help us to investigate the spatial gene flow in different situations of population structure.

**Suggested Reading**

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**Sample Questions**

1) A total of 120 individuals were tested for M, N blood group and the observed genotype frequencies of MM, MN and NN are 34, 62 and 24 respectively. Calculate the gene (allele) frequencies?

2) If 'ì' is the mutation rate ('ì' = $10^{-5}$) per generation for a gene frequency of A then how many generations are required to reduce the gene frequency by a factor of ½.

3) What is Hardy-Weinberg equilibrium? Explain why HWE is important in genetic of populations?

4) In case in a population the observed gene frequencies of a particular bi-allelic locus are in HW equilibrium for the locus, does this imply the population satisfies the assumptions of the HW equilibrium? Explain?

5) What is genetic drift and how it operates in populations? Explain with Examples.

# UNIT 3 GENETIC POLYMORPHISM

**Contents**



**Learning Objectives**

After reading this unit, you will be able to:

- define the concept of genetic polymorphism;
- explain genetic polymorphism with respect to serological, biochemical and molecular markers;
- explain the genetic markers in disease association; and
- discuss the use of polymorphic markers in population and forensic studies.

## 3.1 INTRODUCTION

Genetic polymorphism can be defined as the occurrence together in the same population two or more than two alleles such that the frequency of rare allele is always >1%, and is maintained in the population not merely by the recurrent mutation. Polymorphism can be in a coding region (coding region means the portion of DNA which code for a gene, it may be synonymous or non-synonymous) or more commonly, in the noncoding regions (which does not code for functional region), often vary by ethnicity. Basic information about the types, frequencies and distribution of common polymorphisms are essential not only for the understanding of pathological entities, but also to know our evolutionary past and provide guidance about our biological future. The most common polymorphism in our genome are single base pair sequence variation i.e. SNP

but other types like copy number changes, insertions, deletions, duplications and rearrangements also occur. The methods to asses this diversity is variable. Few examples of polymorphic markers are listed in table 3.1.

**Table 3.1: Example of Genetic polymorphisms**

| Type of marker | Year | No. of loci | Properties |
|---|---|---|---|
| Blood groups | 1910-1960 | ~20 | May need fresh blood, rare antisera. Genotype cannot always be inferred from phenotype because of dominance. No easy physical localization. |
| Electrophoretic mobility variants of serum proteins | 1960-1975 | ~30 | May need fresh serum, specialized assays, no easy physical localization often limited polymorphisms |
| Human Leucocyte Antigens (HLA) | 1970 | 1 (multi locus haplotype) | One linked set highly informative. Can only test for linkage to 6p21.3 |
| DNA RFLPs | 1975 | >105 (potentially) | Two allele markers, maximum heterozygosity 0.5,initially required Southern blotting, now PCR. Easy physical localization |
| DNA VNTRs (minisatellites) | 1985- | >104 (potentially) | Many alleles, highly informative can be typed by southern blotting easy physical localization. Tend to cluster near ends of chromosomes. |
| DNA VNTRs (microsatellites) (di-,tri-, and tetranucleotide repeats) | 1989- | 105 (potentially) | Many alleles, highly informative Can be typed by automated multiplex PCR, easy physical localization. Distributed throughout genome |
| DNA SNPs | 1998- | 106 (potentially) | Less informative than microsatellites. Can be typed on a very large scale by automated equipment, without gel electrophoresis, etc. |

## 3.2 BALANCED POLYMORPHISM

When natural selection favors heterozygotes over both homozygotes, the result is balanced polymorphism. It accounts for the persistence of an allele even though it is deleterious when homozygous. Some of the examples are given below:

### Sickle Cell Disease

It is an autosomal recessive disorder that causes anemia, joint pain, a swollen spleen, and frequent, severe infections. It illustrates balanced polymorphism because carriers are resistant to malaria, an infection by the parasite *Plasmodium falciparum* that causes cycles of chills and fever. The parasite spends the first stage of its life cycle in the salivary glands of the mosquito *Anopheles gambiae.* When an infected mosquito bites a human, the malaria parasite enters the red

blood cells, which transport it to the liver. The red blood cells burst, releasing the parasite throughout the body.

It is known since long that malaria is a quite common in the tropical regions of Africa. Sickle shape red blood cells provide selective advantage as malarial parasite cannot grow in these cells. Therefore, along with malaria the sickle cell anemia also increased in these parts of Africa. The sickle cell disease is less common in Caucasians due to the less frequency of malaria. This shows the heterozygous advantage of sickle cell as it provides protective effect.

The rise of sickle cell disease goes hand in hand with the cultural development with the advent of cultivation of crops gave a breeding ground to *Anopheles* mosquitoes as the malaria rose the selective pressures gave rise to the change in the shape of the RBCs from elliptical to sickle shaped and when it occurred in homozygous condition the disease was caused otherwise it had selective advantage. The spread of sickle cell disease is associated to the migratory events. Africa by people migrating from Southern Arabia and India, or it may have arisen by mutation directly in East Africa.

Settlements with large numbers of sickle cell carriers escaped devastating malaria. They were therefore strong enough to clear even more land to grow food-and support the disease-bearing mosquitoes. Even today, sickle cell disease is more prevalent in agricultural societies than among people who hunt and gather their food.

**G6PD Deficiency**

It is a sex-linked enzyme deficiency. It affects 400 million people throughout the world. It results into hemolytic anemia which is life-threatening. It is under the influence of certain environmental conditions like eating fava beans, inhaling certain types of pollen, taking certain drugs, or catching certain infections. It has been seen in Africa that hemizygous males and heterozygous males for this enzyme deficiency are at less risk for malaria again revealing a selective advantage for heterozygotes. Therefore, natural selection acts in two directions hence it could be one of the example of balanced polymorphism.

## 3.3 TRANSIENT AND BALANCED POLYMORPHISMS

Polymorphism occurs when two or more clearly different phenotypes exist in the same population of a species-in other words, the occurrence of *more than one form* or *morph*.

A transient polymorphism is one that is changing in frequency over time. In transient polymorphism, one form is gradually being replaced by another. As the name implies, it represents a temporary situation as a by-product of directional natural selection.

The phenomenon of industrial melanism occurs in a number of moth species in Europe and the United States. The British ecological geneticist, E. B.Ford, first called attention to this phenomenon as a way of demonstrating the effect of natural selection in nature (as opposed to artificial selection experiments which have long enjoyed success in the lab). Ford noted that a light colored moth species,

*Biston betularia*, occasionally undergoes mutation at a single locus to produce a dark or melanic individual. Since the mutant allele is dominant, any gamete containing this mutant will produce a melanic individual upon syngamy. The first melanic specimen in this species was found in a collection from Manchester, England dated 1848, but by 1895 about 95% of all collected specimens were dark morphs, referred to as the form *carbonaria.* In a series of 12 observations and mark-recapture experiments during the 1950s, H. B. D. Kettlewell demonstrated that the two forms (light and dark) were differentially preyed upon by birds. He found that the birds selectively caught and ate more individuals of the form that did not match its background as compared to the one that was masked. In industrialized areas of England where the substrate (walls and tree trunks) upon which the moths rested were darkened by pollutants in the smoke poured out by factories, the *carbonaria* form possessed a selective advantage. Rural areas, unaffected by pollutants, afforded the light form an adaptive advantage. The environmental change brought on by the industrial revolution did not produce the *carbonaria* form (which presumably appeared from time to time due to recurrent mutation); it only protected the dark moths from bird predation (the agent of natural selection). The fact that the light form still exists in rare numbers in industrialized areas testifies to the amount of time selection requires to eliminate a recessive allele.

**Mendilian Population**

A population is a group of individuals who share a common gene pool where the characters are transmitted in a Mendelian fashion from one generation to the next generation. A group of individuals within which marriages are performed is called a Mendelian population. In a given Mendelian population, which is under Hardy-Weinberg equilibrium, the resultant genotype and phenotype frequencies are more or less permanently established.

## 3.4 SEROLOGICAL MARKERS

Blood groups are the best cited examples of serological markers. Both ABO and Rh are quite important serological markers as they can be used to study population diversity. These blood groups cause newborn hemolytic diseases. They also have a role in blood transfusion and also solid organ transplantation. They follow mendalian inheritance. ABO blood groups were discovered by Landsteiner in 1900 and are cited as a best example of triallelic inheritance. Blood groups can be tested by using antisera and red blood cells using simple agglutination techniques. Presently more advanced molecular techniques are also used.

The ranges of phenotypes in humans are a direct result of genetic variations which act together with environmental and behavioral factors to produce diversity. The identification of gene polymorphisms, which control the blood group antigen expression, contributes to the understanding of the biological significance of blood group systems. In addition to assisting in the characterization of allelic variations, the identification of gene polymorphisms allows us to estimate the processes involved in the formation of different populations (the founder effect, genetic drift, migration, etc.). Thus, blood group gene polymorphisms are valuable predictors of genomic ethnic ancestry.

## 3.5 BIOCHEMICAL POLYMORPHISMS

There is marked difference between individuals on the basis of biochemical markers like G6PD, human enzymes and proteins etc. This has been explained in the above section. However, here we would like to throw some light on the molecular basis of G6PD variants.

### Molecular Basis of G6PD Variants

The G6PD gene, located on chromosome Xq28 region, is 18 Kb long consisting of 13 exons transcribed to a 2.269 Kb messenger RNA with 1.545 Kb of coding regions. The commonest variant in South China, G6PD Canton, has been sequenced and was found to be due to a mutation at nucleotide (nt) position 1376 of cDNA, G to T, resulting in a missense mutation in amino acid position 459, Arg to Leu. With improved DNA technology, the whole cDNA sequence can be amplified and screened for mutation directly. PCR technique and restriction analysis has been used.

### World Incidence and Distribution of G6PD Deficiency

G6PD deficiency in male subjects can be detected easily by a number of screening tests. The simplest one is the fluorescent spot test developed by Beutler and Mitchell which relied on the fluorescence of NADPH, generated by an adequate amount of G6PD enzyme. This test can also be done on blood sample dried on filter paper similar to the Guthrie cards. In Hong Kong, the routine screening of newborns have included test for G6PD deficiency.

## 3.6 MOLECULAR MARKERS

Although ~99% DNA is known to be similar between individuals but still sequence differences exist between individuals in non-coding regions of the genome and such polymorphic regions are useful for various kinds of analyses in population genetic studies. A genetic marker can be a nucleotide sequence of variable length, varying from a single base pair to several hundred base pairs. Selection of markers for any study is dictated by the nature and purpose of the study. The more commonly used markers in population genetics studies can broadly be grouped as follows:

### 3.6.1 Repetitive DNA Sequence Variants

#### Tandem Repeats

Besides the interspersed repeats (SINEs and LINES), Tandem repeats are the other kind of repeated elements found in the genome. These are highly variable tandemly repeated arrays of 2 or more base pair core units in the non-coding regions of the genome and are located adjacent to each other. On the basis of size of the core unit, they are categorised into minisatellites (10-60 bp[1] ), Short Tandem Repeats (STRs) or microsatellites (<10 bp). When the number of nucleotides in the core unit is not known or is variable then it is called Variable Number Tandem Repeats (VNTRs).

#### Insertion/ Deletion Polymorphisms

An InDel or Insertion-Deletion polymorphism refers to insertion or deletion of a DNA sequence of variable length in the genome. The concerned DNA sequence

may vary in length from a single nucleotide to several hundred nucleotides. They are widely spread across the genome and constitute around 1.5 million of more than 10 million polymorphisms known in humans.

***Alu* InDels –** *Alu* Insertion/ Deletion polymorphisms (*Alu* InDels) involve *Alu* sequences that are characterized by the cleavage action of *Alu*I restriction endonuclease.

Properties of Alu sequences such as their known ancestral state, identity by descent, wide occurrence and stability make them ideal markers for human evolutionary and diversity studies.

## 3.6.2 Non- Repetitive DNA Sequence Variants

### Single Nucleotide Polymorphisms (SNPs or *Snips*)

SNP or Single Nucleotide Polymorphism is a single nucleotide (base pair) change in a DNA sequence. As with all polymorphisms, for an alteration to be considered a snip it must be present in ≥ 1% of the population being considered. They make up about 90% of all the human gene sequence variation. SNPs may be present in coding regions (exons) or non-coding regions (introns) or intergenic regions.

**Restriction Fragment Length Polymorphisms (RFLPs)** are the characteristic pattern of fragments of DNA produced when a DNA sequence is cleaved by specific enzymes belonging to endonuclease class of enzymes. The property of these enzymes that enables them to cleave DNA segment only at specific locations known as restriction sites have led to their use in detecting genetic differences on the basis of absence or presence of restriction sites.

## 3.6.3 Lineage Markers

### Mitochondrial Markers

Maternally inherited mitochondrial genome consists of multiple copies of circular mitochondrial DNA or mtDNA. Markers present on this haploid genome are primarily used for tracing maternal ancestral lineage(s) in populations because of their uniparental inheritance.

### Y-chromosomal Markers

Like mtDNA, Y- chromosome has a uniparental inheritance but in the male line and can thus be used for tracing paternal ancestral lineages. In absence of recombination, Y-chromosome is more or less transmitted unchanged from one generation to next and the few changes that may occur usually do not have any effect as around 98% of the DNA is in non-coding region.

### Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium predicts that in the absence of evolutionary forces, both allelic and genotypic frequencies remain constant in a population and that if the equilibrium is disturbed a new equilibrium will be reached within one generation based on the allelic frequencies of the remaining population. The conditions that must be met for the predictions of the Hardy-Weinberg equilibrium to be valid are described below:

1) Random mating: Mating patterns must randomly reflect the entire breeding population, with no dependence on genotype or closeness of relationship (either positive or negative).

2) No sex bias in allelic frequencies: The distribution of alleles must be the same in both sexes.

3) All genotypes equally viable and fertile: There must not be any selective advantages or disadvantages. This is seldom true in a real population, and often must be taken into account in terms of evolutionary pressures.

4) Mutation rate too low to alter ratios: The basic assumption is that alleles are stable through many generations and are not altered or degraded significantly by mutation. In practice this is generally not a serious problem.

5) Closed population (no in or out migration): The "population" that is being considered must be a constant one. Introduction of new genes into the breeding pool or loss of genes from the breeding pool by migration between "populations" can distort trends.

6) Population must be large: The population must be large enough so that there are no confounding effects due to genetic drift (random events altering allelic frequencies by pure chance) or due to "founder" effects, where a recessive gene becomes fixed in a population because too many of its members are descendants of a single individual.

The Hardy-Weinberg law can also be applied to multiple alleles and X-linked alleles. The genotypic frequencies expected under Hardy-Weinberg equilibrium will differ according to the situation.

## 3.7 TOOLS FOR STUDYING POLYMORPHISMS

Both conventional and advanced techniques are used to study polymorphisms. Conventional techniques are blood groups by carrying out simple agglutination techniques or protein electrophoresis for studying the protein polymorphisms etc. Under advanced techniques are the tools for studying molecular markers, and the foremost requirement for carrying out molecular analysis of any kind is the availability of the genetic material. As mentioned earlier, DNA is the focal point of human diversity and disease-association studies by virtue of the fact that it is the blueprint of our existence. There are several techniques for isolating DNA such as manual methods (like Phenol Chloroform, Salting-out) and kits. The technique of DNA isolation or extraction varies depending on the starting material, but, it is the technique of PCR which is the most useful for DNA analysis.

**Polymerase Chain Reaction (PCR)**: It involves cycling of DNA sample through a series of heating and cooling cycles with the required raw materials and enzymes to achieve its exponential amplification. The technique has come a long way since its invention. Instead of having to manually maintain the heating and cooling cycles, automated thermal cyclers are now available; and instead of having to add fresh polymerase (earlier derived from *E. coli*) after every cycle because of its denaturation due to heating, thermally stable DNA polymerases such as *Taq* DNA Polymerase are now made use of.

Amplification of DNA by PCR has found applications in a variety of fields ranging from forensics to archaeology; study of variation and evolution to mutation detection; gene mapping and cloning and DNA sequencing to epidemiology among several others.

**Restriction Digestion:** It is the method of cutting DNA sequences into fragments using restriction endonucleases or enzymes that cut at specific recognition sites. This generates DNA fragments of varying lengths producing a variation pattern known as Restriction Fragment Length Polymorphisms (RFLPs). The variation may be produced in response to absence or presence of particular SNP(s) or an insertion or deletion event in that region and is recognised in the form of banding pattern. Resulting fragments are separated according to molecular size using gel electrophoresis. There are several classes of endonucleases- Type I, Type II, Type III and Type IV but the most commonly used restriction enzymes are of type II and they cleave DNA fragment at specific sites within or close to the recognition sequence. Most of these enzymes cut palindromic sequences.

The technique is useful in detection of mutations/ SNPs. It is also used to detect VNTRs. The technique has been widely used for constructing physical maps of the genome, genetic linkage maps; in forensic testing; and in epidemiological and evolutionary studies.

**Electrophoresis:** It is one of the few techniques that has been in use since the beginning of study of classical genetic markers and is still in use for molecular markers. It is the method of separating macromolecules (both proteins and nucleic acids) on the basis of size, electric charge or other physical properties under the influence of electric field.

**Sequencing**: DNA sequencing refers to establishing the exact sequential arrangement of bases in a stretch of DNA. Knowledge of exact sequence of bases in a gene is crucial especially in ascertaining the function of genes. This is also important as the disease-causing alterations in the genes can then be identified.

The selection of technique and markers depends upon the purpose of study. In the following section we have discussed the uses of polymorphic markers.

Uses of polymorphisms: All the markers listed in table 3.1 can be used for population diversity studies. Now a days most extensively studied markers are Single nucleotide polymorphisms. Genomics and specially SNP research can be used to improve health care through gene therapy, to yield new targets for drug discovery, to renew the process of drug development and to discover new diagnostics.

## 3.8 GENETIC MARKERS AND DISEASE

Understanding the genetic basis of complex human diseases (like hypertension, cardiovascular disease, diabetes etc.) has been increasingly emphasized as a means of achieving insight into disease pathogenesis, with the ultimate goal of improving preventive strategies, diagnostic tools, and therapies. Genetic approaches to complex disorders thus offer great potential to improve our understanding of their pathophysiology, but they also offer significant challenges. These can be studied either using linkage analysis. In linkage analysis we use families and try to find out which polymorphic marker is near to the disease gene and then try to map the gene on the human genome. The other approach is where we study populations of both types of individuals. One would be those suffering with a disease and the other would be who are not suffering with the disease. We take different polymorphic markers and study in these two sets of samples. Then we

compare both the groups and if both the groups differ significantly at these markers we propose that these markers may be associated with the disease.

Association studies can be a very powerful approach for finding genetic determinants of a complex disorder. It has been suggested that if hundreds of thousands of single nucleotide polymorphisms (SNPs) were identified across the genome, then it would be possible to perform genome-wide association studies to identify the regions of linkage disequilibrium around disease susceptibility genes. In addition, they noted that much smaller sample sizes would be required to detect association than to detect linkage. The SNP Consortium is rapidly identifying single nucleotide polymorphisms, and within next several years, genome-wide association studies may become a reality.

These association studies can result into positive association or negative association. Some time they result into false positive or false negative results. The following general guidelines, summarized in Table 3.2, may be useful for genetic association studies. First, are the candidate gene(s) under study should be biologically reasonable. Several factors can determine the appropriateness of a candidate gene. If human genetic linkage studies have identified a chromosomal region linked to a disease, or if an animal model for a disease is influenced by a particular gene or syntenic chromosomal region, positional candidate genes in such genomic regions warrant strong consideration. In addition, the biologic plausibility of a candidate gene for involvement in disease pathogenesis is important. However, obvious limitations of this candidate approach are the large number of potential candidate genes for complex diseases and the reality that only known genes can be investigated. Although candidate genes can be selected for study on this basis, they should not be ruled out on the basis of our current understanding of disease pathophysiology- important new insights may be missed if potential candidate genes must fit into current pathophysiologic models.

**Table 3.2: Evaluation of candidate gene case-control association studies**

| Issue | Key Questions | Possible Solutions |
|---|---|---|
| Selection of candidate | Is candidate gene biologically reasonable? | Demonstration of biologically functional effect |
| Gene polymorphism | Is the candidate gene a positional candidate? | Within linked region in man or systemic from animal model |
| Population stratification | Are cases and controls matched? | Matching on ethnicity<br>Family-based association designs<br>Negative results with multiple unlinked markers |
| Hardy-Weinberg (H-W) equilibrium | Is control group in H-W equilibrium? | Calculation of H-W equilibrium with goodness-of-fit test (2 alleles) or simulation (multiple alleles) |
| Multiple comparisons | How many alleles were tested?<br>How many genetic loci were tested? | Bonferroni correction<br>Estimation of empirical P values |

A second criterion in evaluation of case-control association studies is the careful selection of cases and control subjects. Do the case subjects meet appropriate criteria for disease affection? Are control subjects free from symptoms of disease, associated intermediate phenotypes, and potential confounders? Have control subjects been exposed to relevant environmental influences involved in disease pathogenesis while remaining clearly unaffected? Were the cases and controls matched on demographic and environmental factors? Was consideration of population stratification included, either by attempting to match ethnicity or by typing unlinked markers.

A third criterion in the evaluation of case-control studies is assessment of Hardy-Weinberg equilibrium in the markers studied within the control group. Hardy-Weinberg equilibrium indicates that the genotype frequencies can be determined directly from the allele frequencies; failure to demonstrate Hardy-Weinberg equilibrium could result from genotyping errors, inbreeding, genetic drift, mutation, or population substructure. Hardy-Weinberg equilibrium can be readily assessed with a goodness-of-fit chi square test for biallelic markers; for markers with multiple alleles (such as short-tandem repeat markers), more accurate determination of Hardy-Weinberg equilibrium can be obtained with Markov Chain Monte Carlo methods. Significant deviations from the expected proportions of homozygote and heterozygote classes in a population of case subjects may be caused by association with the disease allele. Lack of consistency with Hardy-Weinberg equilibrium among control subjects should prompt investigation for potential complications, including genotyping errors and population stratification. A final criterion for evaluation of a case-control study is correction for multiple comparisons. This remains a problematic topic requiring additional statistical genetic research. However, an effort to correct for spurious associations, which can result from testing a large number of alleles, is warranted. The multiple comparison issue is especially problematic with markers that have multiple alleles like short-tandem repeat polymorphisms; the conservative Bonferroni approach to use a corrected significance value calculated by multiplication of the observed *P* value by the number of alleles tested. Bonferroni corrections for the total number of alleles at all loci are probably too conservative because the alleles at one locus are not independent of each other and closely linked loci are probably not independent either. A less conservative but more computationally intensive approach is to estimate empirical significance values using simulation approaches.

### Genome Wide Studies

Unlike the direct approach of case-control association with candidate genes, genome scanning (screening) is an indirect strategy that does not rely on conjecture. Basically, either affected individuals, usually siblings, from a number of families or families with two or more affected individuals are genotyped with polymorphic DNA markers that cover the entire chromosome complement. A set of about 400 short tandem repeat polymorphic markers that are spaced at about every 10cM is used for most genome scans. This level of resolution has been enhanced with the assembly of about 3000 simple sequence repeat polymorphic markers that are about 1.5cM apart. Single –nucleotide polymorphic sites (SNPs) are preferred for genome scans because they are uniformly distributed about every 300 bases throughout the genome and easily identified with automated equipment. Eventually, sets of SNPs will supersedes short tandem repeat polymorphic sequence marker systems.

Furthermore, major landmark attempts that have also been made to study various aspects of human genome, and few are listed below.

***Human Genome Project*** (HGP): A National Institute of Health (NIH, US) initiative started in 1990, HGP was a multinational collaborative project aimed at identifying all the genes in the human DNA and determining the sequence of about 3 billion nucleotide pairs that constitute the human DNA to understand the species' genetic makeup.

First draft was released in 2001 followed by the complete draft in 2003. Some of the main findings from the draft sequence are as follows:

- Total number of genes was estimated at 30, 000.
- The average gene was found to consist of 3000 bp but sizes vary greatly.
- Repeated sequences that do not code for proteins ("junk DNA") make up at least 50% of the human genome.
- About 1.4 million locations with SNPs were identified.

Findings from HGP are already having profound impact on diverse areas of research including molecular medicine (improved diagnosis of disease, earlier detection of genetic predispositions to disease, rational drug design etc.), bioarchaeology, anthropology, evolution and human migration, DNA forensics (identification), agriculture, livestock breeding etc.

***Human Genome Diversity Project*** (HGDP): HGDP was formally organised in 1993 under Stanford University's Morrison Institute, and was aimed at understanding the diversity patterns worldwide, the contributing factors and the implications of the observed diversity patterns. Findings from the project could also shed light on the origins and migration patterns of the entire human species. HGDP could also aid in understanding the role played by environmental factors in complex human diseases.

***HapMap Project***: The International HapMap Consortium is an international collaborative venture between Japan, the United Kingdom, Canada, China, Nigeria, and the United States aimed at developing haplotype map of the human genome in a bid to identify genetic determinants of complex diseases. The information made available through the HapMap project is helping researchers find genes that affect health, disease, and individual responses to medications and environmental factors.

***Indian Genome Variation*** (IGV): IGV was the first large scale effort to document and understand the genomic structure of enormously varied Indian populations. The study found high degree of genetic differentiation among the different ethnic groups.

### Genetic Testing and Counseling

Frequently the question may arise as to whether the patient has a certain disease for which there is a genetic basis. Often among the 10,000 conditions for which a genetic basis has been identified, the diagnosis can be made from evaluation of personal and family history, physical examination, and conventional laboratory tests. A useful database for identifying these conditions is available on Online Mendelian Inheritance in Man (OMIM) (www.ncbi.nlm.nih.gov/omim). This

catalog is updated regularly and can be searched using multiple terms. The entries provide information about the clinical signs as well as the genetic basis for the condition, if known, including mutations that have been found to cause the condition. To determine whether genetic testing is available for a given condition and to find a laboratory, a useful link is GeneTests, a free online service (www.genetests.org). The entries in this catalog indicate the test menus and contact information for the laboratories, as well as whether the testing is provided on a routine or research basis. A very useful adjunct in the GeneTests Website is GeneReviews, which provides succinct summaries about many genetic conditions and the ways the genetic testing can be used for diagnosing these conditions, including prediction of natural history.

The clinician is likely to encounter many situations in which a genetic test may be useful. Sometimes genetic testing is required from diagnosis when it cannot be made by clinical criteria alone. The fragile X syndrome is the most common genetic form of mental retardation. Although the diagnosis may be suggested by the presence of the characteristic signs—large ears, protruding chin, and large testes—the only way to diagnose fragile X is by genetic testing. For the various forms of spinocerebellar ataxia, there is considerable overlap. Yet, these can be readily distinguished by their specific mutations. Patients with atypical forms of certain diseases may have a negative gold standard test, but positive genetic test. For most patients with cystic fibrosis, the diagnosis can usually be made by a sweat chloride test. However, a number of individuals have been described with pulmonary disease suggestive of this condition for whom the sweat chloride test is normal. For these patients, the diagnosis has been based on observation of mutations in both copies of their CFTR genes.

For some conditions, the signs of disease may not yet have developed, yet on the basis of one's family history, one may want to know about the risk of developing disease. This is true for the person whose parent(s) may have died from Huntington's disease, a progressive neurodegenerative disease or for the person whose mother and sister may have died from breast or ovarian cancer, suggesting a heritable risk. For these individuals, a positive genetic test result will indicate an increased, although not necessarily absolute, risk for developing the disease.

Genetic testing is used for assessing reproductive risks—by testing the parents for carrier status and by testing the fetus. Individuals with a positive family history of genetic disease (usually autosomal recessive or X-linked) or who come from ethnic groups with an increased prevalence of autosomal recessive or X-linked diseases are candidates for carrier screening. Currently, carrier screening for cystic fibrosis, fragile X syndrome, and spinal muscular atrophy is recommended in the United States. For people of Mediterranean, African, or South Asian ancestry, hemoglobinopathy screening is recommended. For individuals of Ashkenazi Jewish ancestry, screening for Tay-Sachs disease, Canavan disease, cystic fibrosis, Gaucher disease, Bloom syndrome, Fanconi anemia, Niemann-Pick disease, familial dysau-tonomia, maple syrup urine disease, glycogen storage disease, and familial hyperinsulinism is available. An individual who is a carrier for a certain condition may choose not to marry another individual who is a carrier for the same condition. Alternatively, if a carrier couple is identified, they may choose to have prenatal diagnosis to determine whether their fetus is affected with this condition. This can be performed either at 10-11 weeks using the procedure of chorionic villus sampling where a bit of placenta is obtained under ultrasound

guidance. As another option, an amniocentesis can be performed at 15-18 weeks of pregnancy to obtain cells from the amniotic fluid. These couples might also choose to have pre- implantation genetic diagnosis with selection implantation of only those embryos that are deemed unaffected.

Not all genetic testing involves looking for heritable mutations. Sometimes it is used to look for genetic alterations that are confined to a specific population of cells. These alterations may cause certain cells to become cancerous, or if cancerous, to progress to a more aggressive stage. Genetic testing can be used to identify chromosomal translocations between two non-homologous chromosomal segments and in the process diagnose a specific form of leukemia. For example, the translocation between chromosomes 1 and 19 in leukemic cells is diagnostic of the acute promyelocytic form of this disease and the translocation between chromosomes 9 and 22 is diagnostic of the chronic myelogenous form. The expression patterns of RNA transcribed from many genes can be assessed to predict the natural history of the disease. This approach has been used to predict breast cancer outcome and whether more or less aggressive therapies should be used to treat patients.

Individuals might also have genetic tests of identity. These might be voluntary and selected to test specific questions, such as whether they are members of a known patrilineal lineage, such a people with a specific surname. These tests analyze a series of polymorphic genetic markers on the Y chromosome. On the basis of the general pattern of markers, or "haplogroup," they may be told of the geographic region where their Y chromosome originated. According to the number of markers that match with people who are suspected to be of the same lineage, individuals may be advised about the common ancestors or other people in that lineage. Such testing is also possible for matrilineal lineages by testing mitochondrial DNA markers.

## 3.9 GENETIC MAPPING OF DISEASE GENE ON HUMAN CHROMOSOME USING POLYMORPHIC MARKERS

In genetic mapping the diseased gene polymorphic markers play a very important role. These markers could be short tandem repeats, variable number of tandem repeats, blood groups, restriction fragment length polymorphism etc. Mapping can be done in a step wise manner.

Collect all the pedigrees where the disease is found. Analyse all the members against various polymorphisms and perform linkage analysis.

Linkage study entails collecting blood cells from members of several two – and three – generation families or from individuals of a large multiple generation family with a specific genetic disorder. The blood can be cultured and cell lines can be maintained large number of polymorphic markers (probes), representing sites from all parts of all autosomes, are used. A two –point (two – locus) LOD score is calculated for each polymorphic locus and the site of the genetic disease from all informative parent offspring combinations and finally the linkage is established. However, genotyping errors can give –ve or +ve LOD score. Hence perfect genotyping is must to get the correct results.

## 3.10 USE OF POLYMORPHIC MARKERS IN FORENSIC TESTING

Polymorphic markers have great utility in personal identification. As mentioned above no two individuals are alike. These differences are at both phenotypic and genotypic levels. The genetic differences can be identified by testing these markers. This testing is provided by commercial firms that market directly to consumers. Identity genetic analysis may also be involuntary and used for paternity testing of children or fetuses or for identification of forensic samples in murder, assault or rape cases, in which the perpetrator of the crime left a tissue sample of blood, semen, hair, or other tissue type from which DNA can be extracted and the test can be performed. However, it must be kept in mind that there are ethnic differences in the distribution of these markers. Hence every population should have its own genetic profile.

## 3.11 USE OF POLYMORPHIC MARKERS IN POPULATION STUDIES

**Population Diversity Studies**

Human genome varies from individual to individual and therefore no two individuals look alike. This was noted long back. Historically, individual variation was studied on the basis of conventional somatoscopic markers. However, with the advancement of technology various genetic markers were discovered and the gene frequency data for studying the evolution of human races was analyzed using these markers. Initially, the classical serological and biochemical markers have played important roles in various types of human population genetic studies. One of the problems that limited their practical utility results from the limited number of possible genotypes at each of such loci. The discovery of hyper variable DNA loci offers the opportunity to ameliorate this problem. It was later realized that comparison of gene frequencies for one or two loci are not reliable since each locus has a different geographical distribution, hence the differences observed may be because of chance factor. Only when a large number of loci are used, the genetic relationship among populations could be drawn successfully. Recent analysis based upon polymorphic markers reveal that inter and intragroup genetic variation may be of a lesser magnitude and may not be of significance if proper markers are not selected and more so if statistical tools used are not highly powerful. However, it is important to record population variation because it is helpful to know the various mechanisms involved in causing variation and it further enhances our knowledge about the molecular basis of disease susceptibility.

## 3.12 SUMMARY

It is difficult to attribute any functional significance to genetic polymorphisms. However, the non-coding sequences of the genes which are located far away from the functional region of the gene may affect the function of the gene. However, these sequences are otherwise useful in studying population diversity, disease gene mapping, forensic investigations etc. Recently after the advent of microarray genes for many complex disorders have been found by using genome wide association studies.

## Suggested Reading

Gardner, E.D, Simmons, M.J and Snustad, D.P. 2003. *Principles of Genetics,* 8th Edition, New York: John Wiley and Sons.

Simmons, S and Simmons, M.J. 2003. *Principles of Genetics,* 3rd Edition, New York: John Wiley and Sons.

## Sample Question

1) Define polymorphism with few examples.
2) What are the evolutionary forces that affect gene frequency of polymorphic markers?
3) Give some uses of polymorphic markers.
4) What is law of Hardy Weinberg?
5) What is genetic testing?
6) Describe the utility of studying molecular markers in anthropological genetics.

# MANE-001
# Human Genetics

Indira Gandhi
National Open University
School of Social Sciences

Block

# 5

## HUMAN MOLECULAR GENETICS

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## Print Production

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July, 2012



ISBN-978-81-266-6137-4



*Further information on Indira Gandhi National Open University courses may be obtained from the University's office at Maidan Garhi. New Delhi-110 068.*

Printed and published on behalf of the Indira Gandhi National Open University, New Delhi by the Director, School of Social Sciences.

Laser Typeset by : Tessa Media & Computers, C-206, A.F.E.-II, Okhla, New Delhi

Printed at :

# BLOCK 5 HUMAN MOLECULAR GENETICS

Molecular genetics studies the structure and function of genes at molecular level. Research in Molecular genetics employs both the methods of genetics and molecular biology. Application of molecular genetics includes use of molecular information to determine the patterns of descent and understanding genetic mutations that can cause certain types of diseases. Using the techniques of molecular genetics we can discover the reasons why traits are carried on and how and why some may mutate.

The core of Molecular genetics is the structure and function, variations (Polymorphisms), causes for variations in DNA sequences; inter relation between the DNA and RNA molecules, curative measures in default structure and function of genetic material. Many discoveries were made by Human genome project in the areas of human molecular genetics. The draft of Human genome sequence was published in 2001 and the finished version published in 2003. It has thrown light on cell and development, molecular phylogentics, Mendelian genetics, genetics of complex diseases and pharmaco genetics.

Application of Human molecular genetics cover three major areas of research - Molecular genetics information in anthropological and historical research useful in tracing human origins, prehistoric migrations and demographic history; the accuracy of DNA based tests to identify people in Forensic analysis; and Molecular genetics information used to identify genetic variants of multi-factorial and complex diseases, which can explain differences between human populations with respect to increased susceptibility or resistance to certain diseases.

This block consists of 3 units. Unit 1 introduces you to Molecular Genetics and the second unit describes on DNA polymorphisms and their use in variation, evolution and disease manifestation. The third unit deals with Human Genome Project.

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# UNIT 1 INTRODUCTION TO MOLECULAR GENETICS

**Contents**



**Learning Objectives**

After reading this unit, you would be able to:

- discuss what is molecular genetics and how is it useful to mankind;
- describe structure and function of nucleic acids;
- imagine how the genome is organised in humans; and
- explain how genes are expressed and the ways in which gene expression is regulated.

## 1.1 INTRODUCTION

DNA is the master molecule which carries the genetic information from one generation to the other. Study of Molecular Genetics accelerated since April 25th, 1953 when James Watson and Francis Crick proposed the structure of DNA which was published in Journal called 'Nature'. Molecular Genetics deals with the flow of genetic information and its regulation. In simple terms it can be defined as the field of biology which studies the structure and function of genes at molecular level.

## 1.2 SCOPE OF MOLECULAR GENETICS

Development of techniques like nucleic acid hybridisation, cloning, sequencing etc. brought a revolutionary change in Molecular Genetics. It is of major interest to the students of biology and medicine. Though it has a lot of significance in many fields, we'll confine ourselves to the applications of molecular genetics to the mankind. They are as follows:

i) Diagnosis of infectious diseases**:** Normally microorganisms are detected in the laboratory using biochemical methods. In case of molecular techniques, microorganisms are detected by using probes (short DNA or RNA sequence) which are complementary to a part of genome of the microbe. The advantage of using molecular methods is:

- Identification of pathogen is done within a short time;
- No need to cultivate the microbes;
- Latent infections can also be identified when no antibody is formed; and
- The technique can be used even when the microorganism cannot be cultured.

ii) Diagnosis of genetic diseases : Before the advent of the above techniques, counselors used to give risk estimate like one fourth risk of getting the disease, if the parents are heterozygous for an autosomal trait. But now by directly testing for the mutation, they are able to confirm the presence or absence of mutation in the fetus. It is of immense help in prenatal diagnosis.

iii) Individuals can be identified and relationship can be determined by DNA fingerprinting.

iv) Mouse models for genetic diseases have been developed by creating transgenic mice.

v) Production of vaccines, antibodies and therapeutic proteins using recombinant DNA technology. Eg; Insulin, Human Growth Hormone.

vi) It has great potential for treating disease. Gene therapy is a process where the cells of a patient are genetically modified to alleviate disease.

vii) With the development of recombinant DNA technology, identification of disease genes became much easier. Once the disease gene is identified, a molecular test can be designed for diagnosis of genetic disease.

**DNA Fingerprinting**

Just like no two individuals have identical finger prints, no two individuals have identical genetic information, except monozygotic twins. Unlike finger prints which are present only on the tips of the fingers, the genetic information which is unique to the individual is present in each and every cell. Alec Jeffrey discovered repetitive sequences called minisatellites to be unique to every individual. DNA fingerprinting is a technique which makes use of these sequences to evaluate genetic information. It's a quick way to compare the DNA sequences of any two living organisms. It is used for personal identification, identification of the parents, when babies are switched in hospital, identification of criminals etc.

## 1.3 DEOXYRIBONUCLEIC ACID (DNA)

DNA is the thread of life. It is the hereditary material in all organisms except in certain RNA viruses. All the information that is needed for the development, behavior, well being etc. of an individual is encoded in its structure. The genetic information that's stored in DNA flows through RNA to proteins. This flow of genetic information is referred to as central dogma of molecular biology. Though the information is present in the DNA, it is the activity of proteins that is responsible for the inherited traits. The function of DNA is to direct its own replication and to direct transcription.

The number of DNA molecules present in a cell is equal to the number of chromosomes per cell. When compared to the length of the chromosome, the length of the DNA is very very long. It's a matter of interest, how this long DNA fits into the cell whose diameter is in microns (like a long snake fitting into a small basket). This is possible because of the winding of the DNA around histone proteins into structures called nucleosomes. Nucleosomes are further coiled and coiled to form chromosome. So each chromosome is nothing but a single DNA molecule along with proteins.

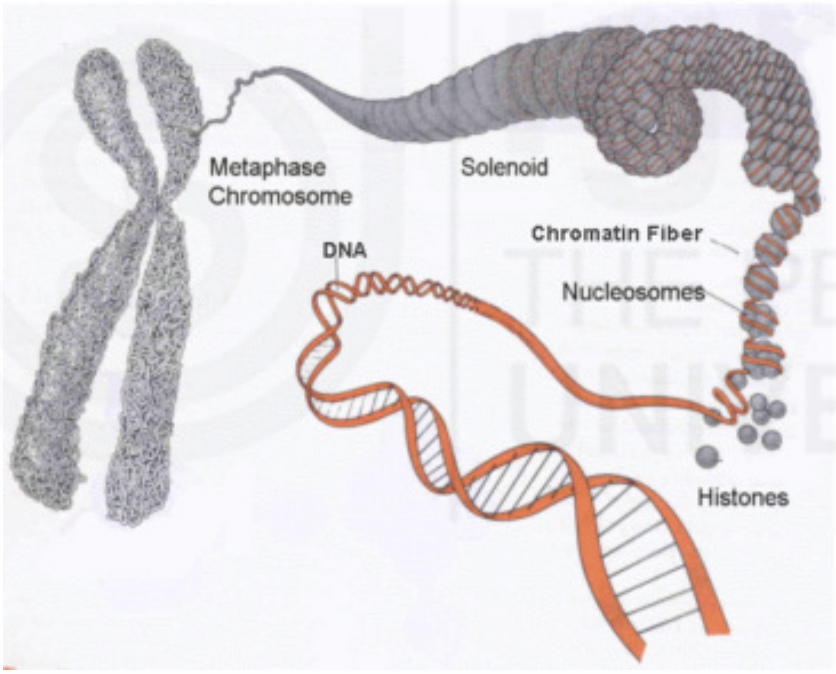



**Fig.1.1: Packing of DNA into chromosomes (Source:http://www.prism.gatech.edu/~gh19/b1510/dnarep.htm)**

### 1.3.1 Structure of DNA

The structure of DNA was proposed by Watson and Crick in the year 1953 for which they won Nobel Prize in 1962. The structure of DNA molecule resembles a gently twisted ladder. Two long polynucleotide chains represent the rails of the ladder. They're coiled around a central axis to form right handed double helix. The rungs are made up of nitrogen bases which are held together by Hydrogen bonds. The basic unit of DNA is nucleotide. It's composed of three subunits-nitrogen base, sugar and phosphate.

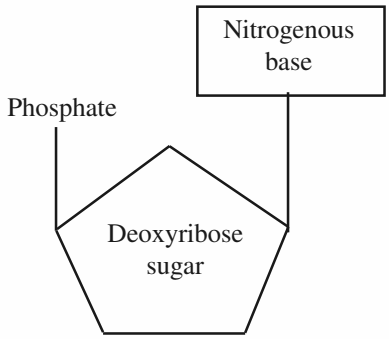



**Fig. 2.2: Nucleotide**

There are two kinds of bases- purines (double ringed) and pyrimidines (single ringed). A purine (Adenine and Guanine) always pairs with a pyrimidine (Thymine and Cytosine). Therefore, the amount of purines present in a DNA molecule is equal to the amount of pyrimidines. Adenine always pairs with Thymine with two Hydrogen bonds whereas Guanine always pairs with Cytosine with three Hydrogen bonds. The two strands of the DNA can be separated easily during replication because of the weak Hydrogen bonds.

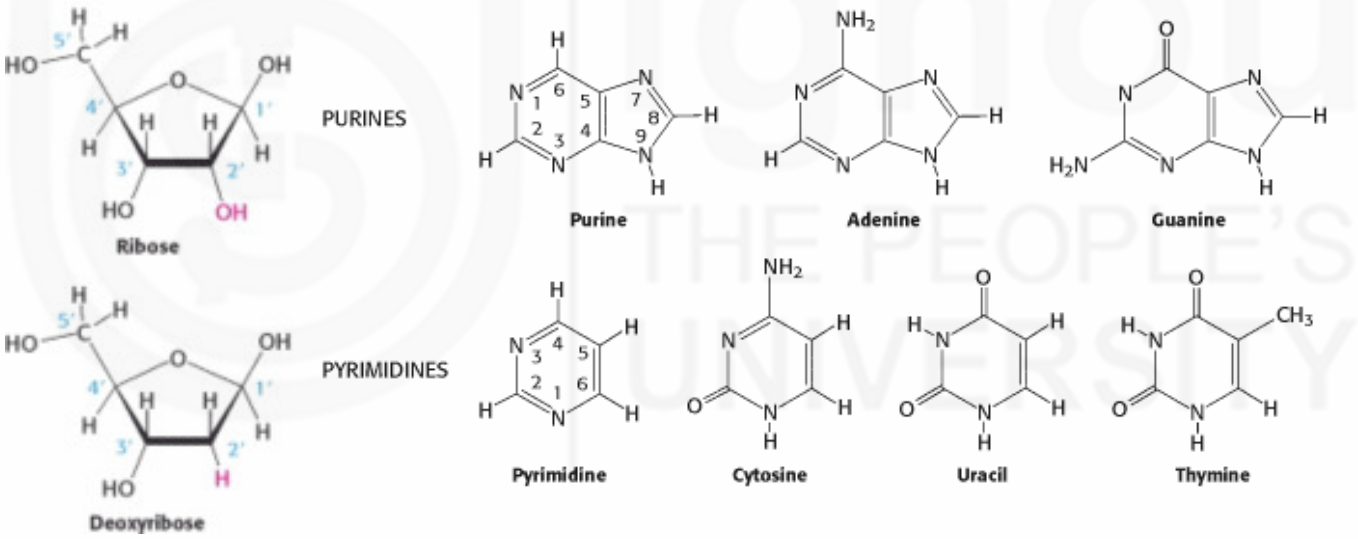



**Fig. 1.3: Pentose sugar ; Purines and pyrimidines (Source : Berg JM et al, 2002 *Biochemistry* WH Freeman & Co.)**

The sugar is a pentose sugar which is deoxyribose in DNA because it lacks Oxygen at second Carbon position. To distinguish between the Carbon atoms present in the base and sugar, the Carbon atoms in the sugar are given a prime (‘). The bases attach to the sugar (at 1’C) by glycosidic bond.

The phosphate group links the 3’C atom of one sugar with the 5’C atom of adjacent sugar by a phospodiester bond. Because of the negative charges present on the phosphate groups, DNA is a polyanion. *In vivo*, these charges are neutralised by the positively charged histone proteins.

## 1.3.2 Features of Double Helix

The two strands of the double helix are antiparallel ie., the 5’ end of one strand aligns with 3’ end of other strand.

Because of the specific base pairing, the two strands are complementary to each other which means that if we know the sequence of one strand we can infer the sequence of the other.

The bases are stacked on one another 3.4A$^o$ apart and are perpendicular to the axis of the double helix. The diameter of the helix is about 20A$^o$ and each complete turn of helix measures 34A$^o$, thus accommodating 10 base pairs in each turn.

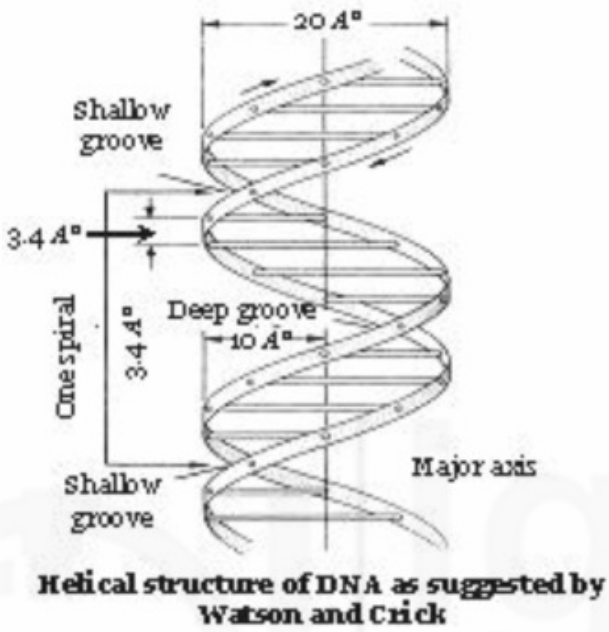



**Fig. 1.4: Structure of DNA (Source:http://www.transtutors.com/chemistry-homework-help/biomolecules/dna-structure.aspx)**

Stacking of base pairs (bp) results in major and minor grooves in DNA. Major groove is rich in chemical information and is recognised by sequence specific DNA binding proteins.

### 1.3.3 How DNA Decides the Hereditary Features

The structure of DNA is the same in all organisms with same four nitrogenous bases-A,T,G and C. Then what makes the difference between plants and animals or how does a zygote know to develop into a monkey or a human? It's the order of the base sequence that makes all the difference. It's not the same in all. The bases are present in different amounts in different species.

## 1.4 GENOME

A genome is the total genetic information present in a cell. Basing on the complexity of humans, if you assume that among all species, the human beings have the largest amount of DNA, you're mistaken. This is because many plant species have much more DNA per cell compared to humans. Even among vertebrates, it's the amphibians which have the greatest amount of DNA per cell. The organisation of human genome is very complex. It comprises of two genomes (Nuclear and Mitochondrial).

### 1.4.1 Organisation of Nuclear Genome

The nuclear genome constitutes more than 99% of the total genome. The haploid genome contains 3 billion bp. The haploid genome is distributed in 23 different types of chromosomes (22 autosomes and 1 allosome). Each chromosome contains many genes. The genes are not uniformly distributed on the chromosomes. A certain area of the chromosome may be rich in genes while areas like centromere and telomeres are largely devoid of genes. Some chromosomes are rich in genes ($22^{nd}$ chromosome) some are gene poor ($4^{th}$ chromosome). The genes which are part of same metabolic pathway may be on different chromosomes and genes which are no way connected to metabolic pathway may be side by side on the chromosome.

There is tremendous variation in the size of the gene, size of the exon as well as intron. On an average an exon may contain < 200bp. Size of the intron may vary from 100bp to >100,000bp. About 1.5% of the total genome is coding (Exon is the coding region and Intron is non coding).

A number of protein coding genes in the human genome form gene families. A set of genes which code for similar protein sequences or which have nucleotide sequence similarity form a gene family (just like related individuals make a family). They arose by duplication of the ancestral gene and accumulation of independent mutations over a period of time. Eg: members of beta globin gene family. An individual won't have same beta globin throughout his development. Apart from beta globin (â) there are different genes like ä, Gã, Aã and å which code for slightly different polypeptides. They express during different stages of development of an individual and forms a gene family. Members of a gene family generally appear as a cluster or they may be dispersed.

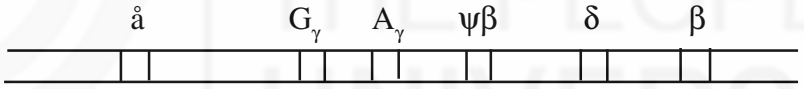



**Fig. 1.5: Human beta globin gene cluster**

More than half of the genome contains repetitive sequences. Basing on the number of copies per genome, the DNA sequences are classified into-unique sequences (1-10 copies), moderately repetitive sequences (10-$10^5$copies) and highly repetitive sequences (>$10^5$copies). Unique sequences include most of the genes which code for proteins. Example for moderately repetitive sequences is the genes which code for ribosomal RNA and histone proteins. Highly repetitive sequences are tandemly arranged and are transcriptionally inactive. They are once again classified into mega satellite, satellite, mini satellite and micro satellite according to the decreasing size of the repeat. Mega satellites are very few in number. Satellite DNA is present in the centromeric region of the chromosomes. The length of the mini satellite DNA is quite variable among individuals and is the basis for DNA fingerprinting. Microsatellites constitute single base runs, di, tri and tetra nucleotide repeats.

Transposons, the DNA sequences which are capable of moving from one part of the genome to other constitute about 45% of the total human genome. Most of them are nonfunctional. Transposition is RNA mediated ie., DNA is first transcribed into RNA and then reverse transcribed into cDNA which is the double stranded form is inserted elsewhere in the genome.

There are pseudogenes in the genome which are nonfunctional copies of a functional gene eg. pseudo beta(øâ) in beta globin gene family. They also arose by duplication of the ancestral gene but in course of time accumulated mutations which rendered them non functional. Few overlapping genes (in class III region of HLA complex present on 6th chromosome.) and genes within genes (presence of two genes within the intron of clotting factor VIII gene) also exist in the genome.

## 1.4.2 Organisation of Mitochondrial Genome

The total amount of mitochondrial genome is <1%. It varies per cell basing on the number of mitochondrial DNA(mt DNA) molecules per mitochondrion and the number of mitochondria per cell.

The size of the human mtDNA is 16,569 bp. It is circular and double stranded. It lies naked in the organelle. mtDNA isn't associated with histone proteins. It contains a light chain and a heavy chain. Heavy strand is rich in guanines and light strand is rich in cytosines. DNA is triple stranded at a region, due to duplication of a section of heavy strand and is called D loop. D loop has no coding sequences. mtDNA has altogether 37 genes out of which 13 code for polypeptides, 22 for tRNAs and 2 for rRNAs. In contrast to nuclear genome, about 93% of the mtDNA is coding. The genome is compact with no introns and presence of overlapping genes. Mitochondria have their own ribosomes on which polypeptides are synthesized. It has a slightly different genetic code when compared to that followed by the nuclear genome.

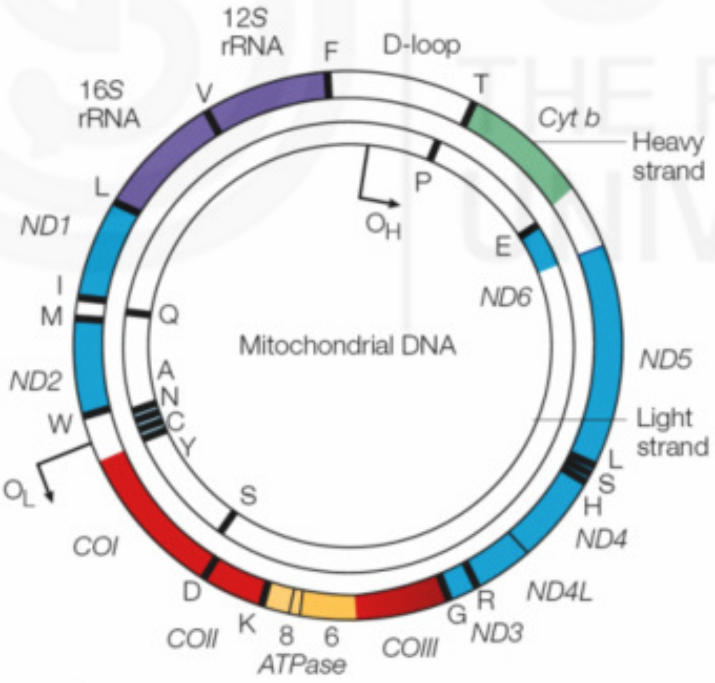



**Fig.1.6: Mitochondrial genome (Source : http://www.nature.com/scitable/content/the-role-of-the-mitochondrial-genome-in-61848)**

The mitochondria synthesize only some of the proteins needed by it, others being synthesized by the nuclear genes. The proteins produced by the nuclear genome are imported into mitochondria. mtDNA shows maternal inheritance because all the mitochondria received by the zygote are from the ovum. Mutations in mtDNA are responsible for certain diseases in humans.

# 1.5 GENETIC CODE

We now know that DNA contains the information that is necessary for the production of proteins. The question is how the information stored in DNA can be decoded into a protein? One of the two DNA strands is transcribed into RNA. This RNA which contains the coded information, acts as a messenger molecule which is further translated into polypeptide. It's essential to understand the nature of genetic code to understand how the coded information in RNA is decoded to protein. Genetic code is a dictionary for the translation of mRNA into protein.

DNA is made up of only 4 different nucleotides (A,T,G and C) and proteins are synthesized from 20 different amino acids. The question is how 4 nucleotides could specify 20 amino acids ? A singlet code (each nucleotide codes for one amino acid) specifies only 4 amino acids, a doublet code (2 bases code for one amino acid) specifies only 16 amino acids ($4^2$). So the minimum number of nucleotides needed to code for 20 different amino acids is 3. This group of 3 nucleotides or nucleotide triplet is called a codon. A triplet code will contain 64 codons ($4^3$) which are in excess of the number of amino acids. The code was deciphered in 1960s by the important contributions made by Nirenberg, Matthaei, Gobind Khorana and Ochoa.

You may have a doubt whether the same genetic code is followed by plants, animals and bacteria as all of them have same 4 bases in their genetic material. Yes, genetic code is universal, except slightly different code is used in mitochondria and by few prokaryotes. Because of this property, we are able to translate mRNA from one species, in a cell of another species (recombinant DNA technology).

## 1.5.1 Properties of Genetic Code

The genetic code is triplet : The code is read in 3 letter words. A group of three nucleotides code for one amino acid.

The code is degenerate : There are 64 codons but amino acids are 20 only which means some amino acids are specified by more than one codon. Eg: GUU,GUC,GUA and GUG code for valine. All these 4 codons are said to be degenerate.

| First base 5' end | Second base U | | C | | A | | G | | Third base 3' end |
|---|---|---|---|---|---|---|---|---|---|
| U | UUU | Phe | UCU | Ser | UAU | Tyr | UGU | Cys | U |
| | UUC | | UCC | | UAC | | UGC | | C |
| | UUA | Leu | UCA | | UAA | Stop | UGA | Stop | A |
| | UUG | | UCG | | UAG | | UGG | Trp | G |
| C | CUU | Leu | CCU | Pro | CAU | His | CGU | Arg | U |
| | CUC | | CCC | | CAC | | CGC | | C |
| | CUA | | CCA | | CAA | Gln | CGA | | A |
| | CUG | | CCG | | CAG | | CGG | | G |
| A | AUU | Ile | ACU | Thr | AAU | Asn | AGU | Ser | U |
| | AUC | | ACC | | AAC | | AGC | | C |
| | AUA | | ACA | | AAA | Lys | AGA | Arg | A |
| | AUG | Start Met | ACG | | AAG | | AGG | | G |
| G | GUU | Val | GCU | Ala | GAU | Asp | GGU | Gly | U |
| | GUC | | GCC | | GAC | | GGC | | C |
| | GUA | | GCA | | GAA | Glu | GGA | | A |
| | GUG | | GCG | | GAG | | GGG | | G |

**Fig.1.7: The genetic code**

The code has polarity: Codons may specify different amino acids when they are read in opposite directions.

Eg.: 5' CCU 3' → proline

3' UCC 5' → serine

As translation occurs in 5'→ 3' direction, it's apt to read the codons in 5'→3' direction only.

The code is non overlapping: Each codon consists of three consecutive nucleotides. That is none of the nucleotide is part of 2 codons. Eg. 5' GCUACCUGC 3'

Non overlapping code will specify only three amino acids. NH2-ala-thr-cys-COOH.

Overlapping code will specify seven amino acids. NH2-ala-leu-tyr-thr-pro-leu-cys-COOH.

The code is comma less: There is no gap or punctuation between 2 codons. After one amino acid is coded, the second one will be coded automatically. If there is any gap between two codons, deletion or addition of one base should not change the reading frame. But a change in reading frame was observed which means that code is comma less.

**Reading Frame**

The possible way in which a nucleotide sequence is read during translation is called the reading frame. Basing on the starting point, a single strand of DNA molecule can be read in three possible ways.

Eg: 5'AGCGCAAGGCGA…..3'

The above sequence has three possible reading frames –one starting with the first base, other two frames starting with second and third bases.

5'AGC GCA AGG CGA….3'

5'GCG CAA GGC GA….3'

5'CGC AAG GCG A…..3'

The code is unambiguous: Though there are few exceptions, a particular codon will always specify the same amino acid. Eg. GCU always codes for alanine.

The code contains "start" and "stop" signals: There is only one start codon (AUG) whereas termination codons (UAA, UAG and UGA) are three in number.

Wobble Hypothesis: Leaving the three termination codons which are recognised by proteins, the remaining 61 codons are recognised by tRNAs. There are only about 30 types of cytoplasmic tRNAs. Then how's it possible to interpret 61 codons? This is possible because of the relaxation of normal base pairing rules when it comes to codon-anticodon recognition. According to Wobble hypothesis of Crick, normal A-U and G-C rules are followed for the first two base positions only but wobbling occurs at third position (G can pair with C or U and U can pair with A or G).

## 1.6 GENE EXPRESSION

Before going to gene expression, let us first understand the meaning of gene, the number of genes in humans, their location and their structure. In simple terms, gene is a stretch of DNA that carries the information necessary for the synthesis of a polypeptide. Actually the definition of gene is much more complex. Today we know that a single gene can give rise to many polypeptides. There are about 25,000 genes in humans according to Human Genome Project. The number of proteins about two lakhs is far greater than the number of genes because of alternative splicing. Genes are located on chromosomes. Each chromosome contains many number of genes arranged in a linear order. Eukaryotic genes are split genes, which means that their coding sequence is not contiguous but it is interrupted by noncoding or intervening sequences called introns. The coding sequences or the expressed sequences are called as exons. In addition to the coding and noncoding sequences, there are flanking regions which are important in regulation and have 'start' and 'stop' signals. These include promoter which is located at the 5' end of the gene and a sequence that is present at the 3' end which provides the signal for the addition of poly A tail to the 3' end of mature mRNA.

**Fig. 1.8: Structure of a gene**

Gene expression is a process in which a protein is synthesized from a gene. It occurs in two major steps. The first step is transcription, in which the linear DNA is transcribed into linear mRNA. The second step is translation during which mRNA associates with the ribosomes present in the cytoplasm and directs the synthesis of proteins.

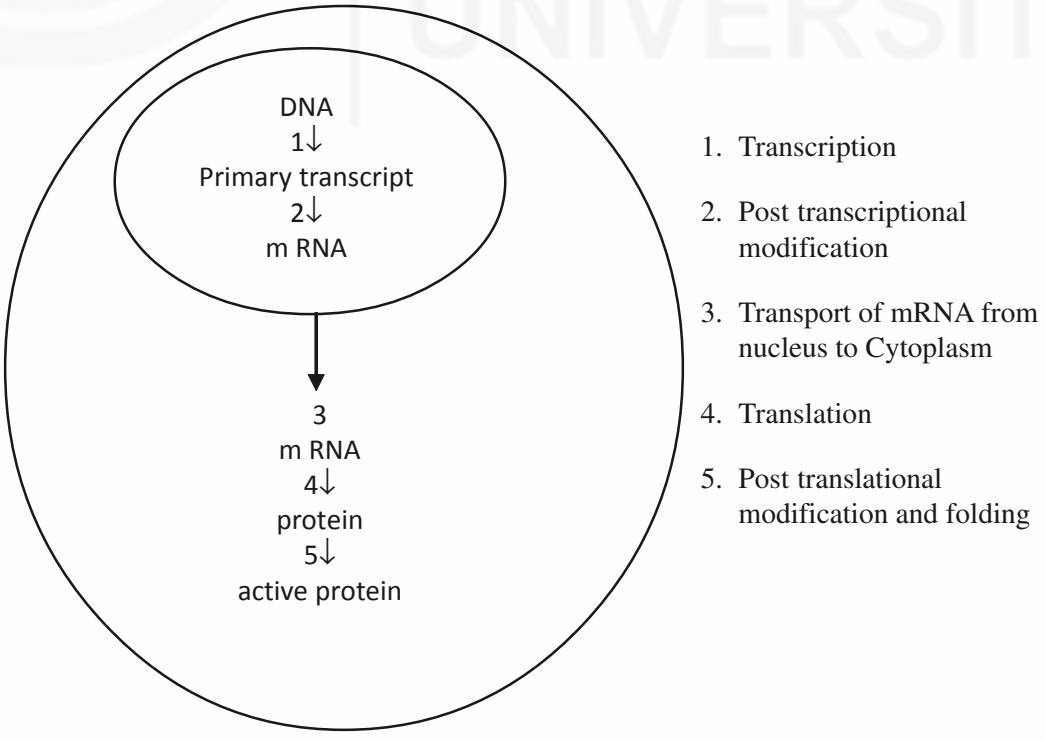



**Fig.1.9: Gene expression**

Here you should note the point that only a small proportion of the total DNA (1.5%) is coding. Moreover, all the genes that are transcribed are not translated that is the end product of some genes is RNA itself eg: tRNA, rRNA etc.

### 1.6.1 Transcription

It's a process in which single stranded RNA is generated from one of the strands of the DNA. It occurs in nucleus in 5'→ 3' direction. It needs RNA polymerase, ribonucleotides and several proteins for initiation. Only one of the two strands of the DNA acts as a template. RNA that is synthesized is complementary to the template but similar in sequence and orientation to that of nontemplate strand (except U is present in place of T). Therefore nontemplate strand is called sense strand and template strand is called antisense strand. Whenever we want to give a gene sequence, it's customary to give the sequence of sense strand in 5' to 3' direction.

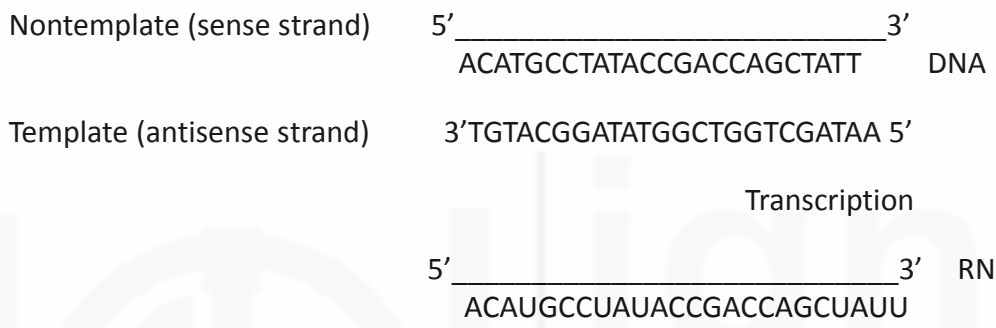



**Fig. 1.10: Process of transcription showing the similarity between RNA and sense strand**

The first base that's transcribed is denoted +1 and the bases that are proceeding in the right side (5'→ 3') are indicated by positive numbers and the direction is called downstream. Conversely the bases towards the left side of +1 are indicated by negative numbers and the direction is called upstream. The promoter which is present in the upstream region of the sense strand contains a group of short sequence elements called TATA box, GC box, CAAT box etc. These elements will be recognised by proteins called transcription factors (TFs). Only when TFs bind to the promoter, followed by binding of RNA polymerase, then transcription occurs. There are three kinds of RNA polymerases in humans. RNA polymerases I and III transcribe the genes which code for tRNA, rRNA and various small RNAs. Structural genes (genes which code for proteins) are transcribed by RNA polymerase II. Termination occurs in them by endonucleolytic cleavage (downstream to a sequence AAUAAA) followed by addition of poly(A) tail.

#### 1.6.1.1 Ribonucleic Acid (RNA) and its Types

RNA molecule is also a polynucleotide chain. The sequence of RNA is determined by the DNA sequence. The difference between DNA and RNA is, it's single stranded, contains ribose sugar (2'OH) in place of deoxyribose sugar (2'-H) and thymine is replaced by Uracil. Occasionally it may fold on itself to give stem loop structures.

Transcription leads to the synthesis of several different types of RNA. Messenger RNA, ribosomal RNA and transfer RNA are the major classes of RNA involved in protein synthesis.

### 1.6.1.2 Messenger RNA (mRNA)

It's this RNA, which carries the message present in the gene to the cytoplasm where synthesis of protein occurs. Only the central part of the mRNA is translated. The region of the first exon and the last exon which are not translated are denoted as 5'UTR and 3'UTR. Each group of three mRNA bases constitutes a codon which specifies an amino acid. The length of different mRNAs vary considerably basing on the length of the gene.

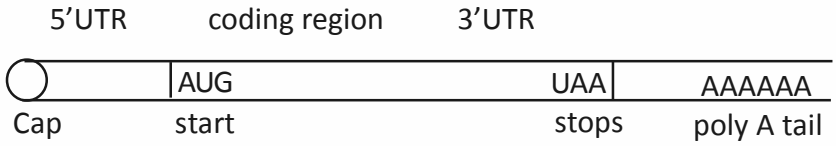



**Fig.11: Structure of mRNA showing coding and untranslated regions**

### 1.6.1.3 Transfer RNA (tRNA)

Transfer RNA molecules interpret the mRNAs with the aid of ribosomal RNAs. They are smallest RNAs (about 80 nucleotides long) which carry the amino acids to the site of protein synthesis. Their one end binds to a codon in the mRNA and the opposite end carries a specific amino acid. Thus it acts like an adaptor. Because of the formation of Hydrogen bonds between some of the complementary bases it forms a clover leafed structure (secondary structure). Its three dimensional structure is however L shaped.

### 1.6.1.4 Ribosomal RNA (rRNA)

About 80% of the total RNA is rRNA. Generally the largest of the RNAs, along with ribosomal proteins it forms the ribosome. A ribosome is made up of two subunits, both of which join at the time of protein synthesis.

Larger subunit: 3 kinds of rRNAs (28S, 5.8S and 5S) + about 50 ribosomal proteins. Smaller subunit: single rRNA (18S) + more than 30 ribosomal proteins

## 1.6.2 Post Transcriptional Modifications

All the above three types of RNAs undergo post transcriptional modifications that is; certain bases present in the RNAs are removed. The RNA that is obtained after transcription is termed primary transcript. In case of mRNA, the introns are removed and the exons are spliced together to form the mature mRNA. Splicing occurs with the help of certain conserved sequences present in the introns. In addition to splicing , in case of mRNA, a cap and a poly A tail are added to the 5' and 3' ends of the mRNA respectively. These two structures help in the migration of the mRNA from the nucleus to the cytoplasm and also in the regulation of gene expression.

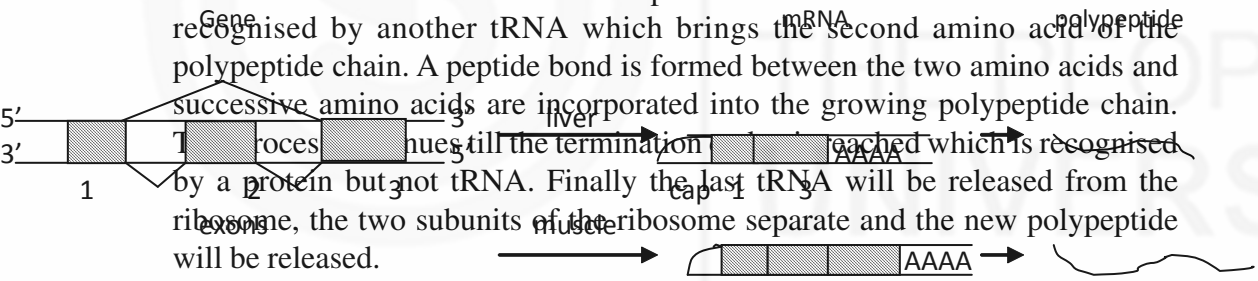
We've already discussed about alternative splicing due to which we are getting about 2,00,000 proteins from the 25,000 genes we have. In simple terms, alternative splicing means getting more number of mRNAs from a single gene. This is possible by differential splicing in different tissues. For eg. a sequence which acts as an intron in one tissue may act as a coding sequence in another tissue thereby changing the sequence of the polypeptide in different tissues.

**Fig. 1.12: Alternative splicing where liver is using only two exons whereas all the three are used in the muscle**

## 1.6.3 Translation

It's a process in which the information present in an mRNA is decoded into the amino acid sequence of a protein. It requires mRNA, tRNA, ribosomes, ATP and various protein factors. It occurs on ribosomes in the cytoplasm. It also occurs in 5'→3' direction. The 5' end of mRNA corresponds to the amino terminus of the protein. Translation starts from the initiation codon and ends with the termination codon. That's the reason why most of the polypeptides start with methionine.

Initiation of translation requires several factors which include a cap binding protein, initiation factors, smaller subunit of the ribosome, initiator methionyl tRNA, all of which bind to the 5' cap region of the mRNA. The initiation complex formed scans the mRNA for the initiation codon. In the elongation step, larger subunit attaches to the initiation complex. The codon next to the AUG is then recognised by another tRNA which brings the second amino acid of the polypeptide chain. A peptide bond is formed between the two amino acids and successive amino acids are incorporated into the growing polypeptide chain. The process continues till the termination codon is reached which is recognised by a protein but not tRNA. Finally the last tRNA will be released from the ribosome, the two subunits of the ribosome separate and the new polypeptide will be released.



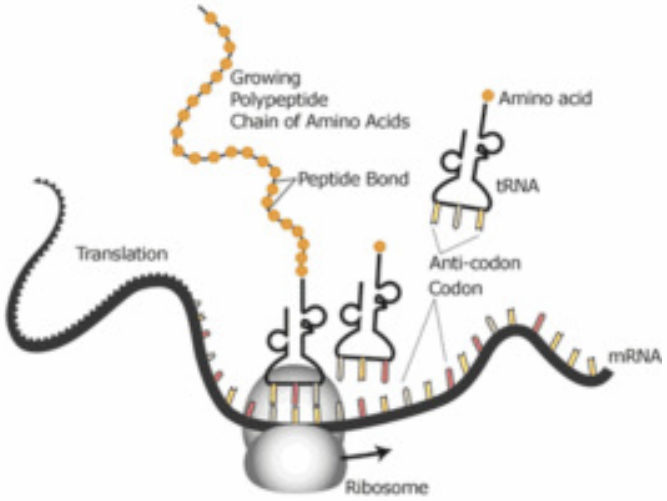




Image adapted from: National Human Genome Research Institute.

**Fig.1.13: Translation of mRNA into polypeptide (Source:http://www2.le.ac.uk/departments/genetics/vgec/diagrams/47-translation.gif)**

### 1.6.3.1 Proteins

A protein is a polymer made up of amino acids. It's the end product of most of the genes. Proteins perform all the metabolic reactions that are carried out in a cell. Though the term protein and polypeptide are loosely used, there is a difference between the two. Polypeptide is the molecule that's formed after translation. After its release, the nascent polypeptide folds up and achieves a three dimensional conformation to become functional protein. Many proteins depend on other proteins called chaperones for folding. In addition to proper folding, polypeptides also undergo post translational modifications (hydroxylation, glycosylation, phosphorylation etc.) to achieve functional status. So a polypeptide is a precursor of protein. Some proteins may have more than one polypeptide, which may be of same kind or of different kinds.

## 1.7 REGULATION OF GENE EXPRESSION

There are 200 different types of cells in human beings. All of them have the same DNA content or to be precise all the genes. Yet all of them are morphologically different and have different function. This depends on the type of genes that are expressed in these cells or in other words differential gene expression is responsible for the diverse properties of different cells. All the genes are not expressed in all the cells or all the times. By this we mean that some genes are expressed only in some cells (tissue specific expression). Similarly some genes are expressed only during a particular time of development. However there are certain genes which are expressed in all cell types (house keeping genes) eg: genes for rRNA, tRNA, DNA polymerases etc. The question is how does a cell know which genes to express, when to express and to what extent. That's what is explained in this topic.

It's a waste of energy for the cell to produce the proteins which are not needed by it. Cells have their own methods, by which they can regulate the expression of genes. Interestingly it's the proteins which are largely responsible for regulation of gene expression. Regulation occurs at three levels.

1) Transcription is the predominant stage at which regulation occurs. Transcription occurs at basal level with the help of TFs. Up regulation and down regulation of gene expression is possible with the help of proteins called activators and repressors respectively. Activators bind to sequences called enhancers whereas repressors bind to silencers. These sequences may be located near the promoter region or far away in the upstream or downstream region of the gene. These regulatory proteins are controlled by signals which determine whether these proteins bind DNA. They determine the amount of the protein to be synthesized. Tissue specific expression is possible by limiting the availability of TFs needed by a gene only to a particular tissue. In some cases, the promoter sequence is methylated in all other tissues except the tissue where it's expressed. Histone proteins also have a role in regulation. Methylation of certain amino acids in histone proteins turns off the expression of a gene.

2) Regulation also occurs at post transcriptional level. Alternative splicing produces different isoforms in different tissues. Isoforms also result due to alternative polyadenylation (same gene uses different polyadenylation signals

in different tissues) and RNA editing (same gene produces different isoforms due to single base substitution, deletion or insertion at the RNA level) .

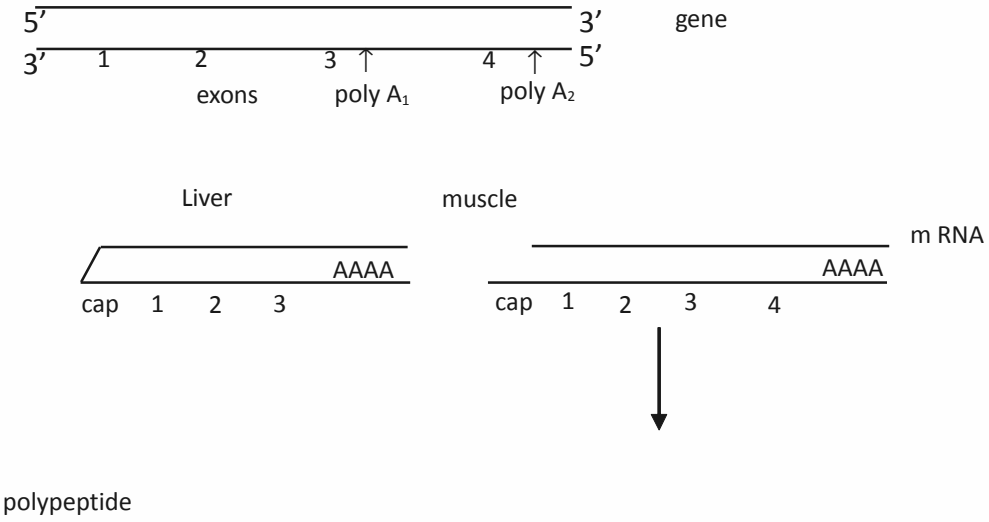



**Fig. 1.14: Alternative polyadenylation where liver uses polyA1 signal and muscle usespolyA2 signal**

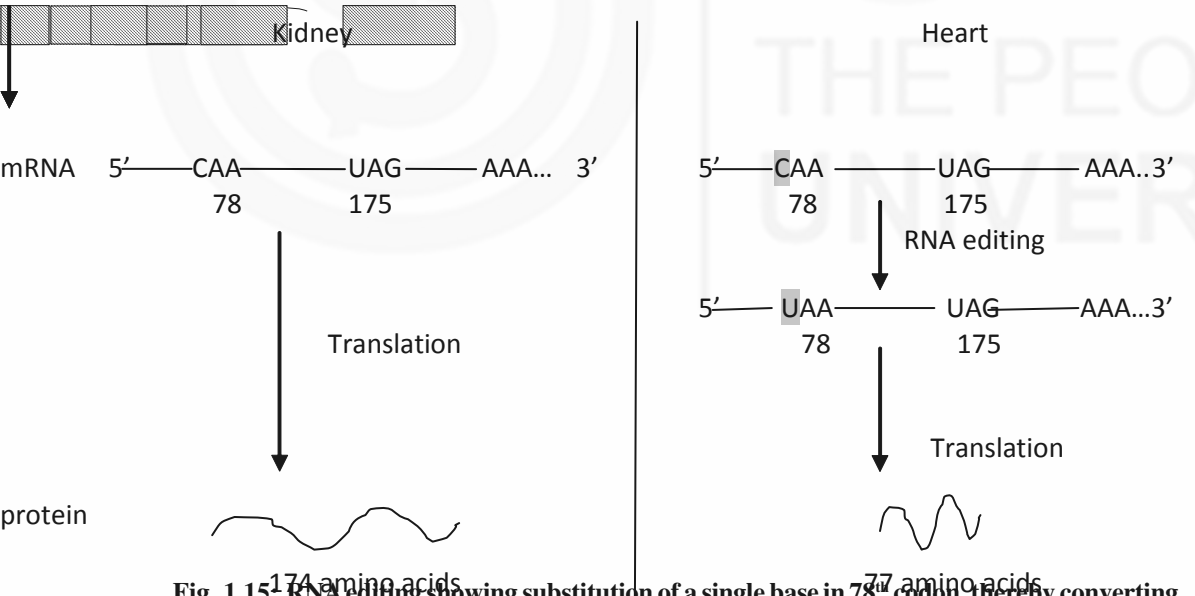



**Fig. 1.15: RNA editing showing substitution of a single base in 78th codon, thereby converting it into a stop codon in heart. Thus, a single gene expresses as 174 amino acid protein in kidney and 77 amino acid protein in heart.**

3) Regulation at translation level includes longevity of mRNA which depends on length of poly A tail (mRNAs without poly A tail are short lived), structure of 3'UTR (many repeats of AUUUA in the 3'UTR makes the RNA short lived) etc. Translation of some mRNAs is regulated by specific RNA binding proteins. Degradation of mRNA is another control point. Rapid degradation of mRNA prevents undesired protein synthesis.

## 1.8 SUMMARY

Molecular Genetics deals with the study of gene expression and its regulation. Revolutionary changes occurred in the field of molecular genetics due to the invention of molecular techniques. It has several applications like diagnosis of genetic as well as infectious diseases, treatment of the disease, personal identification etc.

DNA is the information macromolecule. Each chromosome contains a single DNA molecule. The structure of DNA was deciphered by Watson and Crick. DNA is a right handed double helix with two ribbon like strands constituting the sugar phosphate backbones. The horizontal rungs are made up of nitrogenous bases. The two strands are complementary and antiparallel to each other. They are held together by Hydrogen bonds formed between the opposite bases. The order of the bases in the DNA determines the hereditary features.

The human genome is comprised of two genomes : nuclear genome which is complex and comprises the bulk of the genome and mitochondrial genome. The size of the haploid genome is about three billion bp and it is distributed in 23 chromosomes. The organisation of the genome is complex. Neither the distribution of the genes nor the base composition is uniform throughout. There is high degree of variation not only in the size of the gene but also in the size of exons and introns. In addition, the existence of repetitive sequences, transposons, pseudogenes, overlapping genes and genes within genes make the genome more complex.

The size of the mitochondrial genome is 16,569 bps. It is a circular, double stranded molecule. It shows maternal inheritance. It contains 37 genes. Most of the genes needed for its function are coded by nuclear genome. Mutations do occur in mitochondria which leads to disease.

Genetic code is the relationship between the nucleotide sequence of mRNA and amino acid sequence of protein. The genetic code is triplet, degenerate, nonoverlapping, commaless and universal.

Gene expression is a two step process. First the information present in DNA is transcribed to RNA. Later the information in the mRNA form is decoded to amino acid sequence of the polypeptide by translation. The polypeptide that is formed during translation, undergoes folding and post translational modifications to form a functional protein.

Gene expression is highly regulated. Each cell contains all the genes present in the total DNA. However to save energy, cells express only the proteins needed by it, in required amounts and at needed time. This kind of control over gene expression is achieved largely by proteins. Gene expression is regulated at different levels like transcription, post transcription, translation. Major control occurs at the transcriptional level. Same gene can produce different forms of proteins in different tissues by mechanisms like alternative splicing, alternative polyadenylation and RNA editing.

## Suggested Reading

Strachan, T and Read, A.P. 2004. *Human Molecular Genetics*. Wiley-Liss.

Lewin, B. 2004. *Genes VIII*. Prentice Hall.

Watson, J.D, Baker, T.A, Bell, S.P, Gann, A, Levine, M and Losick, R. 2004. *Molecular Biology of the Gene.* Pearson Education.

McConkey, E. H. 1993. *Human Genetics. The Molecular Revolution*. Jones and Barlett Publishers.

## Sample Questions

1) Describe the salient features of DNA double helix.

2) "The organisation of human nuclear genome is complex". Justify the statement.

3) Define genetic code and write about its properties.

4) Explain how the nucleotide sequence present in a gene is used to synthesize a protein.

5) Describe how gene expression is regulated at different levels.

### Short Notes

i) MtDNA

ii) Genome

iii) RNA and its Types

# UNIT 2 DNA POLYMORPHISMS

**Contents**



**Learning Objectives**

After having studied this unit, you will be able to:

- understand Human DNA polymorphisms;
- explain types of DNA polymorphisms;
- discuss DNA polymorphisms role in disease manifestation; and
- describe Single Nucleotide Polymorphism's role in reconstructing Human evolution and modern Human migrations.

## 2.1 INTRODUCTION

Human genome (entire genetic material present in a cell) consists of 3 billion bases. There are about 10 million Single Nucleotide Polymorphisms. The DNA sequence will carry code of information for carrying genetic information from parent to child (generation to generation). A person or plant or animal phenotypically looking different, means that there are variations in the genetic material of the organism. Any change in the DNA sequence will bring change in the genetic information, inturn brings change in phenotypic expression and biological function. The change in the DNA sequence is called *Mutation*. If the mutation frequency is more than 2 per cent in a population, it is called as *polymorphism.* Hence, we can define DNA polymorphism as DNA having more than one form, with a frequency of above 2 percent in a population.

## 2.2 DIFFERENT FORMS OF DNA POLYMORPHISMS

DNA polymorphisms can be studied in the form of Single Nucleotide Polymorphisms (SNPs), Restriction site Polymorphisms (RSPs) or Restricted Fragment Length Polymorphisms (RFLP) and Variable Number of Tandem Repeats (VNTRs).

**Single Nucleotide Polymorphisms (SNPs):** A single nucleotide is substituted by a different nucleotide.

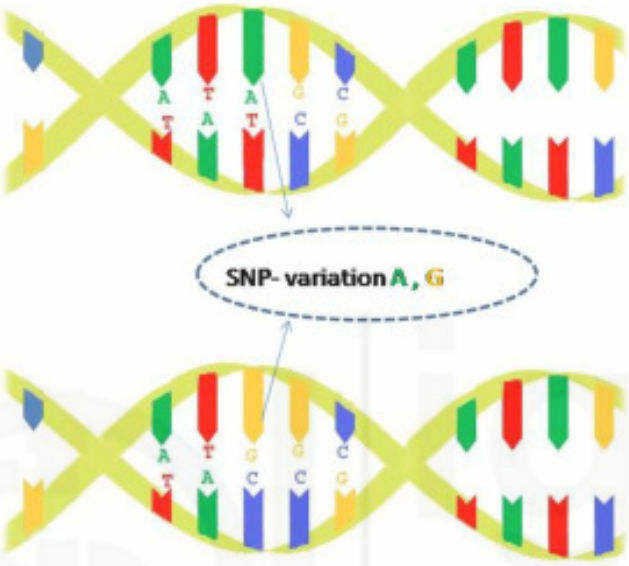



**Fig.2.1: SNP in double strand DNA**

There are two types of nucleotide substitutions resulting SNPs. 1) Transition: A substitution occurs between Purines (A,G) or between Pyrimidines (C,T). This type of substitution constitutes two thirds of all SNPs. 2) Transvertion: A substitution occurs between a Purine and a Pyrimidine.

**Insertions:** A new nucleotide will be inserted in the sequence.

**Deletions:** An existing nucleotide will be deleted in the sequence.

**Restriction site Polymorphisms (RSPs) or Restricted Fragment Length Polymorphisms (RFLP)**: A sub set of SNPs cause a loss or gain of a restriction site (restriction site is the location where a particular enzyme cuts the DNA sequence at particular sequence location into pieces of DNA). Due to change in nucleotide at particular site location, enables the enzyme to cut DNA into pieces. This leads to create different length of DNA piece in an individual and another length of DNA piece in another individual. This is called Restriction site polymorphism. RSPs are described as restricted fragment length polymorphisms (RFLP).

**III.Variable Number of Tandem Repeats (VNTRs):** It is divided into two types:

Microsatellite polymorphism

Minisatellite polymorphism.

Micro-satellite polymorphism is also called as Short Tandem Repeats (STRs). A small array of tandem repeats of a simple sequence (usually less than 10 base

pairs). Ex; GATAA GATAA GATAA GATAA GATAA GATAA in this sample 5 bases repeated 6 times.

a) GA GA GA GA GA – it is dinuleotide repeat

b) TAT TAT TAT TAT- it is trinuleotide repeat

**Mini-satellite polymorphism**: A collection of moderately sized arrays of tandemly repeated DNA sequence which are dispersed over considerable portions of the nuclear genome.

c) TTAGGGTACCGG TTAGGGTACCGG TTAGGGTACCGG –this array of 12 nucleotides repeats from 3-20 Kbp(thousand base pairs).

## 2.3 HUMAN EVOLUTION WITH SPECIAL REFERENCE TO MITOCHONDRIAL DNA AND Y-CHROMOSOME POLYMORPHISMS

DNA polymorphisms particularly SNPs became a powerful tool in reconstructing human origins, evolution and prehistoric migrations. Earlier we used to depend on archaeological and paleontological evidences to reconstruct human evolution. In the absence of these traditional evidences DNA analysis became an alternative tool to reconstruct human evolution.

The hominid fossil record in Africa begins about 4 MYs ago in Early Pliocene with representatives of the genus Australopithecus from Ethiopia and Tanzania. Homo erectus arose more than a million years ago in the Pleistocene, giving rise to our own genus, Homo. Anatomically modern humans began to appear 120000-100000 years ago and co-existed with Neanderthals until the latter became extinct about 30,000years ago. Based on these evidences, two theories have been proposed for the evolution of modern humans: **1.Multiregional evolution**: It proposes that present day worldwide populations are the descendants' of in situ evolution after an initial dispersal of Homo erectus from Africa during the Lower Pleistocene(~650kybp), **2. Uni regional hypothesis** (also called as **Recent African Origin model** or **Out-of-Africa**):- All present day populations have descended from a recent common ancestor that lived in East Africa ~150,000 years ago.

At this juncture, mitochondrial DNA and Non-recombining region of Y-chromosome (NRY) analysis provided an alternative approach to reconstruct Modern Human evolution. mtDNA and NRY Y-chromosome analysis enable us to trace maternal and paternal lineages of modern humans. Along with these DNA markers autosomal and X-linked markers have also been studied.

DNA can also be extracted from bone material of ancient specimens. The ancient mtDNA analysis from Neanderthal specimens reveals that Neanderthals are not immediate ancestors to modern humans. Modern humans diverged from Neanderthals about 400,000 years ago. Neanderthals went extinct without contributing any mtDNA to modern humans.

### 2.3.1 mtDNA Polymorphism-Human Evolution

DNA polymorphisms suggested a recent origin of modern humans from African populations. Initial evidence came from mtDNA, which is transmited maternally. Each human cell cytoplasm contains 10-100 mitochondria. Each mitochondrion will have a circular double strand DNA molecule about 16569 base pair length. The most ancient mtDNA haplotypes (having the same genotype) are L0, L1, L2

and L3. Haplotypes L1 and L2 are specific to the sub-Saharan Africa. L3 is present in North East Africa and Middle East. L3, M & N are parallel branches. M branch is called as Asian branch and N is called as European branch. All branches of M arose in Asia. M branches didn't present in Europe. M1, a branch of M present in East Africa is originated in Middle East and back migrated into Africa. In Asia N branch is also present. M is the oldest branch than N branch. An ancestral branch of Asian might have arisen in North East Africa and subsequently left to colonise Asia (50-70 thousand years ago) and Europe (45-50 thousand years ago).The mtDNA analysis of world populations reveals that modern humans can be traced back to a single mother, 'mitochondrial eve'. The analysis also shows that this individual existed about 100,000-130,000 years ago in east Africa. Of course, the mitochondrial eve was not the only person living on the planet at that time: there are perhaps about 10,000 individuals living at that time but unlike Eve, there mtDNA sequences didn't get transmitted to the present human populations.

SNPs normally exhibit two forms of variation at a nucleotide position or have two alleles. For example Africans (L0, L1 & L2 haplogroups) have Thymine at nucleotide position 3594 where as all Non-Africans will have Cytocine at np3594. Likewise all Asians have four mutations (M lineage) in their mtDNA sequence.

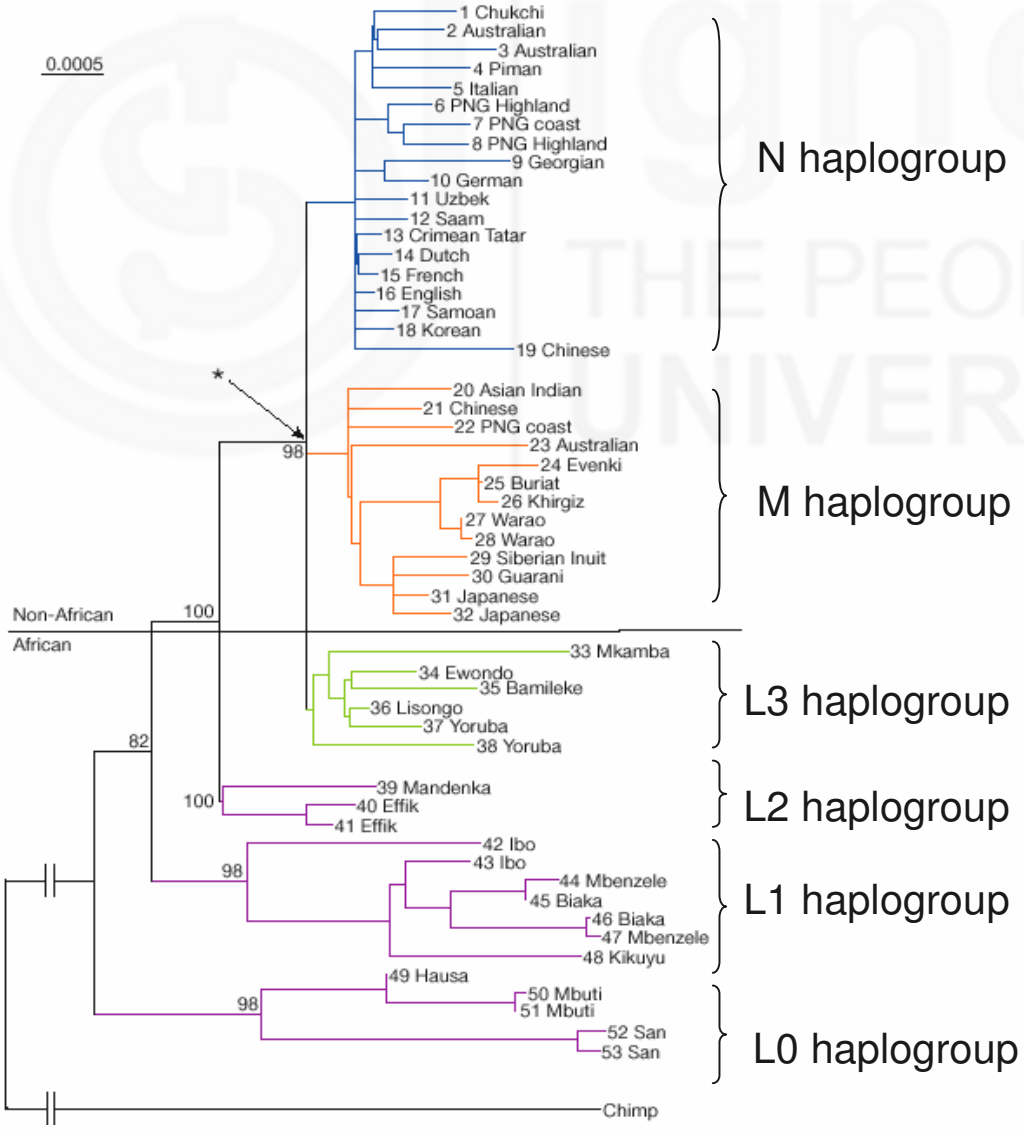



**Fig.2.2: mtDNA phylogenetic tree constructed based on Polymorphisms present in mtDNA of world populations (adopted after Ingman, 2000)**

### Indian scenario

The initial dispersal of modern humans from East Africa en-route North and East of Africa has now been documented, following the African mtDNA haplogroups into Saudi Arabia and then Western India. Indian specific mtDNA branches, M and N encompass all the populations in India irrespective of their social rank, caste or tribe. In India the frequency of M haplogroup ranges from 54 to 97 percent. Indian specific M sub haplogroups are M2,-M6, M18, M25, and M30-M62. M1is present in North East Africa, M31 & M32 are specific for Andaman Islands. M7, M8, M9, M10, M11 & M12 are specific for China and Japan. Most numerous sub haplogroups of European N haplogroup are Indian specific. Ex: N5, R5, R6, R7, R8 T30, R31, U2. Genetic links of Indians with East Eurasians, West Eurasians and Australians are established by mtDNA polymorphisms.

## 2.3.2 Y Chromosome Polymorphism-Human Evolution

The Y chromosome is a suitable tool for investigating the recent human evolution, for medical genetics, DNA forensics and genealogical reconstructions, due to its uniqueness among the other human chromosomes. The Y chromosome has a sex-determining role, it is male specific and constitutively haploid (Single). It is inherited paternally and is transmitted from father to son, and unlike other chromosomes, the Y chromosome escapes meiotic recombination in its NRY (Non Recombining Y chromosome) region. The non-recombining portion of the Y chromosome descends as a single locus. As they change only by accumulating mutations in time, they preserve by far more simple record of their history compared to autosomes.

Y chromosome variation consists of large amount of different types of polymorphisms, which are widely used in evolutionary studies. They may roughly be divided into two large groups: bi-allelic markers and polymorphisms of tandem repeats or multi-allelic markers. Bi-allelic markers include SNPs (Single Nucleotide Polymorphisms) and insertions and deletions (indels). SNPs are the most common type of polymorphisms, constituting more than 90% of total polymorphisms of DNA. Only these bi-allelic mutations that have occurred, only once in history of humans and have a detectable frequency in human populations are used in phylogenetic studies.

Y chromosome DNA polymorphisms are useful to trace paternal lineages. The Y chromosome consortium is formed to document the binary polymorphisms in NRY (non recombining region of Y chromosome). There are about 599 polymorphisms made Y chromosomes in to 311 groups. This information was constructed into a tree and named the main branches starting from alphabets A to T. Major branches are called as Clades and sub branches are called as a haplogroups.

Polymorphisms at P91, M168, M294 distinguishes A, B clades from the rest of clades. Clade A & B are exclusively present among African populations. The majority of branches of the Y chromosome tree outside Africa are composed of a tripartite assemblage of the following haplogroups: a) C; b) D and E, and c) an overarching haplogroup F that defines the internal node of all remaining haplogroups from G to T.

Because the mutation defining haplogroup C (M130=RPS4Y) has not been observed in any African populations, this haplogroup is likely to have arisen somewhere in Asia after an early departure of modern humans from Africa, prior to the arrival of them to Sahul in Southeast Asia. The most western region where

haplogroup C* has been detected is India. This lineage consists of several sub-lineages with irregular phylogeographic patterning, ranging from Central and North Asia to America and in the direction of Southeast Asia up to Australia and Oceania. Differently from hg C, haplogroups E and D share three phylo-genetically equivalent markers.

Calde D* is found in Andaman Islands whereas D1 & D2 are found in Tibetans and Japanese. E is the most frequent and divergent in Africa.The third major sub-clade of M168 lineages is super haplogroup F. It is characterized by mutation M89 at its root from which all other haplogroups deploy. F has been suggested to have evolved early in the diversification and migration of modern humans. Later on, the ancestral trunk of F diversified into many branches by subsequent acquisition of mutations, giving rise to many region-specific haplogroups, such as J and G in Near and Middle East, I in Europe, H in Southern Asia, etc. An expansion of F lineages gave rise also to a population that acquired the M9 mutation (haplogroup K), which defines another major bifurcation in the phylogeny . The branches of this clade probably migrated in different directions (North and East) and gave start to many separate and region-specific haplogroups in Eurasian continent and beyond. Out of descendants of M9 lineage, haplogroup L (M20) has greatest frequency in Southwest Asia and distinctive K lineages and M (M4, M5) haplogroup are restricted to Oceania and New Guinea, whereas haplogroup O with its numerous sub-clades predominates in southern and southeastern Asia, reaching North China, Manchuria and some Siberian populations. The population carrying M9 expanded also in direction of north towards Central Asia characterized by subsequent mutations defining haplogroup P, which encompasses distinctive eastward expanding haplogroup Q (M242) characteristic to Siberian populations and Amerindians and Eurasian haplogroup R lineages that have expanded westward. Thus, one may speculate that multiple independent formations and fragmentations of populations carrying F-related lineages throughout most of Eurasia may have displaced the earlier haplogroup C and D lineages towards the margin in many areas. Among Indians H, O, R1, R2 are the major clades. H haplogroup is nearly restricted to India, Srilanka and Pakistan. Among Austro-Asiatic language speaking groups of India O haplogroup is predominant followed by H group. Indo-European language speakers have 50 percent of O haplogroup followed by H & R.

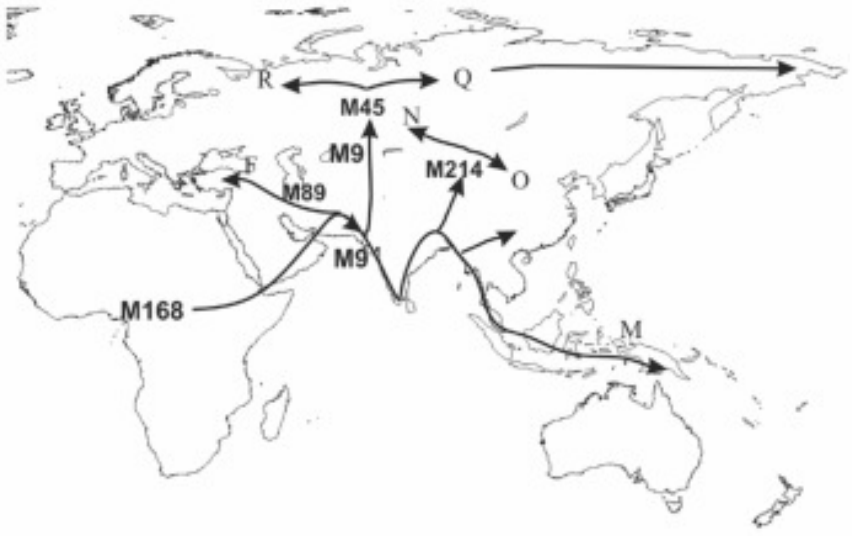



**Fig.2.3: Schematic reconstruction of super haplogroup F (defined by M89) origin, subsequent diversification of M9 lineages and their possible migration routes across the world. Adapted from Underhill (2003).**

**Table 2.1: Number of mutations associated with 20 major Y chromosome clades (Karafet,2008)**

| Clade | Mutations | haplogroups |
|---|---|---|
| A | 47 | 12 |
| BT | 5 | |
| B | 32 | 17 |
| CT | 3 | |
| C | 30 | 19 |
| DE | 8 | |
| D | 23 | 15 |
| E | 101 | 56 |
| C,FT | 1 | |
| FT | 25 | |
| F | 6 | 5 |
| G | 13 | 10 |
| H | 12 | 10 |
| IJ | 7 | |
| I | 35 | 16 |
| J | 42 | 34 |
| KT | 4 | |
| K | 5 | 5 |
| L | 13 | 7 |
| M | 20 | 12 |
| NO | 6 | |
| N | 11 | 10 |
| O | 48 | 31 |
| P | 20 | 1 |
| Q | 18 | 14 |
| R | 50 | 28 |
| S | 8 | 6 |
| T | 6 | 3 |
| **TOTAL** | **599** | **311** |

Based on the above table the tree has been constructed which is as follows.

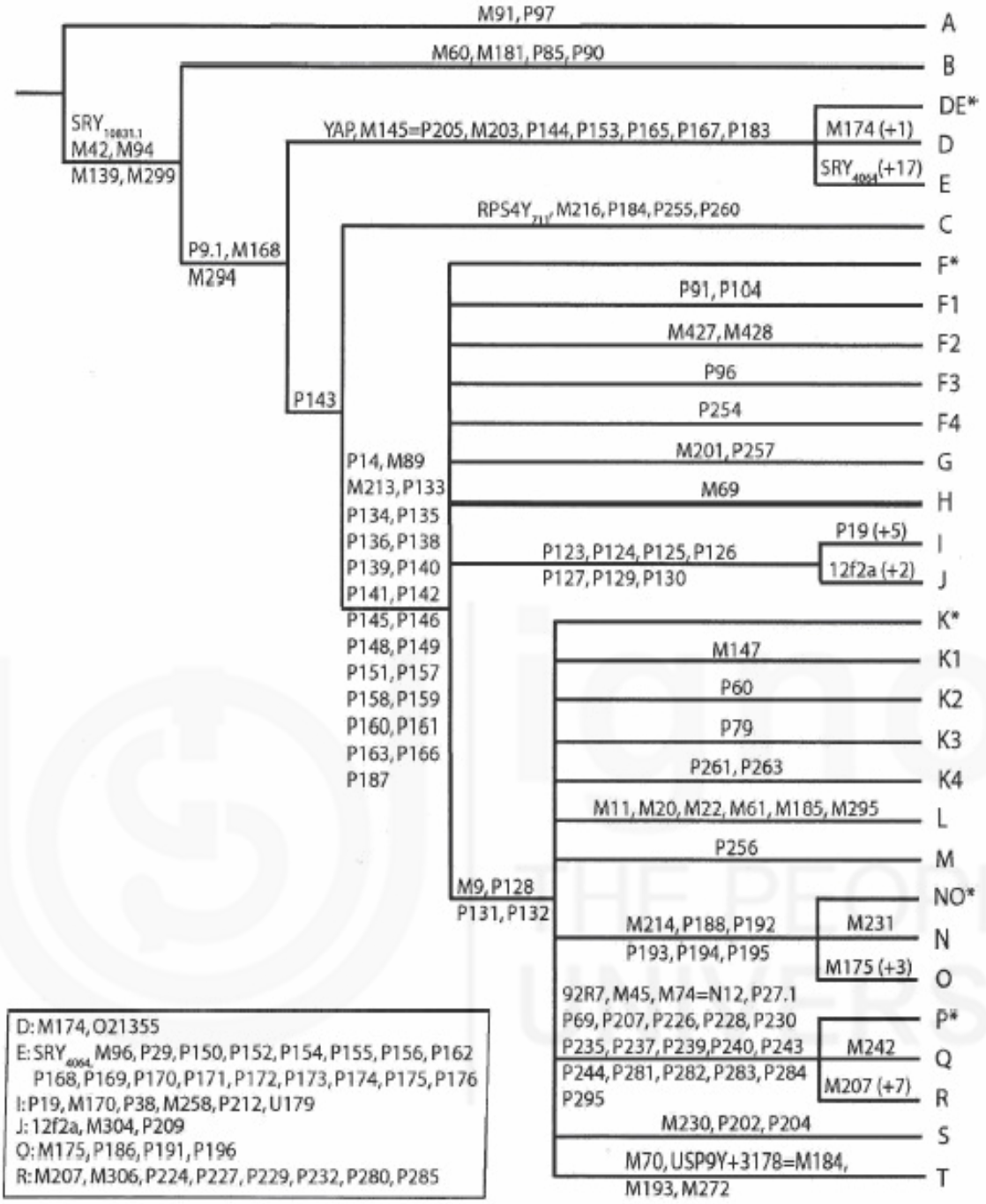



**Fig.2.4: Y-Chromosome tree. Mutation names are indicated on the branches. A to T are branch names. Sub trees are not shown in this figure (Karafet, 2008)**

Apart from bi-allelic polymorphisms, insertions and deletions (indels) persist over generations and are sufficiently common to be considered as polymorphisms. One such example is a 2kb deletion in 12f2 marker, used for defining haplogroup J. Some indels have arisen independently more than once in human history. For example, the deletion or duplication of the 50f2/C region in background of different haplogroups is thought to be arisen at least 7–8 times. Another example is the deletion of DAZ3/DAZ4 region that has been indicated to occur in haplogroup N individuals, widely spread in northern Eurasia.

Another frequent type of polymorphism, present also in Y chromosome, is tandem repeats, mostly in non-coding DNA regions. According to their length, these repeats are classified as satellite-DNAs (repeat lengths of one to several thousand base pairs), mini-satellites or variable number of tandem repeats (VNTRs), ranging from 10 to 100 bp, and microsatellites or short tandem repeats (STRs), with motifs less than 10 bp, mostly 2 to 6 bp long. In Y-chromosomal studies microsatellites are widely used, than mini-satellites. Microsatellites are multi-allelic markers with different allele numbers ranging from 3 to 49 in locus. Their mutation rate is much higher than that for biallelic markers and, therefore, they are widely used in phylogenetic studies to investigate details of demographic events that have occurred in a more recent time scale. In evolutionary studies STRs are valuable in combination with binary haplogroup data, as they enable us to study diversity within a haplogroup. STRs are particularly widely explored in forensic work. So far the number of widely used Y chromosomal STRs has been quite low (about 30) but in a recent study 166 new and potentially useful STRs were described.

Based on the Phylogenetic analysis it was concluded that, all humans have originated from an African ancestor. About 70,000 thousand years ago modern man came out of Africa and peopled all the continents.

Thus the combination of molecular age and geographical structure makes mtDNA and the NRY a sensitive genetic index capable of tracing the micro evolutionary patterns of noval modren human diversity. Mitochondrial DNA and Y chromosome studies in Indian populations reveals affiliation with Europeans, East Asians, Austro-Melanesians and in situ development of deep rooted ancestry whose relative clustering and coalescence ages suggest shaping of Indian gene pool during late pleistocene.

## 2.4 DNA POLYMORPHISMS AND DISEASE ASSOCIATION

Disease is a disordered or incorrectly functioning of organ, part, structure, or system of the body resulting from the effect of genetic or developmental errors, infection, poisons, nutritional deficiency or imbalance, toxicity, or unfavourable environmental factors.

A genetic disease is any disease that is caused by an abnormality in an individual's genome. The abnormality can range from minuscule to major from a discrete mutation in a single base in the DNA of a single gene to a gross chromosome abnormality involving the addition or subtraction of an entire chromosome or set of chromosomes. Some genetic disorders are inherited from the parents, while other genetic diseases are caused by acquired changes or mutations in a pre-existing gene or group of genes. Mutations occur either randomly or due to some environmental exposure. There are about 30,000 genes in humans. Some of the mutations in these genes cause a disease, predispose us to the common diseases in combination with other variants and with the environment. Knowledge of these polymorphisms offers tremendous advantage in the study of disease and variable response to treatment.

### 2.4.1 Monogenetic Disease

It is caused by changes or mutations that occur in the DNA sequence of a single gene, also called Mendelian disorder. There are more than 6,000 known single-gene disorders, which occur in about 1 out of every 200 births. Some examples of monogenetic disorders include: Cystic Fibrosis, Sickle Cell Anaemia, Marfan syndrome, Huntington's disease, and Hemochromatosis. Single-gene disorders are inherited in recognisable patterns: autosomal dominant, autosomal recessive, and X-linked.

Example: Sickle cell anaemia is a disease passed down through families in which red blood cells form an abnormal crescent shape (Red blood cells are normally shaped like a disc.) Sickle cell anaemia is caused by an abnormal type of haemoglobin called haemoglobin S. Haemoglobin is a protein inside red blood cells that carries oxygen. Haemoglobin S changes the shape of red blood cells, especially when the cells are exposed to low oxygen levels. Then the red blood cells become crescent shaped or sickles. The sickling occurs because of a mutation in the haemoglobin gene. The haemoglobin beta(HBB) gene is found in region 15.5 on the short (p) arm of human chromosome 11. In sickle cell haemoglobin (HbS) the glutamic acid in position 6 is mutated to valine in a beta chain. This change allows the deoxygenated form of the haemoglobin to stick to itself and become crescent shape.

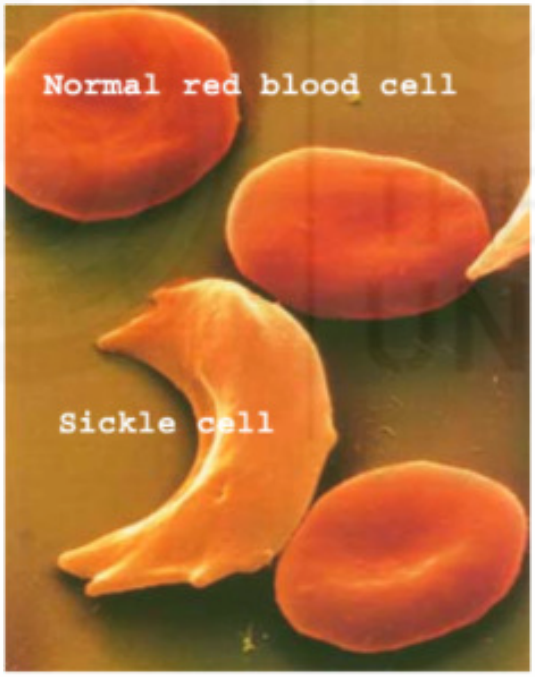
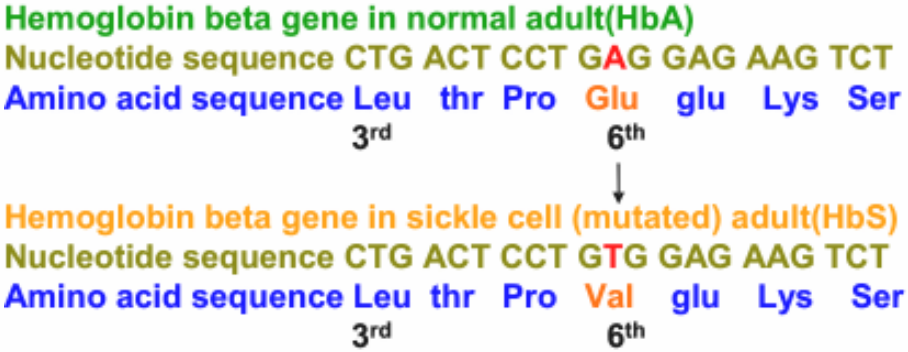



**Fig.2.5: Normal and sickle red blood cells.**



The fragile, sickle shaped cells deliver less oxygen to the body's tissues. They can also get stuck more easily in small blood vessels, and break into pieces that interrupt healthy blood flow.

Sickle cell anaemia is inherited from both parents. If you inherit the haemoglobin S gene from one parent and normal haemoglobin (A) from your other parent, you will have sickle cell trait. People with sickle cell trait do not have the symptoms of sickle cell anaemia. The children of both sickle cell parents will get sickle cell anaemia.

Sickle cell disease is much more common in people of African and Mediterranean descent. It is also seen in people from South and Central America, the Caribbean, and the Middle East.

### 2.4.2 Multifactorial Disease

It is called complex or polygenic disease. Complex diseases are caused by a interaction of environmental factors and mutations in multiple genes. Some common chronic diseases are multifactorial in nature. Examples of complex diseases include: Cardio Vascular diseases, high blood pressure, Alzheimer's disease, arthritis, diabetes, cancer, and obesity. For example, different genes that influence breast cancer susceptibility have been found on chromosomes 6, 11, 13, 14, 15, 17, and 22.

Mutations in BRCA1 gene (BRCA1 Gene is located on chromosome17q21.31) contribute significantly to the development of familial/hereditary breast and ovarian cancer. Founder mutations such as the BRCA1-185delAG and 5382insC are found among Ashkenazi Jews.

Polymorphism in BRCA1 `Chr17 at np` 37043496 is shown in the figure.

`CCGCCCCTACCCCCCC`**`G`**`TCAAAGAATACCCAT(normal form)`

`CCGCCCCTACCCCCCC`**`C`**`TCAAAGAATACCCAT (mutated form)`

Large rearrangements, mostly deletions in regions of Y-specific genes (AZFa, AZFb, AZFc), have been known as causes for many diseases leading to male infertility, causing spermatogenic failure, azoospermia, severe oligo spermia or otherwise severely impair male reproductive fitness.

## 2.5 TECHNIQUES IN MOLECULAR GENETICS

To study DNA polymorphisms initial step is extraction of DNA from cells. We can extract up to 1500 nano grams of DNA from 5ml of blood by Phenol chloroform method.

**DNA Extraction- Principle**

The process of DNA extraction can be divided into three stages: (i) disruption of the cellular membranes, resulting in cell lysis, (ii) protein denaturation, and finally (iii) the separation of DNA from the denatured protein and other cellular components.

About 5 ml of blood is transferred to a sterile 15ml conical bottom polypropylene tube and equal amount of RBC lysis buffer is added. Shake the tube gently for 3 to 5 minutes. The contents of the tube transforms into transparent red colour upon the lysis of RBC. We have to ensure that RBC is completely lysed before proceeding to further step. Once RBC is lysed, transfer the tubes into the centrifuge. Run the centrifuge at 1500 rpm for 15 mts at 20$^{o}$C. Upon the completion of the run carefully, remove the tubes from the centrifuge and decant the supernatant without disturbing the pellet at the bottom. Now add 4 ml of RBC buffer to the tube and break the pellet using hand or vortex. Repeat the centrifugation step as earlier with same settings. Decant the supernatant, if pellet is still red repeat the RBC lysis. If pellets are light pink or white proceed for further step.

Add 1 ml of digestion buffer and 10 ul of Proteinase K to the tube and carefully dislodge the pellet from the bottom of the tube. Adjust the hot water bath at 55$^{o}$C, now transfer the tubes into the hot water bath. Gently dislodge the tubes for every 30 minutes to enhance the digestion process. The content of the tubes turns clear and transparent upon the digestion.

Now add 250ul of 5M sodium per chlorate to the tube and gently mix the contents by partially inverting the tube. Now add 500ul of tris saturated phenol, 500ul of 24:1 chloroform isoamyl alcohol, mix the contents thoroughly and adjust the centrifuge at 4ºC, 4000 rpm and 15 minutes. Take a fresh tube, carefully transfer the supernatant using the 1ml pipette and cut tips into the fresh tube. Now add 500ul of 24:1 chloroform and isoamyl alcohol, and repeat the centrifugation step with same settings. Carefully transfer the supernatant using a cut tip into a fresh tube. Add double the volume of chilled alcohol; gently invert the tube for a minute. A milky white fibrous DNA is visible. Now transfer the DNA into a 1.5ml tube, add 1 ml of 70 % alcohol, spin at 12,000 rpm for 10 minutes, and repeat the step for one more time to eliminate the remaining protein contamination. Dry the pellets and add 200ul of TE buffer and mix the contents thoroughly and transfer into the hot water bath/dry bath for digesting the DNA. This process usually takes 2 hours. Transfer tubes for -80ºC for long term storage.

Details of the buffers and reagents used for DNA extraction are given below.

1) *RBC Lysis Buffer contains* Sucrose, 1M Magnesium Chloride 1M Tris-Hcl and Triton X.

2) *Digestion Buffer constitutes* 1M Tris-Hcl (pH 8.0), 1M Sodium Chloride, 0.5M EDTA (Na salt)

2) *Tris-EDTA Buffer made up of* 1M Tris-HCl (pH 8.0), 0.5M EDTA

After DNA was completely dissolved in the TE buffer, its quantity and quality was checked by both spectrophotometry and gel electrophoresis.

**Determination of DNA concentration by Spectrophotometry**

Prior to any analysis, DNA should be quantified and checked for purity and integrity. Based on its structure, DNA absorbs light in the ultraviolet range,

specifically at a wavelength of 260nm. A value of 1 at $OD_{260}$ is equal to 50ng/µl double stranded DNA. Therefore to calculate the concentration of DNA, the following formula can be used:

$$\text{Concentration of DNA} = 260nm_{abs} \times 50ng/\mu l$$

**Procedure**

2µl DNA sample was diluted to 200 µl with Double Distilled water (Dilution 1:100). Spectrophotometer was set to auto zero with the Double Distilled water. Optical Density (OD) of the diluted DNA aliquot was measured at 260 nm and 280nm using quartz crystal cuvette.

**Quality Assessment**

A ratio of OD values at 260nm and 280nm indicates the purity of the extracted DNA sample. If the ratio is within range of 1.6 to 2.0, then DNA sample is considered as clear and free from contaminants like residual protein and mRNA. An OD ratio less than 1.6 indicate the residual proteins or phenol contamination, whereas ratio of more than 2.0 indicates residual RNA contamination.

**Quantity Assessment**

DNA quantity was estimated as the OD value at 260nm of extracted sample is 1.00 then the concentration of the DNA is 50ug/ml.

Therefore, DNA concentration = OD at 260nm x 50 x Dilution factor.

**DNA quantification and electrophoresis**

Electrophoretic analysis of DNA using agarose gels can confirm DNA integrity. Typically intact genomic DNA will be up to 40KB in size, depending upon the species. Prepare 1% agarose gel has to be by adding required quantity of agarose to 1X Tris-Acetate-EDTA (TAE) buffer and mix well. Heat the mixture in microwave oven until it became clear and take care to avoid over boiling and evaporation. Cool the mixture to ~$50^0$ C and add ethidium bromide to make a final concentration of 0.001ug/ml. Pour the entire mixture into a tray in which combs are fixed to make wells in the gel. After gel formation, place the tray in buffer tank containing 1X TAE buffer for submerged gel electrophoresis and remove the combs with care to avoid rupture of wells. Mix 1µl of each DNA sample with 1µl of loading dye and load the mixture into the wells. Subject the Gel to electrophoresis at 90V for 30 minutes and visualise using gel documentation system where it is exposed to Ultraviolet rays. Under Ultraviolet rays exposure, DNA will give luminance which indicate the presence of DNA in the sample as shown in the below figure.

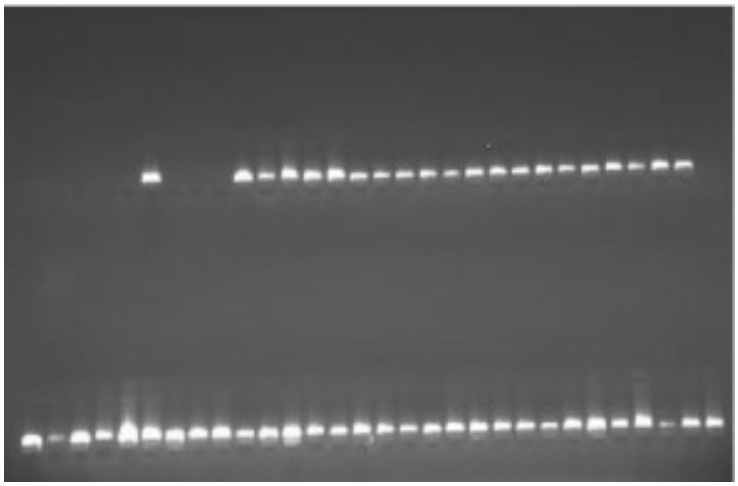


**Fig. 2.6: Electrophoresis gel picture showing DNA bands**

### 2.5.1 Polymerase Chain Reaction

PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in 1980s. It is an essential and ubiquitous tool in genetics and molecular biology. With the use of this technique we can clone DNA *invitro*.

PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre existing 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons). DNA sequencing by dye termination technique requires multiple copies of DNA, hence PCR is performed to generate numerous copies of DNA fragments of interest which were further used for sequencing.

The genomic DNA of each subject can be amplified on thermal cycler with an initial denaturation at $96^0$C for 3 minutes and later on for 35 cycles at $95^0$C for 60 seconds, at estimated annealing temperature of the primer for 45 seconds, extension at $72^0$ C for 2.30 minutes and a final extension at the end of $35^{th}$ cycle at $72^0$C for 7 minutes in a final volume of 10 µl containing 50mM KCl, 10mm Tris (pH 8.3), 1.5mM MgCl2, 75 ng of each primer, 100 µM deoxy-NTP, and 1 U *Taq* polymerase.

**Primer designing**

The main limitation of PCR technique is to provide short pieces of single-stranded DNA (primers) that are complementary to a part of target sequence. With the use of human genome sequence available we can now design primers to any region of interest in the human genome. The most critical step in PCR experiment is designing oligo-nucleotide primers. As poor primers could result in little or even no PCR product, alternatively they could amplify unwanted DNA fragments. Either will affect the downstream analysis. Many of the factors which affect the primers specificity and sensitivity like product size, primer size, tm, GC content, GC clamps and dimer formation can be adjusted as per the user requirement. Primers which fit the specified criteria can be checked for their specificity using NCBI BLAST.

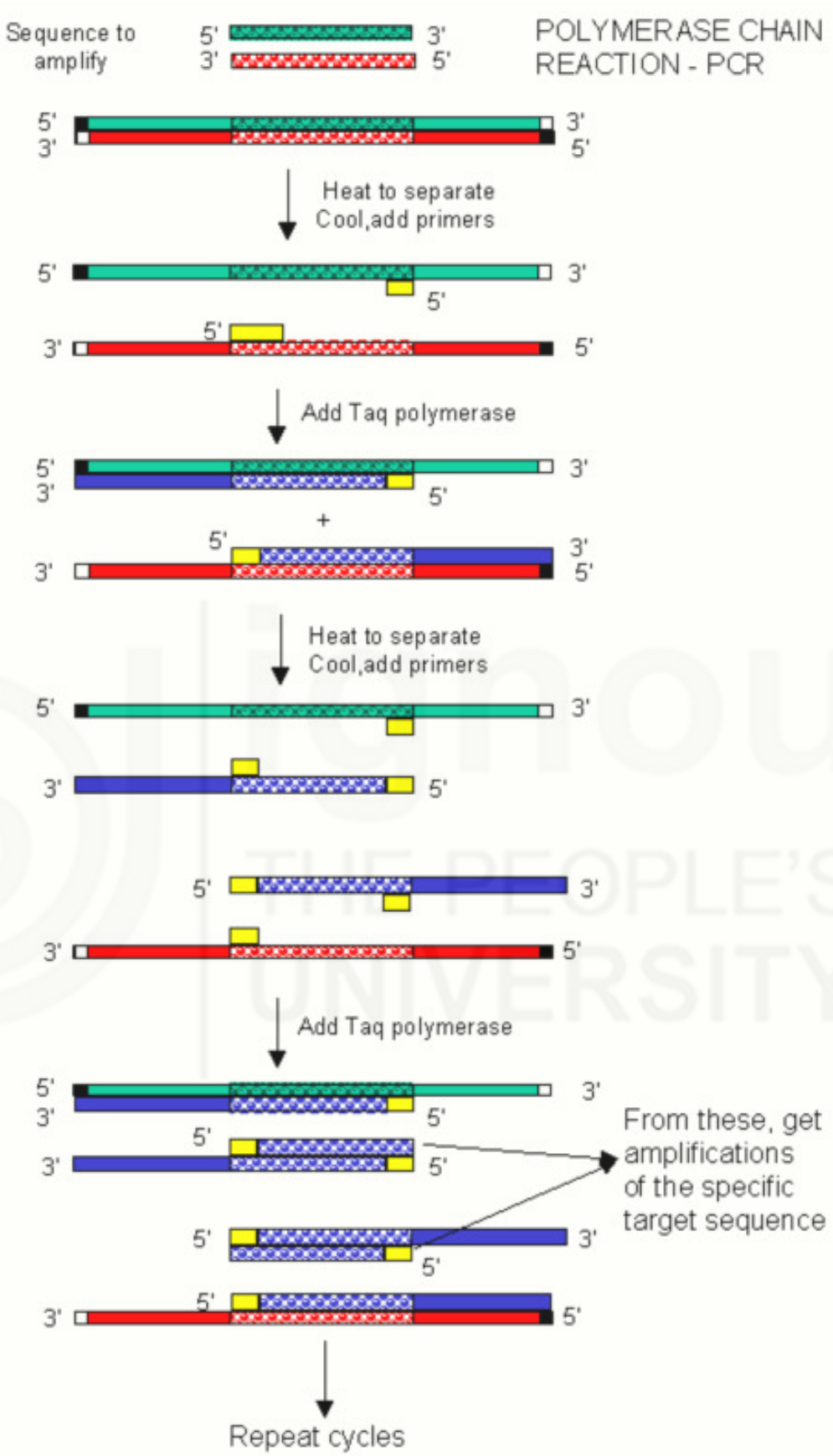



**Fig.2.7: Schematic diagram of Polymerase chain reaction**

## 2.5.2 Restriction Fragment Length Polymorphism

Restriction-Fragment Length Polymorphism (RFLP) was proposed by American geneticist David Botstein, biochemist Ronald W. Davis, population geneticist Mark Skolnick, and biologist Ray White. Restriction fragment length polymorphisms (RFLPs) can be used to produce a linkage map of the human genome and to map the genes that cause disease in humans.

Restriction Fragment Length Polymorphism (RFLP) analysis measures fragments of DNA containing short sequences that vary from person to person, called VNTRs. After extracting DNA from a sample and amplifying it with the technique known as Polymerase Chain Reaction, we can add restriction enzymes that cut the DNA at specific points. The resulting fragments can be sorted by length with gel electrophoresis technology to determine how many times a given VNTR is repeated.

If two different samples show VNTRs of different lengths, the samples could not have come from the same person. On the other hand, two samples showing VNTRs of the same length could have come from the same person, or from two people who happen to have VNTRs of the same length at that location. By comparing enough VNTRs from two individuals, however, the likelihood of a coincidental match can be reduced to nearly zero. RFLP testing requires hundreds of steps and weeks to complete, and it has been largely replaced by newer, faster techniques.


**Fig. 2.8: Restricted enzyme EcoR-I identifies its specific GAATTC sequence and Cuts between G and A of the DNA strand. a) Showing the cutting positions. b) Showing resulted four strand fragments after enzyme digestion**

### Short Tandem Repeats in DNA Analysis

STRs can be amplified and sequenced using PCR and Sequencing techniques. Analysis is based on the number of repeats present in the sample.

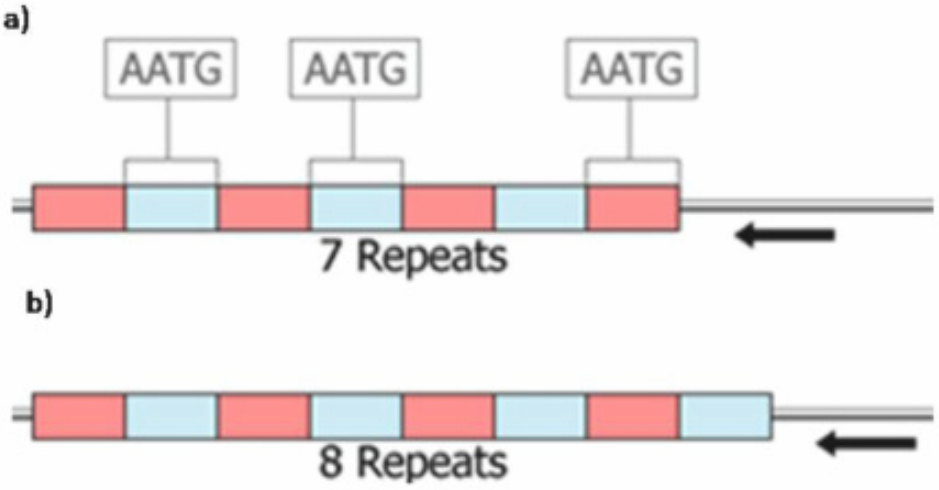



**Fig.2.9: a) One DNA strand showing 7 repeats (STRs) of AATG. b) Another DNA strand showing 8 repeats of AATG.**

### 2.5.3 DNA Sequencing Methods

In the 1960s and 1970s, British scientists Frederick Sanger and Alan Coulson, Alan Maxam and Walter Gilbert in the United States, developed DNA sequencing techniques. Automated equipment makes DNA sequencing a speedy, routine laboratory procedure. Sanger and Gilbert won the 1980 Nobel Prize in Chemistry for their work.

**Sanger method of DNA Sequencing**

In Sanger method, specific terminators of DNA chain elongation 2', 3'-dideoxynucleoside triphosphates are synthesized. These molecules can be incorporated normally into a growing DNA chain through their 5'- triphosphates groups. However, they cannot form phosphodiester bonds with the next incoming deoxynucleotide triphosphates (dNTPs). When a small amount of a specific dideoxy NTP is included along with the four deoxyNTPs normally required in the reaction mixture for DNA synthesis by DNA polymerase, the products are a series of chains that are specifically terminated at dideoxy residue. This forms the basis for Sanger's method.

**Procedure**

Initially single strand DNA is prepared through denaturation process. Then single strand DNA is mixed with a short end labeled piece of DNA (Primer) that is complementary to the end of single strand DNA. Labeling of primer is carried out using enzymes like Alkaline Phosphatase and Polynucleotide Kinase. After primer is annealed to DNA, sample is divided into four portions in four tubes. In each tube, along with DNA, Primer, DNA polymerase, a carefully controlled ratio of one particular dideoxynucleotide with its normal deoxynucleotide, and the other three dNTPs are added.

In each tube, DNA polymerase polymerizes normally from primer by utilizing nucleotides. When ddNTP is incorporated, the growth of that chain will stop. If the correct ratio of ddNTP: dNTP is chosen, a series of labeled strands will result, the lengths of which are dependent on the location of a particular base relative to the end of the DNA.

After suitable time period, the resultant labeled fragments in each tube are separated by size on an acrylamide gel. The separated fragments are detected by exposure of the gel to x-ray film through the process of autoradiography. From

the band developed in each lane of the autoradiograph and knowledge of which lane contain which base, the sequence of the complementary sequence can be obtained. From the complementary sequence, the sequence of the original strand can be easily determined with the help of Watson and Crick base pairing rule. Thus Sanger method is used for DNA sequencing.

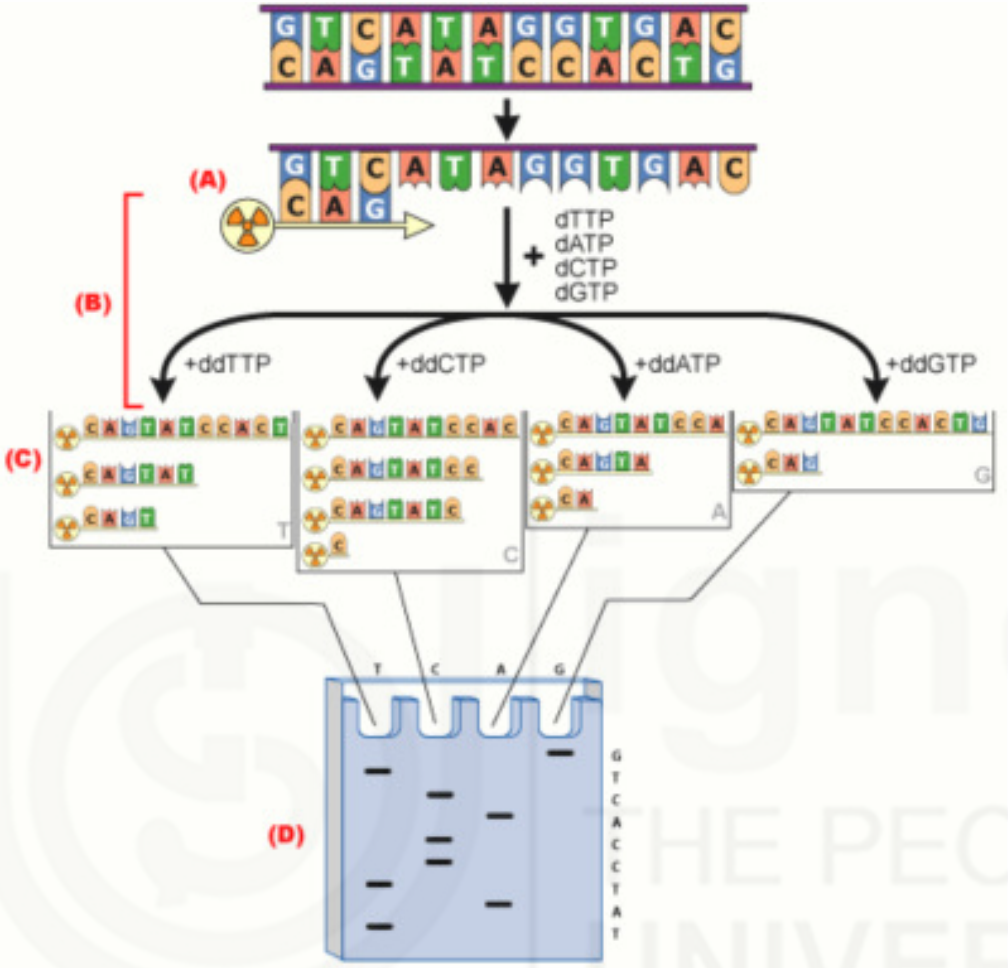



**Fig.2.10: Schematic diagram of Sanger's enzymatic sequencing**

## Automated DNA Sequencing

There are various methods available for DNA sequencing like chemical degradation, chain termination method, sequencing by ligation and micro fluidic Sanger sequencing etc. Advances in automation have opened gates to new fast and reliable automated DNA sequencing technologies. Owing to its greater expediency and speed, dye-terminator sequencing is now the mainstay in automated sequencing. Dye-terminator sequencing is a slight modification of the Sanger's chain termination method it utilizes labelling of the chain terminator ddNTPs, which permits sequencing in a single reaction. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes, with different wavelengths of fluorescence and emission. The dye labelled DNA fragments will be capillary electrophoresed and a detection system will identify the labelled bases when they pass through a laser that activates the dye.

## Cycle sequencing

To read the sequence of the amplified DNA termination with florescent dye labelled ddNTPs, each base PCR amplicon should be subjected to cycle reaction with one primer using BigDye terminator cycle sequencing ready reaction kits following the manufacturer's guidelines.

### Sequencing run

The PCR product should be added 10µl of Hi-Di formamide before feeding it to the sequencer machine.

### Sequence Alignment

The generated sequences can aligned to their respective reference sequences with the use of software called DNA baser. It performs sequence comparisons for variant identifications, SNP discovery and validation. It allows analysis of the re-sequenced data, comparing the consensus sequences to a known reference sequence. The reference sequences for the gene studied are obtained from NCBI Gen bank data base.

### Sequence Editing and Mutation Scoring

We can score the mutations from the aligned sequences by checking electropherograms of the DNA sequences. Genotypes can be exported from the software for further analysis.

Single Nucleotide Polymorphisms or SNPs (pronounced snips") are variations in a DNA sequence that occur when a single nucleotide in the sequence is different from the normal in at least one percent of the population. When SNPs occur inside a gene, they create different variants or alleles, of that gene.

Unlike repeated portions of DNA like STRs and VNTRs, in the case of SNPs it is the sequence itself, not its length that is useful to forensic scientists. SNPs are commonly occurring every 100 to 300 bases along the entire length of the human genome. Mutations in SNPs are very rare, so the sequences tend to be passed unchanged across generations. But because any given SNP is relatively common in the population, an analyst must examine dozens of SNPs to derive a true DNA fingerprint. For this reason, SNP analysis is rarely used in forensic cases.

**Figure:** Sequence showing Homozygous mutation

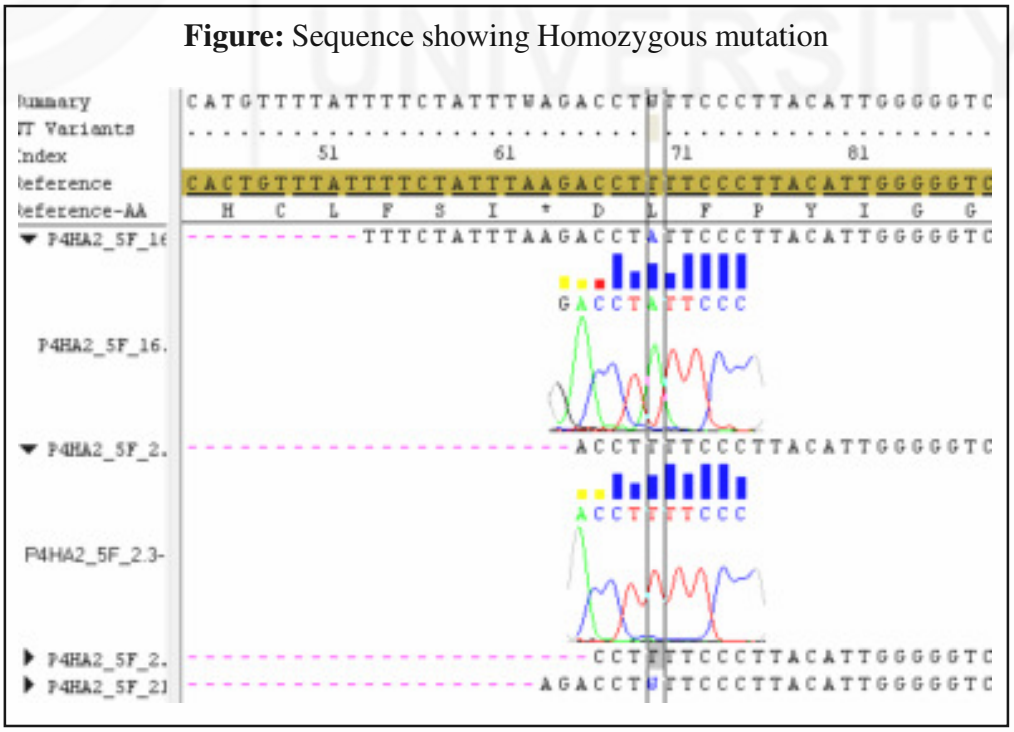



**Fig.2.11: DNA Sequence Electropherograms showing mutations**

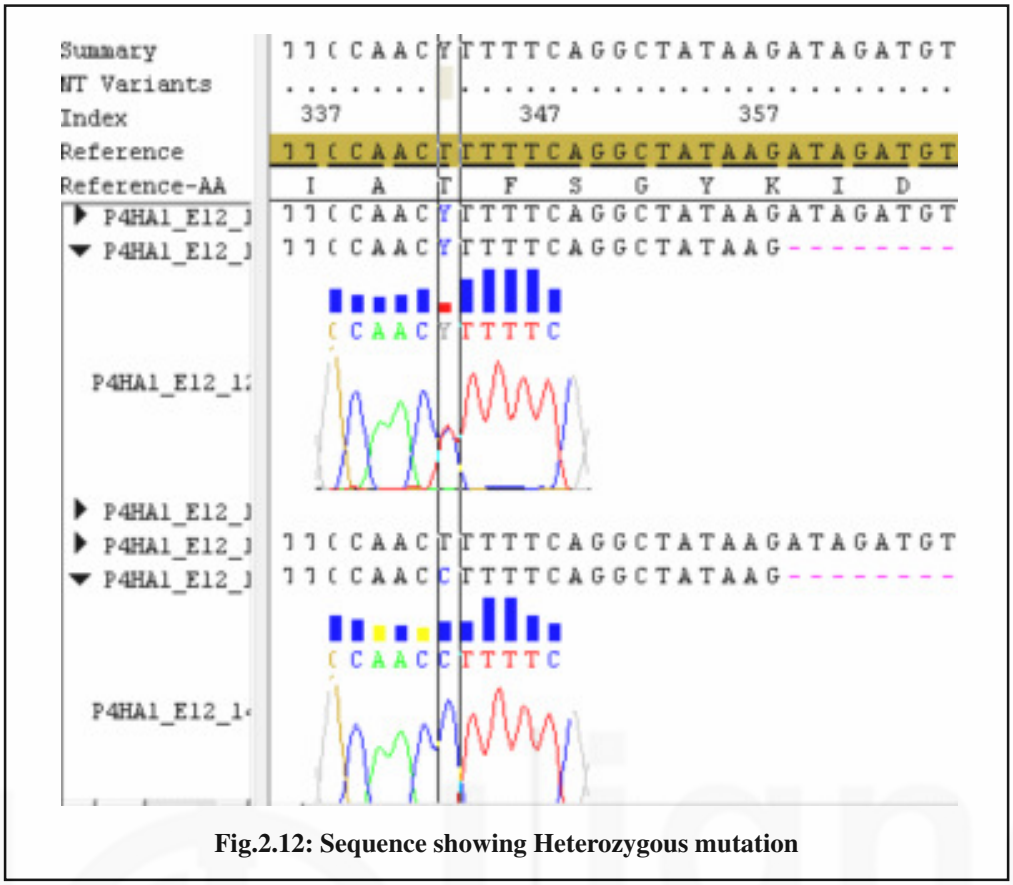



**Fig.2.12: Sequence showing Heterozygous mutation**

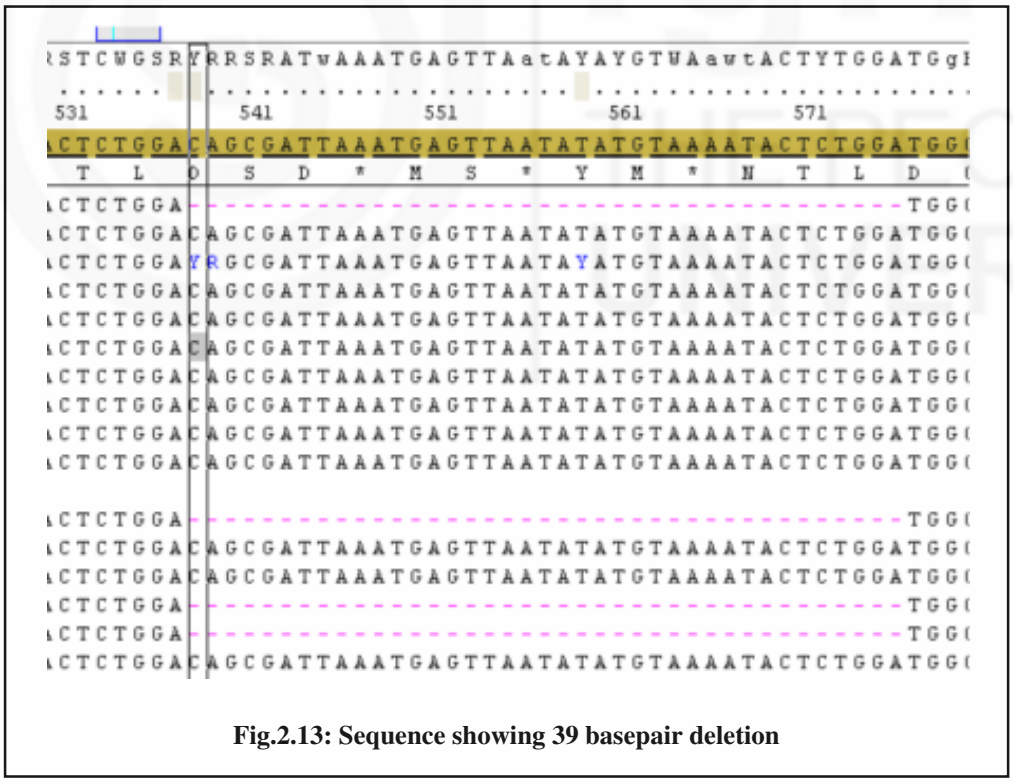



**Fig.2.13: Sequence showing 39 basepair deletion**

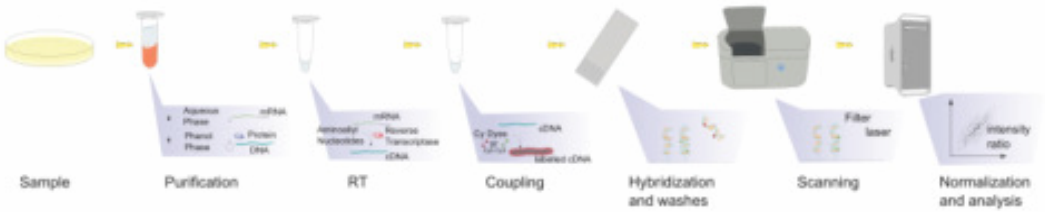
## 2.5.4 Microarray

A DNA microarray (also commonly known as gene chip, DNA chip, or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10-12 moles) of a specific DNA sequence, known

as probes (or reporters). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridisation is usually detected and quantified by detection of fluorophore, silver, or chemiluminescence-labelled targets to determine relative abundance of nucleic acid sequences in the target

DNA microarrays can be used to measure changes in expression levels, to detect single nucleotide polymorphisms (SNPs), or to genotype or resequence mutant genomes (see uses and types section).

**Principle**




**Hybridisation of the target to the probe**

The core principle behind microarrays is hybridisation between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter non-covalent bonding between the two strands. After washing off of non-specific bonding sequences, only strongly paired strands will remain hybridized. So fluorescently labelled target sequences that bind to a probe sequence generate a signal that depends on the strength of the hybridisation determined by the number of paired bases, the hybridisation conditions (such as temperature), and washing after hybridisation. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot. Microarrays use relative quantisation in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position.

Many types of arrays exist and the broadest distinction is whether they are spatially arranged on a surface or on coded beads: (i) The traditional solid-phase array is a collection of orderly microscopic "spots", called features, each with a specific probe attached to a solid surface, such as glass, plastic or silicon biochip (commonly known as a genome chip, DNA chip or gene array). Thousands of them can be placed in known locations on a single DNA microarray. (ii)The alternative bead array is a collection of microscopic polystyrene beads, each with a specific probe and a ratio of two or more dyes, which do not interfere with the fluorescent dyes used on the target sequence.

DNA microarrays can be used to detect DNA (as in comparative genomic hybridisation), or detect RNA (most commonly as cDNA after reverse transcription) that may or may not be translated into proteins. The process of measuring gene expression via cDNA is called expression analysis or expression profiling.

## 2.6 SUMMARY

DNA, or deoxyribonucleic acid, contains genetic information in the form of genetic code that is responsible for physical appearance of the organism, biological development and maintenance of life processes. Any change in the DNA leads to different form of DNA in the population. Occurrence of more than one form of DNA in the population is called as DNA polymorphism. This Polymorphism may be due to variation at single nucleotide base, an array of nucleotide bases or at chromosomal level. DNA polymorphisms are playing an important role in understanding human evolution and in unravelling the genetic basis of diseases.

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Palanichamy MG, Sun C, Agrawal S, Bandelt H-J, Kong Q-P, et al. 2004. *Phylogeny of mitochondrial DNA macrohaplogroup N in India, based on complete sequencing: implications for the peopling of South Asia.* Am J Hum Genet 75: 966–978.

**Web resources:**

http://ycc.biosci.arizona.edu/

http://yhrd.org

http://mitomap.org

http://phylotree.org

http://projects.tcag.ca/variation/decipher

http://ncbi.nlm.nih.gov/projects/SNP/

## Suggested Reading

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Sambrook, J. and Russell, D. 2001. *Molecular cloning: A laboratory manual*, 3rd Edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Human Genome Nature Issue (15 February 2001) *Nature* 409, 813-958. http://www.nature.com/genomics/human/

Human Genome Science Issue (16 February 2001) *Science* 291, 1177-1351. http://www.sciencemag.org/content/vol291/issue5507/index.shtml

U.S. National Human Genome Research Institute: www.ncbi.nlm.nih.gov/

About human genome project: http://www.genome.gov/10001772

Human genome Browsers and Integrated Databases: www.ensembl.org/

## Sample Questions

1) Define what in DNA polymorphism and its forms?
2) Explain how mtDNA polymorphism helped in understanding modern humans in Indian subcontinent?
3) Discuss the Y-chromosome tree?

## Short Notes

1) Polymerase Chain Reaction?

   Sanger method of DNA sequencing?

   RFLP and its uses?

   Microarray and its usage?

# UNIT 3 HUMAN GENOME PROJECT

**Contents**



**Learning Objectives**

After studying this unit, you would be able to:

- explain and appreciate the significance and outcome of the biology's largest programme – The Human Genome Project;
- to discover the secrets of life in terms of genetic makeup of biological systems;

- to understand the resemblance and differences between the humans and other organisms in terms of sequence variations;
- to explain the genetic differences between world populations and evolution of mankind; and
- discuss the applications of sequence information for the benefit of mankind and society in general.

## 3.1 INTRODUCTION

The life processes in any living organism are controlled by a set of genes that are located on chromosomes which are present in numbers that are highly specific for a given species. In humans there are 23 chromosomes present as pairs in all somatic cells (referred as diploid number or 2n) and as a single unit in gametic cells (referred as haploid number or n). Of the 23 pairs of chromosomes present in an individual, one set is inherited from the father and another from the mother along with the genes carried by them. Hence we see the resemblance of characters between the parents and their children. In human system there are trillion cells of different types that are organised into various tissues/organs that carry out myriad functions related to day to day life processes. All the functions carried out by these cells are controlled by genes located on the 23 chromosomes (table-3.1a and 3.2b).

**Table 3.1: Number of Entries in Online Mendelian Inheritance in man (OMIM) as reported on December 9, 2011**

| | Autosomal | X-Linked | Y-Linked | Mito-chondrial | Total |
|---|---|---|---|---|---|
| * Gene with known sequence | 13017 | 638 | 48 | 35 | 13738 |
| + Gene with known sequence and phenotype | 171 | 6 | 0 | 2 | 179 |
| # Phenotype description, molecular basis known | 3048 | 258 | 4 | 28 | 3338 |
| % Mendelian phenotype or locus, molecular basis unknown | 1654 | 136 | 5 | 0 | 1795 |
| Other, mainly phenotypes with suspected mendelian basis | 1800 | 129 | 2 | 0 | 1931 |
| **Total** | **19690** | **1167** | **59** | **65** | **20981** |

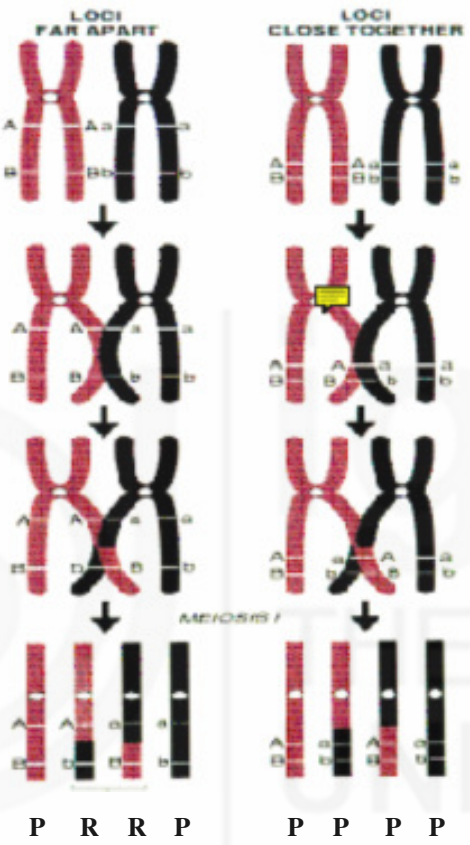
**Table 3.1b: Synopsis of human gene map**

| Chromosome | Loci | Chromosome | Loci | Chromosome | Loci |
|---|---|---|---|---|---|
| 1 | 1288 | 9 | 494 | 17 | 756 |
| 2 | 837 | 10 | 483 | 18 | 187 |
| 3 | 695 | 11 | 809 | 19 | 830 |
| 4 | 503 | 12 | 685 | 20 | 333 |
| 5 | 611 | 13 | 246 | 21 | 143 |
| 6 | 783 | 14 | 419 | 22 | 327 |
| 7 | 598 | 15 | 392 | X | 716 |
| 8 | 466 | 16 | 529 | Y | 46 |
| | | | | **Total** | **13176** |

Physical location of all the genes on different chromosomes of an organism is represented as "Genetic Maps". These maps are generated by determining distances between different genes present on a chromosome by an approach called linkage analysis [Box-3.1]. The distance between two genes is expressed as CentiMorgan (cM) which is a unit of genetic distance. Thus 1cM represents 1% probability of recombination occurring during meiosis i.e. gametogenesis. The genes present on the same chromosome are said to be linked and the group of such linked genes are called linkage groups or chromosomes.

**Genetic Mapping**

**a) Recombination**




**P: Parental combination of genes; R: Recombinants resulting due to crossing over occurring between the loci A and B**

**b) Linkage estimation**

Maximum likelihood estimate (mle) of recombination fraction (referred as è or x or r) is used for determining linkage between two loci on a chromosome based on the relative probability ($P_R$) of having obtained the family. The $P_R$ is determined by calculating the probability of having obtained the various combinations of the particular trait under consideration on the assumption of there being no measurable linkage ($H_0$= No linkage; è = 0.5) and comparing this with the probabilities based on a range of recombination fractions è varying from 0.00 to 0.05, i.e.

$$P_R = \frac{\text{P (FAMILY, GIVEN è = 0.0 to 0.5)}}{\text{P (FAMILY, GIVEN è = 0.0 to 0.5)}}$$

$P_R$ is expressed as its logarithm. The $\log_{10}$ of PR is called as log of the odds or lod score. Lod score value of = 3.0 indicates that the two loci tested are linked, value of 2.0 as evidence of strong linkage, value of 1.0 as evidence for tentative linkage and that of -2.0 absence of linkage. The Lod score of 2.0 that is suggestive of linkage can be further evaluated by analyzing more candidate/marker loci and by screening additional members of the family.

Chemically genes are made up of a macromolecule called Deoxyribose Nucleic Acid (DNA) which exists as a double helical structure resembling a ladder. Chemically, DNA comprises 4 nitrogenous bases- Adenine (A), Guanine (G), Thymine (T) and Cytosine (C) which are arranged as rungs of the ladder and supported by a sugar-phosphate backbone (Fig.3.1). Each base with a sugar and phosphate molecule is referred as a nucleotide. The nitrogenous bases Adenine and Guanine are referred as "Purines" and Thymine and Cytosine as "Pyrimidines". This structure of DNA as described by Watson and Crick (1953) satisfies all the criteria of a genetic material including the segregation of different genes/characters through generations.

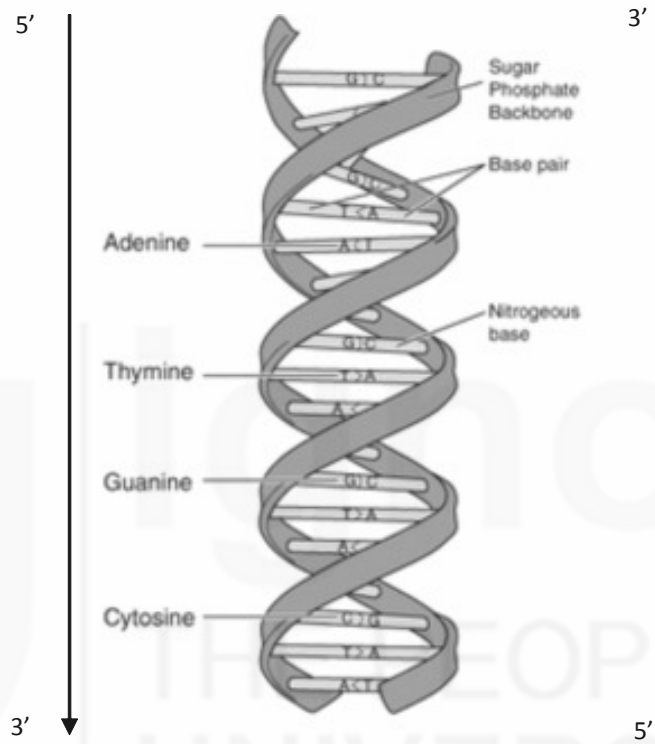



**Fig.3.1: Double helical structure of the genetic material**

The structure of de-oxyribose nucleic acid (DNA) comprising 4 nitrogenous bases (Adenine, Guanine, Thymine, Cytosine) each attached to a sugar and phosphate molecule that form a backbone. Adenine and Guanine are called purines and Thymine and Cytosine as pyrimidines. The base pairing is strictly complementary i.e. Adenine always pairs with Thymine while Guanine always pairs with Cytosine. The bases are held together by hydrogen bonds forming rungs of the ladder. The two strands of the DNA forming twisted double helical structure run in the opposite directions i.e. one strand runs from 5' to 3' and the other from 3' to 5'direction.

In early 1950s human geneticists have attempted to map some disease genes using certain genetic markers like ABO blood groups. One such study established close linkage between the loci for a disease called Nail-Patella syndrome and that of ABO blood groups. In the following years researchers attempted to map several other disease genes using different polymorphic loci [Box-3.2] related to serum proteins, enzymes and leucocyte (HLA) antigens. Later with the discovery of enzymes called restriction endonucleases or restriction enzymes (REs), new markers known as restriction fragment length polymorphisms (RFLPs) were identified which proved to be better markers in genetic analysis experiments.

Restriction enzymes cut the DNA at specific sites breaking them into fragments of different sizes. If a given DNA sequence has one restriction site 2 fragments of different lengths will be generated. The number of fragments generated will be n+1 when n number of restriction enzymes is used to cut the given DNA sequence. In later years certain sequences of nucleotides or base pairs (bps) were found to be repeated in various numbers differing in different individuals thus showing polymorphism. These stretches of repeats (0.1-20kb long) are referred to as variable number of tandem repeats (VNTRs) or minisatellites where the core repeat sequence of DNA carries 15 to hundreds of nucleotides. Initially DNA finger printing – a technique followed in forensic science used certain VNTR markers. Later smaller stretches of repeat sequences (<0.1 kb) called microsatellites with only 1-4 nucleotides (occurring as di, tri and tetranucleotide repeats) in each stretch were identified which are highly polymorphic. Now more than 6000 such markers located on different chromosomes are available for conducting any study. VNTRs and microstaellite markers were extensively used in 1990s in gene mapping studies and studies on their associations with diseases and risk predictions. With the discovery of single nucleotide polymorphisms (SNPs read as snips) which distinguishes individuals at single nucleotide level, research in human genetics took a different turn with the application of genome wide screening for mapping genes, finding differences between population groups, between normal and disease samples which in turn aids in formulating better treatment measures.

**Genetic Polymorphisms**

Genetic polymorphisms is defined as the presence of more than two allelic forms at a given locus found in individuals in such frequencies that the rarest of them does not occur just due to recurring mutations but it is due to a phenomenon called "polymorphisms". The frequency of the rarest allele/form as a rule is taken as > 1.0%. Several genetic loci related to red cell antigens, serum proteins, enzymes, leucocyte antigens and DNA markers have been identified over the years which are used in gene mapping, studies on associations with diseases and risk prediction and also tracing the origin of human population groups and estimating genetic distances. The different polymorphic markers available for such studies are given below

| Type of Marker | No. of Loci | Features |
|---|---|---|
| Blood Group antigens | >25 | Genotype cannot always be inferred from the phenotypes. Difficult for physical localization of genes |
| Serum Proteins | 30 | Often limited polymorphisms. Difficult for physical localization of genes |
| Leaucocyte antigens (HLA) | 1 | HLA system with A,B,C,D and DR loci each harbors hundreds of alleles thus resulting extensive polymorphism. ls highly informative useful in gene mapping |
| Restriction Fragment Length Pol-ymorphisms (RFLPs) | $>10^5$ | Bi-allelic markers, maximum heterozygosity 0.5, genotyped using Southern blotting and PCR techniques. Easy for physical localisation |
| Variable number of tandemn rep-eats (VNTRs) | $>10^4$ | Many alleles at each locus. Highly informative. Easy for physical localization of genes. Tend to cluster near the ends of chromosomes |
| Microsatellites (di, tri and tetra-nucleotide repeats) | $>10^5$ | Many alleles at each locus. Highly informative. Useful for physical localization of genes. More than 6000 markers identified that are distributed throughout the genome. Sometimes unstable. |
| Single Nucleotide Polymorphisms (SNPs) | $>4x10^5$ | Shows variation between any two individuals at single nucleotide level. Are most stable but less informative than microsatellites. Can be genotyped on a very large scale using automated sequencing. Useful for genome wide screening, gene mapping and risk prediction for diseases |

In spite of the availability of thousands of polymorphic markers (RFLPs, VNTRs, Microsatellites and snips), generation of human genetic map by mapping all the estimated 30,000 genes one by one appeared to be a Herculean task in that it is both tedious and time consuming. Hence to overcome this difficulty, the idea of determining the entire sequence of nucleotides in the DNA was floated which was discussed in depth at several scientific meetings before it was finally approved and the stage to undertake the Human Genome Project (HGP) was set.

**What is a genome?**

A genome represents total set of different DNA molecules (DNA content) including all of its genes along with spaces between them in an organelle (like mitochondria), cell or an organism. The genome in a species is organised in a specific manner with features to co-ordinate various functions and also reproduction to keep up with continuity of the species. Each genome is a blue print that contains all of the information needed to build and maintain an organism. Human genome is more complex with variation in its organisation found in the nucleus and mitochondrial components. A complete sequence of human mitochondrial (mt) genome was published in 1981 By Fred Sanger and his colleagues.

## 3.2 HUMAN GENOME PROJECT (HGP)

The human genome project was an international effort to sequence every nucleotide in the human genome and to identify all the genes contained within the genome. This effort was coordinated by United States Department of Energy and National Institutes of Health (NIH). It was the highest ever funded programme in biology and laboratories from UK, Japan and Germany were also associated with it.

### 3.2.1 History of HGP

The implementation of HGP was not instantaneous, but it was the outcome of careful efforts put forward by the scientists after several deliberations. In a way the idea of human genome sequencing was initiated in 1977, when the dideoxy DNA sequencing method (Fig 3.2a and 3.2b) that was simple and efficient was discovered by Sanger and his colleagues from Cambridge, UK. In 1980 it became apparent that better understanding of biology of organisms will be achieved when the detailed structure of DNA base-by-base is understood. In 1984 for the first time US Department of energy (DOE) held a workshop at Alta, Utah to address the problem of detecting low frequency of very rare mutations in humans exposed to radiations and other environmental hazards. The meeting focused on the methods and technologies needed for the detection and characterisation of the mutations (sudden heritable changes occurring in a gene/DNA) for which, it was felt that the entire genome sequencing required. In the following years, the meetings held led to the formal proposal of need to sequence human genome to derive benefits in furthering cancer research (Mc Conkey, 1993).

A dedicated Human Genome Project was conceived in 1986 by DOE at Mexico Conference and objectives, cost involved, time needed etc. were discussed. In 1987 DOE's repot on human genome initiative has fore seen three major objectives 1) Generation of refined physical maps of human chromosomes 2) Development of support technologies and fecilities for human genetic research

and 3) expansion of network and increasing the computational and database capabilities. DOE responded to Santa Fe Meeting's report in 1987 and in 1988 National Institutes of Health (NIH) set up an office for Human genome research to co-ordinate genome research activities of NIH and other organisations. US congress authorized NIH and DOE to allocate funds for HGP. 3-5% of the budget was allocated for the programmes on ethical, legal and social implications (ELSI). In the same year an International Human Genome Organisation (HUGO) was founded by the efforts of independent group of scientists to coordinate national efforts, facilitate exchange of research resources, public debate and availability of information on the implications of human genome research and also sequencing and mapping of cDNA. While not involved in any research by itself, HUGO organises workshops for the benefit of researchers in the field.

Finally on 1st October, 1990 a 15 years programme with the budget of $3-billion to sequence Human Genome was officially launched by US DOE and NIH. In addition to US, universities and research centers from United Kingdom, France, Germany and Japan were involved in the project. As the commencement of the project picked up, altogether 18 different countries and companies participated in the programme. In the following year The Genome Data Base (GDB) repository was established for human DNA mapping data and it was made available for all working in the field. James D. Watson, co-investigator of DNA double helical structure was recruited as director of National Institute for Human Genome research (NIHGR) who visualised the creation of complete catalogue of three billion base pair in the human genome and mapping of all the genes. He continued to direct the project till 1992 and was replaced by Francis Collins who took over the charge of HGP project in 1993 which was renamed as National Human Genome Research Institute (NHGRI) in 1997. A parallel initiative was undertaken by Celera Corporation in 1998 and Dr. J. Craig Venter working for the corporation led the programme of sequencing Human Genome. To catch up with public funded government programme the company started a faster and cheaper approach with the cost of $300 million as against government funding of $3.0 billion.

The public and private ventures competed neck to neck and announced the first draft of human genome sequence simultaneously on June 26th, 2000. The first rough working draft was published in Nature by the public funded government project (2001) and in Science by Celera (Venter et. al., 2001). Before that in 1999 an international consortium of HGP comprising geneticists from UK, France, Germany, Japan, China and India announced the first complete sequence of human chromosome 21 (Hattori et. al. 2000). The draft covered about 83% of the genome, and with 90% of the euchromatin regions with 150,000 gaps and order and orientation of many segments still to be established. Finally ~ 92% of the human genome sequence was completed in 2003 two years earlier than the target date set at 2005. This was possible mainly because of the development of advanced and efficient technologies for automated sequencing and networking programmes that were supported by the project.

With additional funding, HGP also focused to sequence several other nonhuman organisms including the bacteria Escherichia*. coli.*, nematode, *Caenorhabditis elegans* the fruit fly, *Drosophila melanogaster*, the yeast *Saccharomyces cerevisiae*, the mouse *Mus musculus.* etc. Information on the sequences of different organisms facilitates comparative mapping studies and understanding the differences and similarities (homology) with human genome and sequences that are conserved among the species during evolution. Originally HGP developed haploid reference genome that comprises 3.2 billion nucleotides.

Any scientific problem starts with a hypothesis and objective followed by stepwise protocol finding the cause including sequencing of gene(s) concerned. But in case of HGP, it reverses the way in which any scientific project is conducted. It first aims at sequencing and then interprets the results later. In other words it first identifies the putative gene(s) based on the nucleotide sequence but will not identify their functions. Thus human genome studies do not end with sequencing the putative gene(s). It has to go through the tedious and challenging process of identifying the boundaries between the genes and other features from the raw DNA sequence which is called "Genome Annotation". The future lies in knowing the functions of the genes, assessing the interaction between genes and the environment and also correlating the observations made with developmental, biochemical and physiological processes going on in an organisms.

### 3.2.2 Goals of Human Genome Project

**The goals set by Human Genome Project were:**

- Identifying all of the estimated 30,000 genes in human DNA and mapping each gene to a site on one of the 23 chromosomes.
- Production of a variety of physical maps of all human chromosomes and that of selected organisms.
- Determination of the complete sequence of human nuclear genome and that of selected model organisms.
- Development of the capabilities for collecting, storing, distributing and analyzing the data generated.
- Creation of necessary technologies to meet the goals of the project.

**In 1998 the following new 5 year goals were set**

- Identification of the human genome variation between persons (i.e. single nucleotide variations between any two persons) since such variations are expected to play an important role in individual's response to infections, drugs and toxins.
- Comparison of human genomes with that of model organisms like bacteria, mouse, yeast, nematode, fruit fly, etc.
- Developing advanced computational capability to collect, store and analyse sequencing data.
- Addressing the ethical, legal and social implications (ELSI) concerned to the use of genetic tools and data.
- Developing interdisciplinary training programmes for future genomics researchers.

### 3.2.3 Strategies of Sequencing

The basis for the human genome sequencing was the dideoxy sequencing method developed by Fred Sanger and his colleagues in 1977. The basic principle of the technique remained the same in HGP programme but with improvements made regarding the efficiency by using the fluorescence labeled automated sequencers and capillary sequencers which helped in obtaining much higher sequencing throughputs (Fig. 3.2a and 3.2b). Dedicated computer programmes like PHRED, PHRAP were developed simultaneously which helped sequence interpretation, scanning for overlapping regions and data assembly.

Two different approaches were used to determine the first draft of genome sequence 1) Public funded project planned by International Human Genome

Sequencing Consortium (IHGSC) headed by Francis Collins and 2) Planned by the Celera Genomics led by Craig J. Ventor (Fig.3.3).

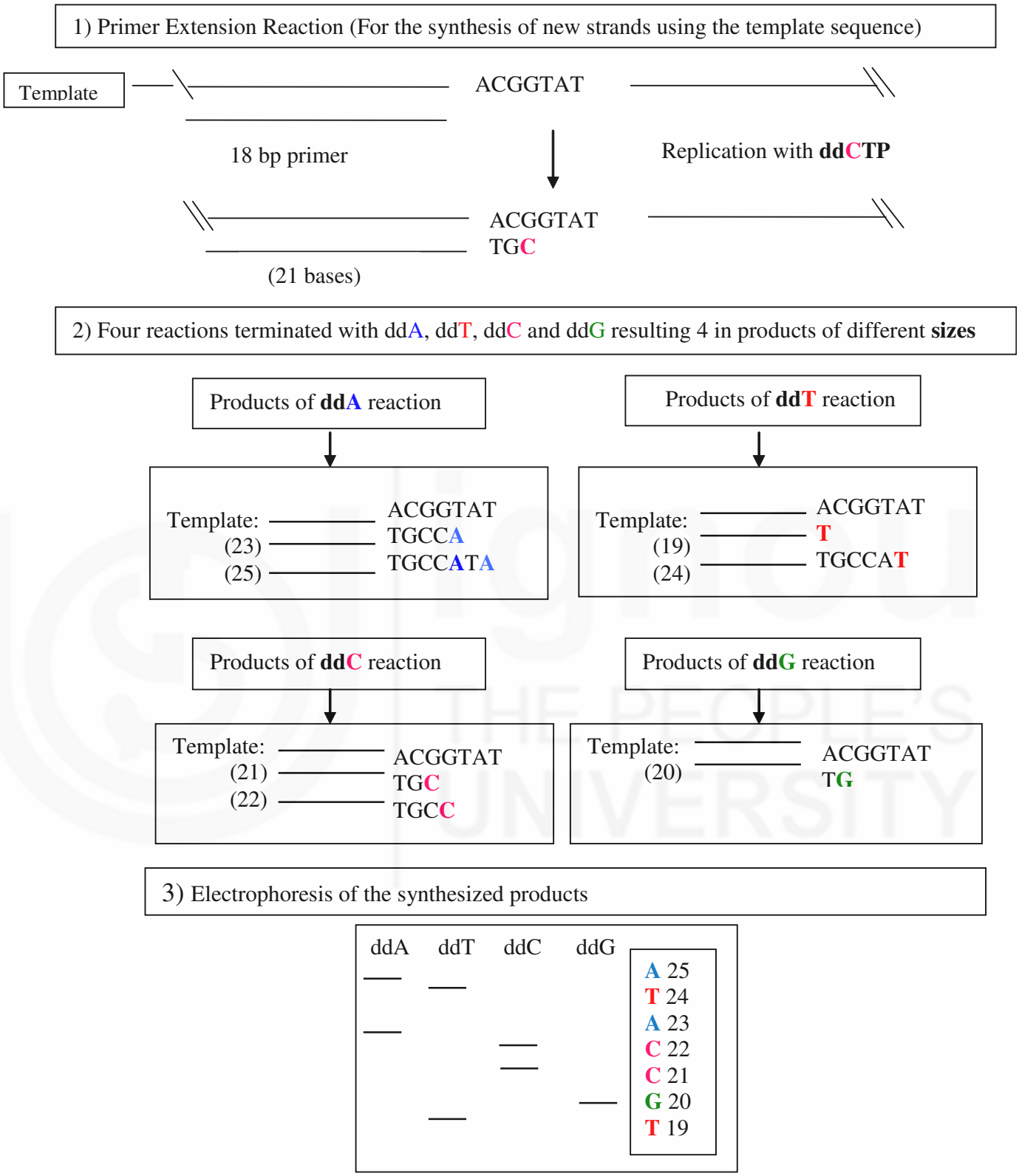



**Fig.3.2a: Sanger's Method of DNA sequencing**

Using the target sequence ACGGTAT, primer with 18 bps, radiolabelled dideoxy nucleotides (ddA, ddT, ddC and ddG) and polymerase enzyme new strands are synthesised. In the presence of dideoxy nucleotides the synthesis of new strands of DNA are terminated whenever the specific ddNTP is added. Thus products of different sizes are generated (that are of 19 to 25 bps length) which can be separated by gel electrophoresis. The original sequence (TGCCGT) can be read from the order/ladder of electrophoresis bands developed on the gel.

### 3.2.3.1 Approach of International Consortium

The public funded HGP was based on the "hierarchical shotgun" sequencing which involves random cleaving by sonification of starting DNA (from a chromosome) into several hundreds of fragments (150,000 bps in length) followed by end repair. These fragments are then cloned into a vector known as "Bacterial Artificial Chromosomes" or BACs which are derived from genetically engineered bacterial chromosomes. These vectors containing the genes or DNA fragments can be inserted into bacteria where they multiply using the bacterial DNA replication machinery. The BAC contents were known to correspond to specific locations on the chromosomes called sequence tagged sites (STSs). Several copies of each BAC were cut or 'shotgunned", into approximately 80 overlapping pieces which were then sequenced. A powerful computer programme was used to assemble the overlapping pieces into overall sequence for each chromosome. This process is nothing but mapping. The entire procedure is referred as "hierarchical shotgun" since the genome is first broken into relatively large pieces which are then mapped to chromosomes before being selected for sequencing.

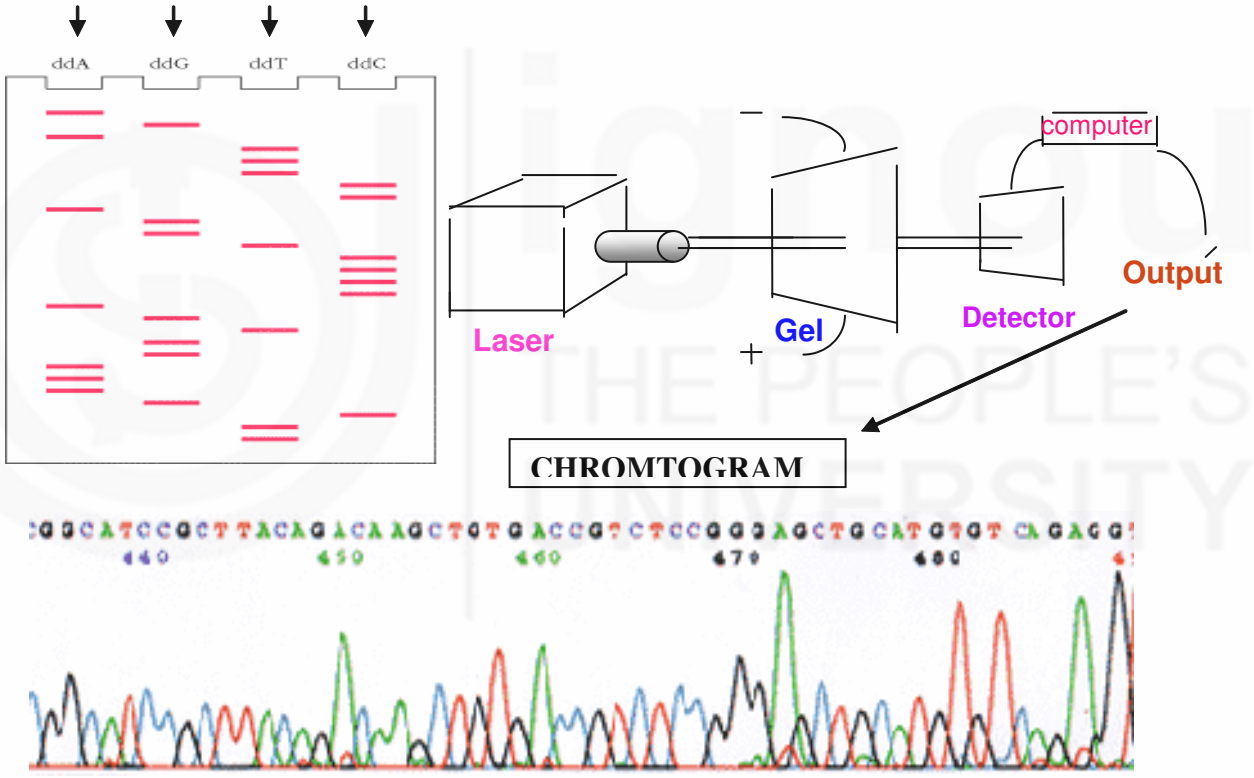



**Fig .3.2b: Automated DNA Sequencing**

The primer extension reactions carried out in automated sequencing is similar to that of Sanger's method except that the primers in each reaction are labeled with a different fluorescent staining molecule that emits light of a distinct color i.e. red for thymine, green for adenine, blue for cytosine and black for guanine. The different primer extension reaction products separate according to size upon gel electrophoresis. The bands are color coded. A laser beam that passes through the gel excites the fluorescent tag on each band and the detector analyses the color of the resulting emitted light. This information is converted into a sequence of bases and is stored in a computer. Print outs can be taken from the computer and the chromatogram will give the sequence details as peaks of different colors corresponding to the color of the fluorescence dye used for each base. In the

above diagram the sequence of nucleotides in 440- 446 positions are TCCGCTT that can be read by the color of the peaks.

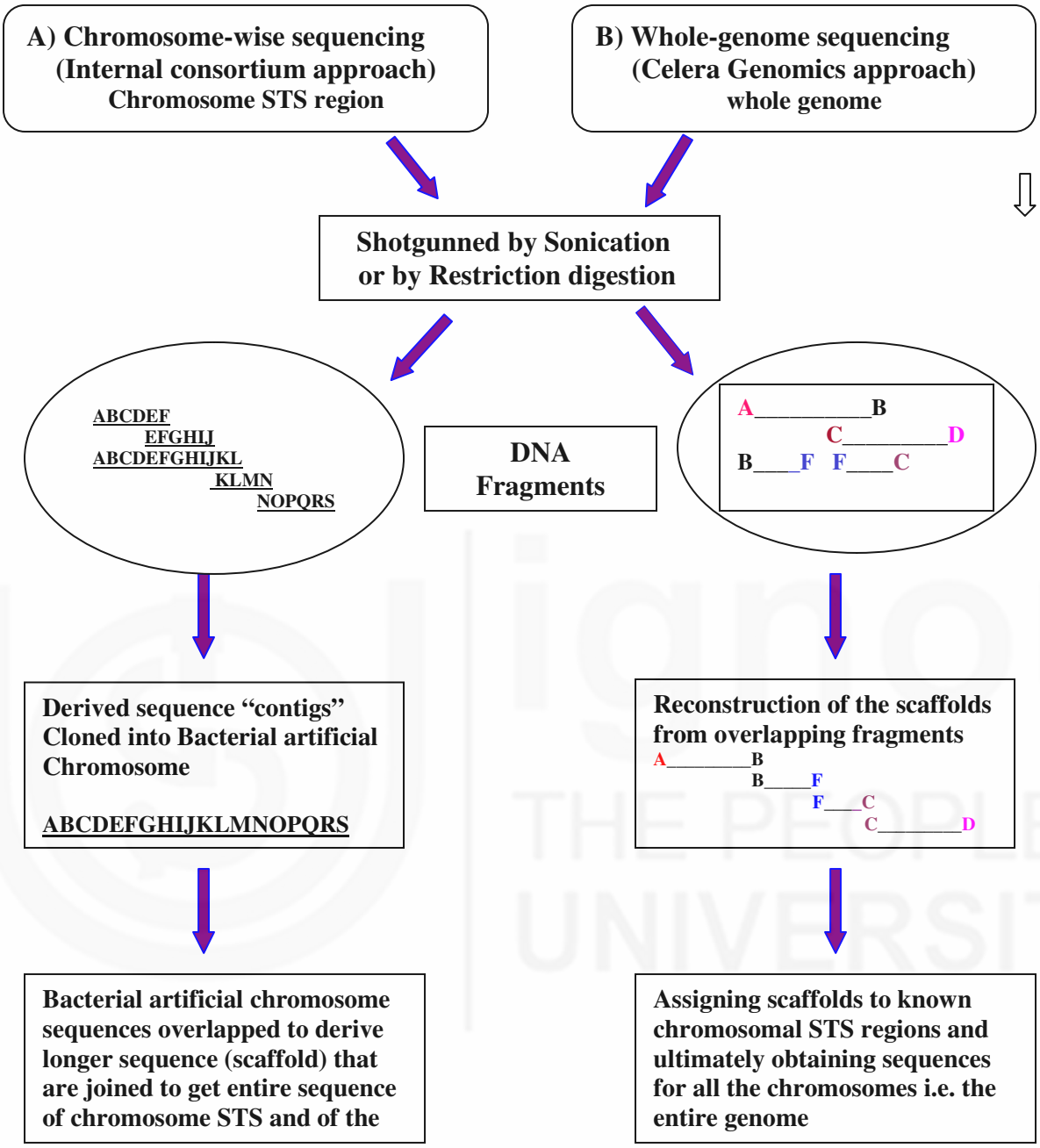



**Fig. 3.3 : Startegies of human genome sequencing. Methods adopted by A) International human genome consortium and B) Celera Genomics. Instead of nucleotide symbols A,G,T and C alphabets A to S are used for convenience.**

### 3.2.3.2 Approach of Celera Genomics

Celera Genomics headed by J. Venter followed "whole-genome shotgun" technique to sequence the human genome employing pairwise end sequencing. This technique was used to sequence bacterial genome of up to 6 million base pair in length, but not for large genome of 3.2 billion base pairs found in human genome. The technique skipped the BAC stage and used shotgunning multiple copies of the genome into small pieces. These pieces were then assembled into large overlapping sequences called "scaffolds" (frame work) using powerful computer programmes. There were 119,000 scaffolds which were assigned to

chromosomal sequence tagged sites (STSs). Celera company used information from public database but denied the access to any one to the private database generated by it. Celera's approach was rapid and of low cost involving only $ 3 millions as compared to publicly funded project of $3.2 billions.

### 3.2.4 Genome Donors for Sequencing

In the IHGSC, an international public-sector HGP, researchers collected samples of blood from females and that of sperm from males from large number of donors. Only a few of many of these (2 male and 2 female samples out of 20 each) were processed for DNA sequencing. Neither the donors nor the scientists knew the source of the samples and thus identity of the donors were protected. Much of the sequence (>70%) of the reference genome produced by the public HGP came from a single anonymous male donor from Buffalo, New York. For the Celera Genomics private-sector project samples were collected from 21 different individuals and only DNA of 5 individuals were used for sequencing.

### 3.2.5 Genome Assembly

Genome assembly which is a difficult computation method, is the process of arranging a large number of short sequences of DNA together to create a representation of the original chromosomes from which the DNA originated. In a shotgun sequencing project, all the DNA from an organism is first broken into millions of small pieces. These pieces are then "read" by automated sequencing machines, which can read up to 1000 nucleotides (with the bases adenine, guanine, thymine and cytosine). A genome assembly algorithm picks up all the pieces of DNA and aligns them to one another by detecting all regions where two of the short sequences, or "reads" overlap. These overlapping reads can be merged together, and the process continues. The draft genome sequence is produced by combining the sequenced contigs (ordered arrangement of cloned overlapping fragments) information and using linking information to create "scaffolds" (frame work). Scaffolds are then positioned or assigned to known chromosomal sequences tagged sites (STSs) creating a path.

### 3.2.6 Genome Annotation

Once the draft sequence is ready, Genome annotation has to be followed. Genome annotation is the process of attaching biological information to the sequences obtained. It is a major challenge for the HGP and covers a) structural annotation that deals with identification of genomic elements like open reading frames (ORFs), gene structure, coding regions and location of regulatory motifs and b) functional annotation that deals with attaching information about biological function, biochemical function, gene regulation and interactions and gene expression to the genomic elements. These steps involve both biological experiments and in silico analysis (bioinformatics).

Automatic annotation tools perform all the annotation by computer analysis.The basic level of annotation is using basic local alignment search tool (BLAST) for finding similarities between the sequences studied and then annotating genomes based on that. Genome annotation is an active area of investigation undetaken by different organisations which publish the results of their efforts in publicly available biological databases accessible via the web and other electronic means.

The HGP catalogued the information on the sequence of nucleotides in thousands of DNA fragments in a public database called GenBank maintained by US National Center For Biotechnology Information (NCBI) and sister organisations in Europe and Japan. From GenBank data base, sequences of known and hypothetical genes and proteins can be retrieved. The data bases are open for every one through internet. Other organisations like, Genome Bioinformatics group from University of California, Santacruz and Ensemble provide additional data and annotation and powerful tools for visualising and searching it.

### 3.2.7 Observations Drawn from Human Genome Sequencing

The draft genome sequence published in 2001 and the complete genome sequence published in 2004 reported the following findings:

- The human genome contains 3.1647 billion chemical nucleotide bases (A, C, T, and G).
- The total number of genes estimated at 30,000.
- The average gene consists of 3000 bases, but sizes vary greatly, with the largest known human gene being dystrophin at 2.4 million base.
- Almost all (99.9%) nucleotide bases are exactly the same in all people and the functions are unknown for over 50% of discovered genes.
- The number of genes in human beings is of same range found as in mice and round worms. Understanding how these genes express themselves and function would help to know how human diseases are caused.
- About 1.1% to 1.4% of the genome's sequence codes for proteins that carry out required functions in an organism.
- 98% of the genome is non-coding for proteins and misnamed as "junk DNA". Now much of the junk DNA is found to code for RNA which regulates other genetic and cellular functions.
- The human genome has high level of segmental duplications i.e. nearly identical, repeated sections of DNA than the other mammalian genomes. These repeated sequences may underline the creation of primate specific genes.
- Repetitive sequences are thought to have no direct functions, but they shed light on chromosome structure and dynamics.

Over time, the repeats reshape the genome by rearranging it, creating entirely new genes, and modifying and reshuffling existing genes. During the past 50 million years, a dramatic decrease seems to have occurred in the rate of accumulation of repeats in the human genome.

### 3.2.8 How is the Human Genome Arranged or Organised?

Genome sequencing facilitated better understanding of a) Nature of the genes controlling several traits b) Nature of Mutations resulting in altered functions of proteins c) Manipulation of the genome and predicting the consequences.

The human genome has gene-dense "urban centers" that are predominantly composed of the DNA building blocks G and C. In contrast, the gene-poor

"deserts" are composed richly of DNA building blocks A and T. Under the microscope GC- and AT-rich regions can be observed as light and dark bands on chromosomes representing euchromatin and heterochromatin regions. Genes appear to be concentrated in random areas along the genome, with vast expanses of long stretches of non-coding DNA between them. Stretches of up to 30,000 C and G bases repeating over and over often occur adjacent to gene-rich areas, forming a barrier between the genes and the "junk DNA." These are called CpG islands and are believed to help regulation of gene activity.

Unlike the human's seemingly random distribution of gene-rich areas, genomes of many other organisms are more uniform, with genes evenly spaced throughout. Humans have on an average three times as many kinds of proteins as the fly or worm because of "alternative splicing" of messenger RNA (mRNA). This process can yield different protein products from the same gene that transcribes mRNA. Humans share most of the same protein families with worms, flies, and plants, but the number of gene family members has expanded in humans, especially in case of proteins involved in development and immunity. The human genome has a much greater portion (50%) of repeat sequences compared to other organisms for e.g., mustard weed (11%), the worm (7%), and the fruit fly (3%).

Scientists have proposed many theories to explain evolutionary contrasts between humans and other organisms, including those of life span, litter sizes, inbreeding, and genetic drift.

## 3.3 BENEFITS OR APPLICATIONS OF HUMAN GENOME PROJECT

Benefits derived from human genome sequencing are enormous and a few of the applications are mentioned below. There are exceptional opportunities to develop genomic research commercially with production and sale of DNA-based products and technologies that are useful for the following fields.

### 3.3.1 Molecular Medicine

New era of molecular medicine and biotechnology have emerged from the knowledge derived from HGP. Molecular medicine instead of treating a disorder based on the symptoms, it digs at the root causes of diseases. It aims at developing rapid and more accurate diagnostic tests or genetic screening for early detection of many diseases that enables effective treatment especially for single gene disorders. In addition it looks into genetic factors causing susceptibilities to common complex conditions (like diabetes, hypertension, heart disease, etc.,) in conjecture to environmental conditions and habits/addiction of the persons to smoking etc. Such information will help in assessing the extent of risk and predict the likely onset of a disorder even before it is expressed in an individual. That is it enables "Preclinical" or "Pre-symptomatic" diagnosis by using DNA probes (short stretches of DNA sequences synthesized with base sequence that is complementary to the target gene sequence) that are specifically designed for the detection of different diseases/disorders even before they are expressed. It is also possible to replace the defective genes by the normal genes by the method called "Gene Therapy". In this method the normal gene or a target DNA sequence is incorporated into a vector (bacterial plasmid/a virus/liposome etc.,) and then transferred to patient's tissue grown in culture. Once the target sequence is transfected i.e. incorporated into the recipient cells they are tested for expression

of the transferred sequence or gene and then the tissue is grafted back into the patient where the incorporated normal gene will start functioning and the disease symptoms would disappear. Further, using genome sequence data novel therapeutic regimen can be developed using new classes of drugs, immunotherapy techniques and supplementing with the missing or defective protein.

### 3.3.2 Risk Assessment

It is understood from human genome analysis that nucleotide differences exist between different individuals which may be associated with their susceptibility or resistance to disease causing factors. Such an information will also be useful in assessing health damage and risks caused by exposure of individuals to radiations including long term low dose exposures and exposure to chemicals and toxins that induce harmful mutations and cancers and infections. This knowledge will help in modulating necessary preventive measures to maintain general health status and healthy society.

### 3.3.3 Energy and Environment

DOE initiated in 1994 for the Microbial genome Programme to sequence the genomes of bacteria which provide knowledge to benefit human health and environment apart from improving economy from industrial applications. Characterisation of complete microbial genomes will lead to the development of new energy related biotechnologies a) like photosynthetic systems, b) production of biofuels, c) microbial systems that work in extreme environments and also d) organisms that can metabolise readily available renewable resources and waste material. It is possible to develop diverse new products, processes and test methods that would help in maintaining pollution free environment. Above all knowledge of bacterial genomes helps pharmaceutical industries to identif how the pathogenic microbes cause diseases, in detecting new drug targets, identify the minimum number of genes necessary for maintaining life process and stand as models for understanding biological interactions and evolutionary history.

### 3.3.4 Anthropology, Evolution and Human Migration

Genomic information facilitated a) understanding of human evolution through germ line mutations in lineages b) knowing common biology the humans share with all other life c) study migration pattern of different population groups based on female genetic inheritance d) trace lineage and migration of males through the study of Y chromosome e) identify mutations and compare breakpoints in the evolution of mutations with ages of populations and historical events.

### 3.3.5 Forensic Science

Genome sequences are species specific and unique to different individuals. Hence genome sequence information used in forensic science is used for a) identifying victims who committed crimes, b) exonerate persons who are wrongly accused, c) identify crime and catastrophic victims d) establish paternity and identify relatives in cases of disputed parentage and e) matching the organ donors with that of recipient for organ transplantation. In addition identification of endangered and protected species among the wild life can be identified by analyzing their genomes of such species. It is possible to detect bacteria and other organisms that may pollute air, water, soil and food. The genomic information also helps in determining pedigrees of plant and livestock in breeding experiments.

### 3.3.6 Agriculture and Livestock Breeding Drought

Understanding of plants and human genomes allows the creation of disease resistant plants and more nutritious and pesticide free foods. Already the bio-engineered seeds that are insect, pests and drought resistant are being marketed. Similarly disease resistant live stocks and those that are more productive for meat and milk yield are also being developed using genome information.

## 3.4 DISADVANTAGES OF HGP

The HGP which yielded enormos benefits for scientific research and mankind also led to fears and concerns about the information generated specially about an individual affected with genetic disease for which diagnostic or predictive tests are availble. The major disadvantage is the discrimination by the fellowmen and society which an individual suffers when affected with a genetic disorder. Such individuals are deprived of insurance coverage and will have to face difficulties to meet the medical bills which could be exorbitant. Further they may lose employment opportunities and those employed may be fired by the employers as they fear that an affceted employee may create safety risk at the work place, to the customers and also other employees specially when the genetic condition affects the coordination and judgement as in case of some neurological disorders. While the genetic screening can benefit a family by providing measures for preventing the recurrence among other memebers, it can also destroy the marriages and family relationships. There are also chances of misusing the genomic information by persons with selfish motives and destructive attitude. This will have a tremendous negative effect. In addition to the government, researchers and scientists, people from all walks of life should realise the negative effects and curb them as HGP offers heaps of benefits to the mankind and we have to reap the benefits it offers.

## 3.5 POST GENOMIC ERA STUDIES

With the availability of genomic sequences from microbes to man, focus is laid on "functional genomics" that provides greater understanding of secrets of life. Research in post genomic era is being focused on:

- Transcriptomics - that involves large-scale analysis of messenger RNAs transcribed from active genes to follow when, where, and under what conditions such genes are expressed.
- Proteomics - that involves study of protein expression and function that explains actual happenings in the cell. This has direct application in designing drugs to treat several genetic diseases/conditions.
- Structural genomics - that generates the 3-D structures of one or more proteins from each protein family that offers clues to function and biological targets for drug designing.
- Experimental methods - for understanding the function of DNA sequences and the proteins they encode including knockout studies to inactivate genes (that are defective or undesirable) in living organisms and monitor changes that could reveal their functions.

- Comparative genomics - that involves analysis of DNA sequence patterns of humans and well studied model organisms for identifying human genes and interpreting their function

## 3.6 NEED FOR INDIVIDUAL DIPLOID HUMAN GENOME SEQUENCE

Originally HGP aimed at developing haploid reference genome that comprises 3.2 billion nucleotides. Other groups like International HapMap project, Appied Biosystem, Illumina, J. Craig Venter Institute(JCVI), Personal genome Project and Roche undertook the extension of obtaining reference sequence of diploid human genomes. On September 4th, 2007 Craig Venter's complete DNA sequence was published unveiling for the first time the 6 billion nucleotide genome (diploid) of a single individual. His genome was sequenced from the 32 million sequence reads or more than 20 billion base pairs of DNA produced. The diploid genome sequences uniquely catalogued the contributions of the parental chromosomes (in which two sets of chromosomes one from his father and the other from his mother are represented) showing the amount of variation existing between the two. The human reference genome (HuRef) analysis now revealed that:

- The human to human variation is 5-7 times greater as compared to that reported in the earlier haploid genome analysis. This works out to a difference of 15-30 million base pairs between individuals.
- There are 4.1 million DNA variants in an individual of which 22% are non-SNP variants (RFLPs, VNTRs and microsatellites) but they account for about 74% of all the variants found in the DNA.
- There are 3.2 million SNPs and nearly non-SNP variants that include indels (insertion/deletion of nucleotides), copy number variants, block substitutions and segmental duplications. In Venter's genome there were 1.2 million variants that were never before reported.

Analysis of diploid genome generated more informed haplotype assemblies. Haplotypes are linked variations found along the chromosomes (i.e. a set of alleles of different genes located on the same homologue with defined distance). The average occurrence of several haplotypes is reported in populations but not in individuals. Information on individual haplotypes enables study of rare or "private" variants which helps in predicting the traits and diseases in that person. This allows personalised medicine for treating a disease in an individual.

## 3.7 SPIN OFF OF HGP

### 3.7.1 1000 Genomes Project

Any two humans are considered to be more than 99 percent the same at the genetic level. However, it is important to understand the small fraction of genetic material that varies among people because it can help explain individual differences causing susceptibility to disease, response to drugs or reaction to environmental factors. To meet this end the "1000 Genomes Project" an international research effort to establish the most detailed catalogue of human genetic variation was launched in January 2008. The project aimed to cover

sequencing the genomes of at least one thousand anonymous participants from a number of different ethnic groups within three years time. With the expertise of multidisciplinary research teams, the 1000 Genomes Project will develop a new map of the human genome that will provide a view of biomedically relevant DNA variations at a greater resolution. The data generated from the 1000 Genomes Project is made swiftly available to the worldwide scientific community through freely accessible public databases. The consortium is expected to generate a valuable tool for all fields of natural science, especially genetics, medicine, pharmacology, biochemistry and bioinformatics.

In 2010, the 1000 genome project finished its pilot phase and increased the sample target to 2000 individuals to be studied by the end of 2010. Still larger project proposed by Wellcome Trust to sequence 10,000 human genomes in three years time to evaluate variantion specially related to diseases. To expand the link of genomic data to observable traits, Church from Harvard Institute, launched "Personal Genome Project" that ultimately aims to sequence 100,000 individuals who voluntarly share their medical records and lifestyle facts. These attempts would generate enoromous information about sequence variations in humans which has lot of applications in treating genetic disaeses, devloping new drugs, population diversity and human evolution.

### 3.7.2 Haplotype Map or HapMap

One of the projects that emerged as an off shoot of HGP is the "haplotype map" or "HapMap" project which is a tool that allows detection of genes and genetic variations that affect health and disease. The concept of HapMap was based on the view that though any two unrelated persons appear similar in that they share about 99.5% of their DNA sequence, the small fraction of difference between them may greatly affect the risk of an individual to develop a disease. Variation between any two persons are observed to occur at a single nucleotide level i.e. if one has an Adenine (A) nucleotide at a particular site on a chromosome other person may have a Guanine (G) nucleotide at the same position. Such a site is referred to as a single nucleotide polymorphism (SNP), and each of the two possibilities i.e. presence of A or G is called an "allele". Sets of nearby SNPs on the same chromosome are inherited as blocks. This pattern of SNPs on a block is called a "haplotype". While the blocks contain a large number of SNPs, a few SNPs are enough to uniquely identify the haplotypes in a block. The HapMap is a map of these haplotype blocks and the specific SNPs that identify the haplotypes are called "tag SNPs".

The "International HapMap Project" was set up in October 2002 with the collaborations of researchers at academic centers, non-profit biomedical research groups and private companies in Canada, Japan, Nigeria, the United Kingdom, and the United States. The target set for the completion of the project was three years and the information generated by the project is made freely available to researchers around the world through the database. The project was conducted in phases and the complete data obtained in Phase-I were published on 27$^{th}$ October, 2005 and that of Phase II was published in October, 2007 and Phase III dataset was released in spring 2009. The HapMap project focuses only on common SNPs, those occurring with a frequency of at least 1% of the population.

The HapMap is considered as a valuable tool since it facilitates reduction of number of SNPs required to examine in the entire genome for association with a

disease/phenotype from studying the 10 million SNPs to roughly 500,000 tag SNPs. This makes the genome scan approaches easier in detecting regions of interest or with the genes that are linked to diseases much more efficiently. The advantage is that there is no need to study more number of SNPs than necessary and all regions of the genome can be covered. Initially four populations were selected for inclusion in the HapMap: 30 adult-and-both-parents trios from Ibadan, Nigeria (YRI), 30 trios of U.S. residents of northern and western European ancestry (CEU), 44 unrelated individuals from Tokyo, Japan (JPT) and 45 unrelated Han Chinese individuals from Beijing, China (CHB). Although the haplotypes revealed from these populations should be useful for studying many other populations, parallel studies are also foreseen in additional populations in the project.

The HapMap provides a powerful resource for comparing the genetic factors of two groups of people with and without their response to environmental factors, susceptibility to infection and in the effectiveness of and adverse responses to drugs and vaccines. Using just the tag SNPs, researchers are able to find chromosome regions that have different haplotype distributions in the two groups of people, those with or without a disease or response to environment, drugs etc. This helps greatly in the therapeutic management of diseases.

### 3.7.3 Protein Structure Initiative

To understand how the genes function, we need to know the structure of the proteins produced by them. Such a study referred as "Structural Genomics" is a large scale study that requires several weeks and is also expensive even to determine a single protein structure. The NIH conducts "Protein Structure Initiative" to understand protein structural families, structural folds and the relation of structure to its function. Several companies are also working on this aspect concentrating on the proteins that are medically useful [Pollack, 2000).

### 3.7.4 Human Epigenome Consortium

Apart from knowing about the genome products (proteins etc), it is also necessary to know when and in which tissue the genes are swithched on or off to start their function or stop it. Such functions are presumed to be controlled by the epigenetic factors. "Epigenetic regulation" refers to regulatories processes that are not mediated by DNA codes but are carried out by mechanisms such as methylation of DNA and histone modification that is presumed to affect the access of transcription mechanisms of DNA, coding for a protein. Epigenetic regulation in clinical disorders is an emerging area of research. A consortium led by Sanger Center from UK, Max Plank Institute for Molecular Genetics from Berlin and a company called Epigenomics has initiated the study of every methylation site within the human genome – a project which could be as large as HGP itself (Hagman, 2000).

### 3.7.5 Human Genome Diversity Project (HGDP)

Human genome diversity project (HGDP) aims at finding and understanding the diversity and unity of the entire human species. The HGDP was thought of in 1991 by Luigi Luca Cavalli-Sforza, a population geneticist from Stanford University, USA. He and many genetiscists and anthroplogists were already collecting data and samples from several populations around the world mainly

to understand how the human populations are related or differ from each other. These samples stored in different laboratories spread over the world are of immense value and need to be analysed with proper planning. Cavalli-Sforza and his colleagues (1991) state that 'The populations that can tell us the most about our evolutionary past are those that have been isolated for some time, are likely to be linguistically and culturally distinct and are often surrounded by geographic barriers". Such isolated populations are getting rapidly merged with neighbouring groups and the information needed to reconstruct our evolutionary history is being lost. Apart from this, keeping in view the danger of some populations becoming extinct, Cavalli-Sforza and other population geneticists expressed the urgency of implementing the project - HGDP. Finally HGDP was planned in 1993 under the auspices of HUGO with estimated cost of $23-35 millions with a time scale of 5 years for its completion. The project focused on two objectives 1) to trace the evolution and migration of different human populations 2) to identify genes which confer resistance and volunerability to diseases along with the devlopment of traetment modalities and tests required.

The project involved collection, preservation and analysis of human DNA samples from various ethnic groups from around the world specially from small indigenous endangered groups. Blood, skin and hair samples from hundreds of ethnic groups around the world. New tools are used to store genetic information indefinitely by devloping cell lines and DNA segments using polymerase chain reaction (PCR) technique. Any researcher can have access to these samples for the future studies.

From 5000 populations groups across the world the initial documentation from HGDP planning workshops listed 700 target groups. After facing several criticism and scentific debates, HGDP changed its approach indicating that samples should be collected from minority and majority ethnic groups in industrial countries and emphasised that all groups should agree to participate in the project. It also emphasized that selection of the indigeneous groups should depend largely on with which groups the anthropologists have been working or members of the groups who mediate with the study group and the outside world.

Coming to the benefits foreseen by the HGDP project, it enables researche into 1) human origins, 2) migratory and mating patterns, 3) adaptation, 4) disease identification and 5) forensic anthropology. The anthropologists and archeologists are concerned about the origins of human species. Scientists now claim that humans evolved only in Africa, then spread themselves around the world. But there are also possibilities for the simultaneous evolution from several other locations and HGDP may throw light on this claim. The project is also expected to help in measuring the geneological raltionships between the populations by providing information on the ancient migratory patterns like settlements of America and Australia from Asia apart from providing cues about the evolution, dispersal and current distribution of languages. Further comparing the genetic variations in the neighbour hood populations of indigeneous groups, it is possible to understand to what extent these groups are inbred and how long ago these populations have reached the terrotories which are now occupied by them. Maping the "geography" of human genes will be of great value not only to the population geneticists but also to linguists, anthropologists, archeologists and historians. This makes the implementation of HGDP a valid one.

## 3.8 ETHICAL, LEGAL AND SOCIAL IMPLICATIONS (ELSI)

A project like HGP is expected to be associated with several serious ethical, legal and social implications (ELSI). Hence 3-5% of the budget allocated for HGP was divereted to meet ELSI. Ethical issues are those that raise questions about what is moral and right, legal issues are those that are concerned with the protection of laws and regulations that should be provided and social issues are those that affect the individuals and society at large. These three aspects are interdependent and should be dealt with promptly. Discussions on these aspects emphsised that clear written consent should be obtained from the paricipants after they are explained about the project, pros and consequences and risks if any. The participants should willingly co-operate with the project proceedings and no force should be imposed. Guide lines have been developed taking care to cover several such points.

## 3.9 SUMMARY

Human Genome project (HGP) is an international initiative implemented in October 1990 to sequence the entire human genome comprising 3.2 billions, base by base with a cost of $3.0 billions within the time frame of 15 years. The project also supported sequencing of several model organisms including that of fruit fly, yeast, mice, bacteria, nematode etc., since the comparative sequence data can help in identifying the new genes and disorders in the human system. The project also supported the development of technologies for high throughput sequencing and of capabilities of computing and storing the sequenced data in to the free data bases like NCBI, Ensembl, GenBank etc. Two different strategies were used for sequencing 1) Strategy employed by the public fundended International human genome consortium where STS regions of each chromosome were shotgunned and the fragments were cloned in to bacterial artificial chromosomes (BACs). Later the sequences from the BAC clones were arranged into longer fragments by joining the overlaps to obtain entire sequence. 2) Strategy proposed by the company Celera Genomics involved shotgunning of the entire genome, developing scaffolds, arranging the overlapping sequences and then assigning them to known chromosomal STS sites. The HGP though discriminates individuals affected with genetic disorders, it offers several benefits like diagnosing diseases, drug designing leading to personalised medicine, assessing the genotypes offering risk or resistance to infections and environmental factors, reducing environmental pollution in developing economically beneficial plants and live stock etc. HGP information culminated in the development of new approaches in the post genomic era like proteomics, transcriptomics etc., which is expected to help in identifying the functions of genes, effect of epigenetic factors in modifying the functions of the genes and phenotypes in establishing biological relationships, in understanding evolutionary process etc., The efforts put by the researchers in this direction led to the development of additional projects like haplotype map (HapMap) project, Human Genome Diversity projects (HGDP). Keeping in view several sensitive issues like discrimination of individuals and population groups, the project also emphasised and allocated the budget to address the ethical, legal and social implications (ELSI). The project in general opened up several avenues for the researchers to answer the questions raised to benefit the humaninty and society at large.

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### Sample Questions

1) What is a genome? What do you know about the Human genome Project, its origin, development and implementation?
2) What are the objectives proposed by the Human Genome Project?
3) What were the strategies adopted to sequence the human genome?
4) Enumerate the benefits of the implementation of Human Genome Project.
5) What do you understand by Human Genome Diversity Project? How does it help in understanding the evolution of mankind?
6) Explain briefly the devlopments foreseen in post genomic era.

# GLOSSARY

**1000 genome project** : **1000 Genomes Project**, launched in January 2008, is an international research effort to establish by far the most detailed catalogue of human genetic variation. Scientists plan to sequence the genomes of at least one thousand anonymous participants from a number of different ethnic groups within the next three years, using newly developed technologies which are faster and less expensive.

**Alternative Splicing** : Various ways of splicing out introns in eukaryotic pre-mRNAs resulting in one gene producing several different mRNAs and protein products.

**Bacterial Artificial Chromosome (BAC)** : A **bacterial artificial chromosome (BAC)** is an engineered DNA molecule used to clone DNA sequences in bacterial cells (E. coli). Segments of an organism's DNA, ranging from 100,000 to 300,000 bps can be inserted into BACs. The BACs with inserted DNA are then taken up by bacterial cells and as they grow and divide, they amplify the BAC DNA which can then be isolated and used in sequencing.

**cDNA** : DNA synthesized by reverse transcriptase using RNA as a template.

**Copy number variations:** are alterations of the DNA of a genome that results in the cell having an abnormal number of copies of one or more sections of the DNA.

**CpG island** : CpG islands or CG islands are genomic regions that contain a high frequency of CpG sites.

**Diploid** : The state of having each chromosome in two copies per nucleus or cell. A cell having two chromosome sets, or an individual having two chromosome sets in each of its cells.

**Epigenesis** : The theory that an individual is developed by successive differentiation of an unstructured egg rather than by a simple enlarging of a preformed entity. The theory holding that development is a gradual process of increasing complexity. For example, organs are formed de novo in the embryo rather than increasing in size from pre-existing structures.

**Epigenetic factors** : Any factor that is responsible for gene activity/ inactivity without altering the base sequence by way of substitution, insertion or deletion. This factor may alter histones or/ and DNA methylation.

**Gene therapy** : The correction of a genetic deficiency in a cell by the addition of new DNA and its insertion into the genome.

**Genetic mapping** : Done based on co-segregation of disease and marker loci & determination of lod scores (likelihood ratios) and is called Linkage Analysis.

**Haploid** : The state of having one copy of each chromosome per nucleus or cell. A cell having one chromosome set, or an organism composed of such cells.

**Haplotype** : set of closely linked genetic markers present on one chromosome which tend to be inherited together (not easily separable by recombination).

**HGP** : The **Human Genome Project** (**HGP**) is an international scientific research project with a primary goal of determining the sequence of chemical base pairs which make up DNA, and of identifying and mapping the approximately 20,000–25,000 genes of the human genome from both a physical and functional standpoint.

**In silico analysis** : Analysis performed using the computers in conjunction with informatics capabilities.

**Linkage** : is the tendency of certain loci or alleles to be inherited together. Genetic loci that are physically close to one another on the same chromosome tend to stay together during meiosis, and are thus genetically *linked*.

**Methylation** : The modification of a strand of DNA after it is replicated, in which a methyl ($CH_3$) group is added to any cytosine molecule that stands directly before a guanine molecule in the same chain.

**mRNA** : An RNA molecule transcribed from the DNA of a gene, and from which a protein is translated by the action of ribosomes. The basic function of the nucleotide sequence of mRNA is to determine the amino acid sequence in proteins.

**Mutation** : Mutation is a permanent change in the DNA sequence of a gene. Mutations in a gene's DNA sequence can alter the amino acid sequence of the protein encoded by the gene.

**ORF** : A section of a sequenced piece of DNA that begins with an initiation (methionine ATG) codon and ends with a nonsense codon. ORFs all have the potential to encode a protein or polypeptide, however many may not actually do so.

**Personal genome project :** The **Personal Genome Project** (PGP) is a long term, large cohort study which aims to sequence and publicize the complete genomes and medical records of 100,000 volunteers, in order to enable research into personalised medicine.

**Polymorphism** : Genetic Polymorphism is the presence of more than two allelic forms at a given locus in such frequencies in a population that the rarest of them is not just due to recurring mutations but is due to a phenomenon called "polymorphisms". The frequency of the rarest allele/form as a rule is taken as > 1.0%.

**Pre-symptomatic** : Relates to the early phases of a disease when accurate diagnosis is not possible because symptoms of the disease have not yet appeared.

**Recombination** : is a process by which a molecule of nucleic acid (usually DNA, but can also be RNA) is broken and then joined to a different one. Recombination can occur between similar molecules of DNA, as in homologous recombination, or dissimilar molecules, as in non-homologous end joining.

**Regulatory motifs** : A sequence motif is a nucleotide or amino acid sequence pattern that is widespread and has, or is conjectured to have, a biological significance.

**Scaffold** : The eukaryotic chromosome structure remaining when DNA and histones have been removed; made from nonhistone proteins. The central framework of a chromosome to which the DNA solenoid is attached as loops; composed largely of topoisomerase.

**Segmental duplication** : Segmental duplications are segments of DNA with near-identical sequence.

**Sequence tagged site (STS)** : Any site in a chromosome or genome that is identified by a known unique DNA sequence. STSs can be used to form genetic maps by standard mapping procedures.

**Sequencing** : Determination of the order of nucleotides (base sequences) in a DNA or RNA molecule or the order of amino acids in a protein.

**Shotgun** : Cloning a large population of different DNA fragments, known to contain a fragment of interest, as a prelude to selecting or screening for that one particular clone containing the fragment of interest for intensive study.

**Sonication** : The process of dispersing, disrupting or inactivating biological material (e.g. viruses) by sound waves.

**YAC** : A **yeast artificial chromosome** (**YAC**) is a human engineered DNA molecule that acts as vector. Segments of an organism's DNA, ranging one million bps can be inserted into YACs. The YACs, with inserted DNA are then taken up by the yeast cells. As the yeast cells grow and divide, they amplify the YAC DNA, which can then be isolated and used for the physical mapping of complex genomes and for the cloning of large genes.

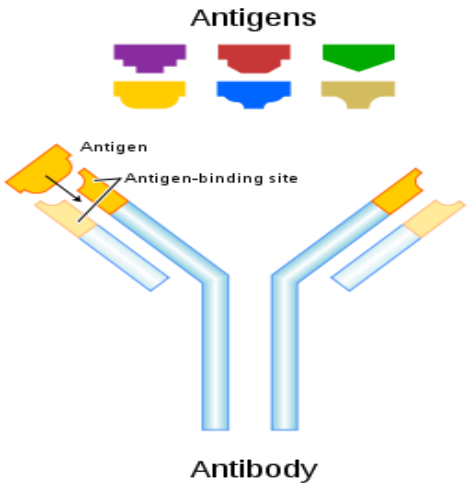
In the following years researchers attempted to map several other disease genes using different polymorphic loci [See Box-2] related to serum proteins, enzymes and leucocyte (HLA) antigens. In later years DNA or molecular markers like restriction fragment length polymorphisms (RFLPs), variable number of tandem repeats (VNTRs) or minisatellites, 1-4 nucleotides repeats (di, tri and tetra nucleotide repeats) called microsatellites and single nucleotide polymorphisms (SNPs or snips) were discovered and were used in gene mapping studies.

**BT3352- IMMUNOLOGY**

**UNIT I ANTIGENS**

An **antigen** is a substance/molecule that when introduced into the body triggers the production of an antibody by the immune system which will then kill or neutralize the antigen that is recognized as a foreign and potentially harmful invader. These invaders can be molecules such as pollen or cells such as bacteria. Originally the term came from **anti**body **gen**erator and was a molecule that binds specifically to an antibody, but the term now also refers to any molecule or molecular fragment that can be bound by a major histocompatibility complex (MHC) and presented to a T-cell receptor"Self" antigens are usually tolerated by the immune system; whereas "Non-self" antigens are identified as intruders and attacked by the immune system. Autoimmune disorders arise from the immune system reacting to its own antigens.

**Antigen**




Each antibody binds to a specific antigen; an interaction similar to a lock and key.

Similarly, an **immunogen** is a specific type of antigen. An immunogen is defined as a substance that is able to provoke an adaptive immune response if injected on its own.Said another way, an immunogen is able to induce an immune response, while an antigen is able to combine with the products of an immune response once they are made. The overlapping concepts of **immunogenicity** and **antigenicity** are thereby subtly different. According to a current text book:

Immunogenicity is the ability to induce a humoral and/or cell-mediated immune response

Antigenicity is the ability to combine specifically with the final products of the [immune response] (i.e. secreted antibodies and/or surface receptors on T-cells). Although all molecules that have the property of immunogenicity also have the property of antigenicity, the reverse is not true."

At the molecular level, an antigen is characterized by its ability to be "bound" at the antigen-binding site of an antibody. Note also that antibodies tend to discriminate between the specific molecular structures presented on the surface of the antigen (as illustrated in the Figure). Antigens are usually proteins or polysaccharides. This includes parts (coats, capsules, cell walls, flagella, fimbrae, and toxins) of bacteria, viruses, and other microorganisms. Lipids and nucleic acids are antigenic only when combined with proteins and polysaccharides. Non-microbial exogenous (non-self) antigens can include pollen, egg white, and proteins from transplanted tissues and organs or on the surface of transfused blood cells. Vaccines are examples of immunogenic antigens intentionally administered to induce acquired immunity in the recipient.

Cells present their immunogenic-antigens to the immune system via a histocompatibility molecule. Depending on the antigen presented and the type of the histocompatibility molecule, several types of immune cells can become activated.

### *Related concepts*

- **Epitope** - The distinct molecular surface features of an antigen capable of being bound by an antibody (a.k.a. *antigenic determinant*). Antigenic molecules, normally being "large" biological polymers, usually present several surface features that can act as points of interaction for specific antibodies. Any such distinct molecular feature constitutes an epitope. Most antigens therefore have the potential to be bound by several distinct antibodies, each of which is specific to a particular epitope. Using the "lock and key" metaphor, the antigen itself can be seen as a string of keys - any epitope being a "key" - each of which can match a different lock. Different antibody **idiotypes**, each having distinctly formed complementarity determining regions, correspond to the various "locks" that can match "the keys" (epitopes) presented on the antigen molecule.

- **Allergen** - A substance capable of causing an allergic reaction. The (detrimental) reaction may result after exposure via ingestion, inhalation, injection, or contact with skin.

- **Superantigen** - A class of antigens which cause non-specific activation of T-cells resulting in polyclonal T cell activation and massive cytokine release.

- **Tolerogen** - A substance that invokes a specific immune non-responsiveness due to its molecular form. If its molecular form is changed, a tolerogen can become an immunogen.

- **Immunoglobulin binding protein** - These proteins are capable of binding to antibodies at positions outside of the antigen-binding site. That is, whereas antigens are the "target" of antibodies, immunoglobulin binding proteins "attack" antibodies. Protein A, protein G and protein L are examples of proteins that strongly bind to various antibody isotypes.

### *Origin of the term antigen*

In 1899 Ladislas Deutsch (Laszlo Detre) (1874–1939) named the hypothetical substances halfway between bacterial constituents and antibodies "substances immunogenes ou antigenes". He originally believed those substances to be precursors of antibodies, just like zymogen is a precursor of zymase. But by 1903 he understood that an antigen induces the production of immune bodies (antibodies) and wrote that the word antigen was a contraction of "Antisomatogen = Immunkörperbildner". The Oxford English Dictionary indicates that the logical construction should be "anti(body)-gen"[6].

### *Classification of antigens*

Antigens can be classified in order of their class.

#### Exogenous antigens

Exogenous antigens are antigens that have entered the body from the outside, for example by inhalation, ingestion, or injection. The immune system's response to exogenous antigens is often subclinical. By endocytosis or phagocytosis, exogenous antigens are taken into the antigen-presenting cells (APCs) and processed into fragments. APCs then present the fragments to T helper cells ($CD4^+$) by the use of class II histocompatibility molecules on their surface. Some T cells are specific for the peptide:MHC complex. They become activated and start to secrete cytokines. Cytokines are substances that can activate cytotoxic T lymphocytes (CTL), antibody-secreting B cells, macrophages, and other particles.

Some antigens start out as exogenous antigens, and later become endogenous (for example, intracellular viruses). Intracellular antigens can be released back into circulation upon the destruction of the infected cell, again.

### Endogenous antigens

Endogenous antigens are antigens that have been generated within previously normal cells as a result of normal cell metabolism, or because of viral or intracellular bacterial infection. The fragments are then presented on the cell surface in the complex with MHC class I molecules. If activated cytotoxic $CD8^+$ T cells recognize them, the T cells begin to secrete various toxins that cause the lysis or apoptosis of the infected cell. In order to keep the cytotoxic cells from killing cells just for presenting self-proteins, self-reactive T cells are deleted from the repertoire as a result of tolerance (also known as negative selection). Endogenous antigens include xenogenic (heterologous), autologous and idiotypic or allogenic (homologous) antigens.

### Autoantigens

An autoantigen is usually a normal protein or complex of proteins (and sometimes DNA or RNA) that is recognized by the immune system of patients suffering from a specific autoimmune disease. These antigens should, under normal conditions, not be the target of the immune system, but, due to mainly genetic and environmental factors, the normal immunological tolerance for such an antigen has been lost in these patients.

### *Tumor antigens*

*Tumor antigens* or *neoantigens* are[*citation needed*] those antigens that are presented by MHC I or MHC II molecules on the surface of tumor cells. These antigens can sometimes be presented by tumor cells and never by the normal ones. In this case, they are called tumor-specific antigens (TSAs) and, in general, result from a tumor-specific mutation. More common are antigens that are presented by tumor cells and normal cells, and they are called tumor-associated antigens (TAAs). Cytotoxic T lymphocytes that recognize these antigens may be able to destroy the tumor cells before they proliferate or metastasize.

Tumor antigens can also be on the surface of the tumor in the form of, for example, a mutated receptor, in which case they will be recognized by B cells.

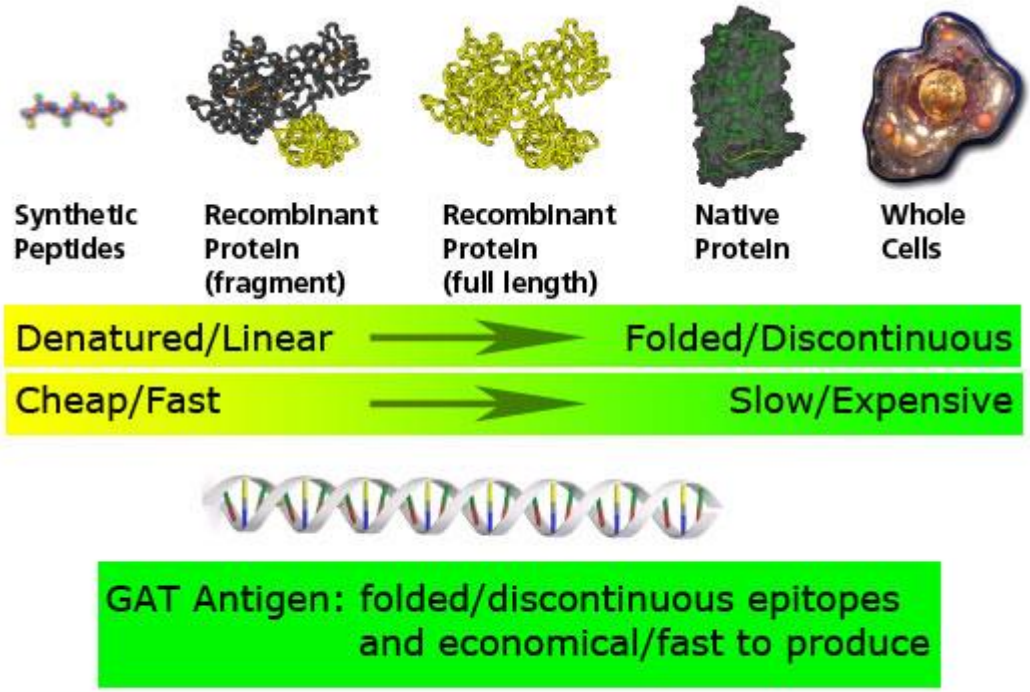
### *Nativity*

A **native antigen** is an antigen that is not yet processed by an APC to smaller parts. T cells cannot bind native antigens, but require that they be processed by APCs, whereas B cells can be activated by native ones.

### *Antigenic specificity*

**Antigen(ic) specificity** is the ability of the host cells to recognize an antigen specifically as a unique molecular entity and distinguish it from another with exquisite precision. Antigen specificity is due primarily to the side-chain conformations of the antigen. It is a measurement, although the degree of specificity may not be easy to measure, and need not be linear or of the nature of a rate-limited step or equation.

### Structure of antigen




**Preparation Of Antigens For Raising Antibodies**

**Polyclonal antibodies** (or antisera) are antibodies that are obtained from different B cell resources. They are a combination of immunoglobulin molecules secreted against a specific antigen, each identifying a different epitope.

***Production***

These antibodies are typically produced by immunization of a suitable mammal, such as a mouse, rabbit or goat. Larger mammals are often preferred as the amount of serum that can be collected is greater. An antigen is injected into the mammal. This induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen. This polyclonal IgG is purified from the mammal's serum.By contrast, monoclonal antibodies are derived from a single cell line.

Many methodologies exist for polyclonal antibody production in laboratory animals. Institutional guidelines governing animal use and procedures relating to these methodologies are generally oriented around humane considerations and appropriate conduct for adjuvant (agents which modify the effect of other agents while having few if any direct effects when given by themselves) use. This includes adjuvant selection, routes and sites of administration, injection volumes per site and number of sites per animal. Institutional policies generally include allowable volumes of blood per collection and safety precautions including appropriate restraint and sedation or anesthesia of animals for injury prevention to animals or personnel.

The primary goal of antibody production in laboratory animals is to obtain high titer, high affinity antisera for use in experimentation or diagnostic tests. Adjuvants are used to improve or enhance an immune response to antigens. Most adjuvants provide for an injection site, antigen depot which allows for a slow release of antigen into draining lymph nodes.

Many adjuvants also contain or act directly as:

1. surfactants which promote concentration of protein antigens molecules over a large surface area, and

2. immunostimulatory molecules or properties. Adjuvants are generally used with soluble protein antigens to increase antibody titers and induce a prolonged response with accompanying memory.

Such antigens by themselves are generally poor immunogens. Most complex protein antigens induce multiple B-cell clones during the immune response, thus, the response is polyclonal. Immune responses to non-protein antigens are generally poorly or enhanced by adjuvants and there is no system memory.

Antibodies are currently also being produced from isolation of human B-lymphocytes to produce specific recombinant polyclonal antibodies. The biotechnology company, Symphogen, produces this type of antibody for therapeutic applications. They are the first research company to develop recombinant polyclonal antibody drugs to reach phase two trials. This production prevents viral and prion transmission.

### *Animal selection*

Animals frequently used for polyclonal antibody production include chickens, goats, guinea pigs, hamsters, horses, mice, rats, and sheep. However, the rabbit is the most commonly used laboratory animal for this purpose. Animal selection should be based upon:

1. the amount of antibody needed,

2. the relationship between the donor of the antigen and the recipient antibody producer (generally the more distant the phylogenetic relationship, the greater the potential for high titer antibody response) and

3. the necessary characteristics [e.g., class, subclass (isotype), complement fixing nature] of the antibodies to be made. Immunization and phlebotomies are stress associated and, at least when using rabbits and rodents, specific pathogen free (SPF) animals are preferred. Use of such animals can dramatically reduce morbidity and mortality due to pathogenic organisms, especially *Pasteurella multocida* in rabbits.

Goats or horses are generally used when large quantities of antisera are required. Many investigators favor chickens because of their phylogenetic distance from mammals. Chickens transfer high quantities of IgY (IgG) into the egg yolk and harvesting antibodies from eggs eliminates the need for the invasive bleeding procedure. One week's eggs can contain 10 times more antibodies than the volume of rabbit blood obtained from one weekly bleeding. However, there are some disadvantages when using certain chicken derived antibodies in immunoassays. Chicken IgY does not fix mammalian complement component C1 and it does not perform as a precipitating antibody using standard solutions.

Although mice are used most frequently for monoclonal antibody production, their small size usually prevents their use for sufficient quantities of polyclonal, serum antibodies. However, polyclonal antibodies in mice can be collected from ascites fluid using any one of a number of ascites producing methodologies.

When using rabbits, young adult animals (2.5–3.0 kg or 5.5-6.5lbs) should be used for primary immunization because of the vigorous antibody response. Immune function peaks at puberty and primary responses to new antigens decline with age. Female rabbits are generally preferred because they are more docile and are reported to mount a more vigorous immune response than males. At least two animals per antigen should be used when using outbred animals. This principle reduces potential total failure resulting from non-responsiveness to antigens of individual animals.

### *Antigen preparation*

The size, extent of aggregation and relative nativity of protein antigens can all dramatically affect the quality and quantity of antibody produced. Small polypeptides (<10 ku) and non-protein antigens generally need to be conjugated or crosslinked to larger, immunogenic, carrier proteins to increase immunogenicity and provide T cell epitopes. Generally, the larger the immunogenic protein the better. Larger proteins, even in smaller amounts, usually result in better engagement of antigen presenting antigen processing cells for a satisfactory immune response. Injection of soluble, non-aggregated proteins has a higher probability of inducing tolerance rather than a satisfactory antibody response.

Keyhole limpet hemocyanin (KLH) and bovine serum albumin are two widely used carrier proteins. Poly-L-lysine has also been used successfully as a backbone for peptides. Although the use of Poly-L-lysine reduces or eliminates production of antibodies to foreign proteins, it may result in failure of peptide-induced antibody production. Recently, liposomes have also been successfully used for delivery of small peptides and this technique is an alternative to delivery with oily emulsion adjuvants.

## Antigen quantity

Selection of antigen quantity for immunization varies with the properties of the antigen and the adjuvant selected. In general, microgram to milligram quantities of protein in adjuvant are necessary to elicit high titer antibodies. Antigen dosage is generally species, rather than body weight, associated. The so called "window" of immunogenicity in each species is broad but too much or too little antigen can induce tolerance, suppression or immune deviation towards cellular immunity rather than a satisfactory humoral response. Optimal and usual protein antigen levels for immunizing specific species have been reported in the following ranges:

1. rabbit, 50–1000 μg;
2. mouse, 10–200 μg;
3. guinea pig, 50–500 μg; and
4. goat, 250–5000 μg.

Optimal "priming" doses are reported to be at the low end of each range.

The affinity of serum antibodies increases with time (months) after injection of antigen-adjuvant mixtures and as antigen in the system decreases. Widely used antigen dosages for "booster" or secondary immunizations are usually one half to equal the priming dosages. Antigens should be free of preparative byproducts and chemicals such as polyacrylamide gel, SDS, urea, endotoxin, particulate matter and extremes of pH.

## Peptide Antibodies

When a peptide is being used to generate the antibody, it is extremely important to design the antigens properly. There are several resources that can aid in the design as well as companies that offer this service. Expasy has aggregated a set of public tools under its ProtScale page that require some degree of user knowledge to navigate. For a more simple peptide scoring tool there is a Antigen Profiler tool available that will enable you to score individual peptide sequences based upon a relation epitope mapping database of previous immunogens used to generate antibodies. Finally, as a general rule peptides should follow some basic criteria.

When examining peptides for synthesis and immunization, it is recommended that certain residues and sequences be avoided due to potential synthesis problems. This includes some of the more common characteristics:

- Extremely long repeats of the same amino acid (e.g. RRRR)

- Serine (S), Threonine (T), Alanine (A), and Valine (V) doublets
- Ending or starting a sequence with a proline (P)
- Glutamine (Q) or Asparagine (N) at the n-terminus
- Peptides over weighted with hydrophobic residues (e.g. V,A,L,I, etc…)

**Reactivity**

Investigators should also consider the status of nativity of protein antigens when used as immunogens and reaction with antibodies produced. Antibodies to native proteins react best with native proteins and antibodies to denatured proteins react best with denatured proteins. If elicited antibodies are to be used on membrane blots (proteins subjected to denaturing conditions) then antibodies should be made against denatured proteins. On the other hand, if antibodies are to be used to react with a native protein or block a protein active site, then antibodies should be made against the native protein. Adjuvants can often alter the nativity of the protein. Generally, absorbed protein antigens in a preformed oil-in-water emulsion adjuvant, retain greater native protein structure than those in water-in-oil emulsions.

**Asepticity**

Antigens should always be prepared using techniques that ensure that they are free of microbial contamination. Most protein antigen preparations can be sterilized by passage through a 0.22u filter. Septic abscesses often occur at inoculation sites of animals when contaminated preparations are used. This can result in failure of immunization against the targeted antigen.

### *Adjuvants*

There are many commercially available immunologic adjuvants. Selection of specific adjuvants or types varies depending upon whether they are to be used for research and antibody production or in vaccine development. Adjuvants for vaccine use only need to produce protective antibodies and good systemic memory while those for antiserum production need to rapidly induce high titer, high avidity antibodies. No single adjuvant is ideal for all purposes and all have advantages and disadvantages. Adjuvant use generally is accompanied by undesirable side effects of varying severity and duration. Research on new adjuvants focuses on substances which have minimal toxicity while retaining maximum immunostimulation. Investigators should always be aware of potential pain and distress associated with adjuvant use in laboratory animals.

The most frequently used adjuvants for antibody production are Freund's, Alum, the Ribi Adjuvant System and Titermax.

**Freund's adjuvants**

There are two basic types of Freund's adjuvants: Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA). FCA is a water-in-oil emulsion that localizes antigen for release periods up to 6 months. It is formulated with mineral oil, the surfactant mannide monoleate and heat killed *Mycobacterium tuberculosis*, *Mycobacterium butyricum* or their extracts (for aggregation of macrophages at the inoculation site). This potent adjuvant stimulates both cell mediated and humoral immunity with preferential induction of antibody against epitopes of denatured proteins. Although FCA has historically been the most widely used adjuvant, it is one of the more toxic agents due to non-metabolizable mineral oil and it induces granulomatous reactions. Its use is limited to laboratory animals and it should be used only with weak antigens. It should not be used more than once in a single animal since multiple FCA inoculations can cause severe systemic reactions and decreased immune responses. Freund's Incomplete Adjuvant has the same formulation as FCA but does not contain mycobacterium or its components. FIA usually is limited to booster doses of antigen since it normally much less effective than FCA for primary antibody induction. Freund's adjuvants are normally mixed with equal parts of antigen preparations to form stable emulsions.

**Ribi Adjuvant System**

Ribi adjuvants are oil-in-water emulsions where antigens are mixed with small volumes of a metabolizable oil (squalene) which are then emulsified with saline containing the surfactant Tween 80. This system also contains refined mycobacterial products (cord factor, cell wall skeleton) as immunostimulants and bacterial monophosphoryl lipid A. Three different species oriented formulations of the adjuvant system are available. These adjuvants interact with membranes of immune cells resulting in cytokine induction, which enhances antigen uptake, processing and presentation. This adjuvant system is much less toxic and less potent than FCA but generally induces satisfactory amounts of high avidity antibodies against protein antigens.

**Titermax**

Titermax represents a newer generation of adjuvants that are less toxic and contain no biologically derived materials. It is based upon mixtures of surfactant acting, linear, blocks or chains of nonionic copolymers polyoxypropylene (POP) and polyoxyethylene (POE). These copolymers are less toxic than many other surfactant materials and have potent adjuvant properties which favor chemotaxis, complement activation and antibody production. Titermax adjuvant forms a microparticulate water-in-oil emulsion with a copolymer and metabolizable squalene oil. The copolymer is coated with emulsion stabilizing silica particles which allows for incorporation of large amounts of a wide variety of antigenic materials. The adjuvant active copolymer forms hydrophilic surfaces, which activate complement, immune cells and increased expression of class II major histocompatibility molecules on macrophages. Titermax presents antigen in a highly concentrated form to the immune system, which often results in antibody titers comparable to or higher than FCA.

**Specol**: Specol is a water in oil adjuvant made of purified mineral oil. It has been reported to induce immune response comparable to Freund's adjuvant in rabbit and other research animal while producing fewer histological lesions

**Adjuvants and their modes of action**

The trend towards the use of peptides and subunit proteins in modern vaccine design has necessitated the use of immunological adjuvants to achieve effective immunity. Aluminium hydroxide, a component of the diphtheria, tetanus and hepatitis B vaccines, was first described as an adjuvant over 60 years ago and is the only adjuvant currently approved for use in humans. It is also a common component of many veterinary vaccines. While this adjuvant is effective at enhancing antibody titres to antigens, the effectiveness of aluminium hydroxide is limited due to its inability to promote cell mediated immunity. Freund's Complete Adjuvant (FCA) has been used experimentally and does stimulate cellular immunity, but is unsuitable for human and veterinary use as it promotes, amongst other toxic side effects, local inflammation and granuloma formation at the site of injection. Thus, in recent years there has been a great deal of interest in developing novel, cheap, effective and safe adjuvants which stimulate cellular, as well as humoral immunity to be used with medical and veterinary vaccines. In addition, the recent unravelling of numerous immunological pathways has facilitated the rational development of new adjuvants and allowed a better understanding of the modes of action of traditional adjuvants.

**Mode of action of immunological adjuvants: some physicochemical factors influencing the effectivity of polyacrylic adjuvants.**

The adjuvant effects of different polyacrylic products and monomers were tested. Influenza vaccine was used as a model antigen. Addition of monomers resulted in a decrease in the antibody response, though adjuvant activity of the monomers should be expected according to some theories on adjuvant action. The particle size of the polymer adjuvants proved to be a very important parameter for adjuvant activity. Particles of 0.1 to 0.2 micron yielded a good adjuvant effect, whereas conglomerates or particles bigger than 0.5 micron yielded only poor or no adjuvant effects. The adjuvant effect of 0.1- to 0.2-micron particles was much more reproducible than rat of Al(OH)3. Attention is drawn to the importance of using physiochemically reproducible materials, such as polymer particles, for experimental work.

**ANIMAL HANDLING AND RESTRAINT**

**Animal Handling Skills-Professionalism and Safety**

_ The public watches us to learn how to properly handle animals.
_ Being professional means being SAFE and HUMANE.
_ Good animal handling skills prevent staff from being injured.
_ Good animal handling skills reduce stress for the animal.

**Examples of Safe Animal Handling:**

_ Be aware of the special stressors for animals in the clinic setting.
_ The clinic is extremely chaotic for any animal-there are an incredible number of smells and other stimuli and animals are likely to be confused and distressed.
_ Many of our patients have lived entirely outdoors and have not been handled or examined before. They may not have any experience on a leash and may panic in response.
_ Even the most social animal may exhibit aggression toward other animals, particularly in a strange environment and may redirect to nearby people when over-stimulated.
_ Never put your face directly into the face of a dog or cat.
_ Do not move in behind or crowd around a dog.
_ Concentrate on the animal you are handling without being distracted by other activities.
_ NEVER sit on the floor while handling/examining a dog. If the animal becomes aggressive or aroused you will be unable to move away or protect yourself and risk serious facial bites.
_ Always be prepared to protect yourself or move away quickly in the event an animal becomes aggressive unexpectedly.

Safe and effective animal handling requires a thorough understanding of the normal behavior and responses of each species. Below is some general information on animal behavior and handling techniques. There is no substitute, however, for careful observation and experience.

**Communication**

Any animal exhibiting potentially aggressive behavior should have a kennel sign (CAUTION) posted to alert others who may be handling the animal. Specific alerts or recommendations should be written on the sign and in the medical record to provide staff and other volunteers with as much information as possible when handling the animal.

**Restraint or Control**

The first rule to keep in mind when handling any kind of animal is that the least restraint is often the best restraint. This does not mean that you give up your control, just that you use as little restraint as necessary while maintaining control of the situation. Every animal and every situation is different so as to what method works best in which situation.

Before attempting to restrain an animal you should take a moment to allow the animal to become comfortable with you:

_ Crouch down so that you are on their level. Do not sit on the ground as you will be unable to move away or protect yourself if necessary.
_ Avoid direct eye contact but maintain safe visual contact with the animal
_ Talk in soothing tones. Avoid high-pitched, excited talk.
_ Try patting your leg or the ground, motioning the animal towards you.

**TYPES OF RESTRAINT**

**VERBAL RESTRAINT:**

Many dogs know some commands or can at least recognize authority, even if the command is unfamiliar.Commands such as SIT, STAY, COME, DOWN, NO or even HEEL may be useful tools to encourage a dog to cooperate. Also, soft quiet words can calm a frightened animal. Yelling or screaming should never be used as it can cause the animal to become more fearful or aggressive.

**PHYSICAL RESTRAINT: TOOLS AND EQUIPMENT**

**Leash:** The most common tool used to handle animals in the clinic is the leash. Placed around a dog's neck it normally controls even the largest dog. In the event a dog refuses to cooperate with a leash - carry him. Some dogs have never seen a leash and will freeze up to the sensation around a

sensitive area like the neck. Leashes can be abused; never drag or strangle an animal with a leash; if the animal starts to struggle, pulling and jerking away from you, she is probably not leash trained. Pause and let the dog calm down and try again after reassuring her. Sometimes a quick tug on the leash will encourage a fearful dog to walk. If the dog refuses to walk, apply a muzzle (if necessary) and carry her.

When handling cats, a leash should be used as a back-up in the event the cat should become frightened and resist restraint. Make a figure-eight harness by looping the free end of a slip lead back through the metal ring. The looser loop is placed around the chest behind the cat's front legs and the other loop placed around the neck with the metal ring/handle on top between the shoulders. This will prevent the cat from escaping or injuring someone should she get loose from your restraint. The harness should be put on at intake and can be left on the cat throughout their stay.

**EVERY animal being transported or handled in the clinic must ALWAYS wear a slip-lead.** This includes puppies, cats and sedated animals. It is too easy for a frightened animal to get loose and escape. Animals presented on leash/collar should be transferred to a slip lead and the leash returned to the client so that it is not lost during the animal's stay.

**Your hand:** A very effective form of restraint, your hands are sensitive to the amount of pressure that is being exerted on the animal and can be quickly modified to the situation. Hands can be used to gently stroke a dog or to firmly grasp a struggling cat. Although hands can be the most versatile, they are also the most vulnerable to injury. Recognizing when they would not be effective is very important.

**Towels:** A towel or blanket is a very useful tool for cats and small dogs. A towel can be used to decrease an animal's arousal by covering the head and body and can help protect from sharp claws.

**Come-a-long or control pole:** The control pole is used to safely handle extremely aggressive dogs.Used appropriately it is an effective tool. Inappropriate or unskilled use can cause serious injury to the animal. The control pole may further distress an upset animal and should only be used when the handler or other's safety is genuinely threatened. Volunteers are NOT to use the control pole unassisted. If an animal is aggressive enough to warrant the use of a control pole an experienced staff member should be consulted for assistance as the animal will also be evaluated for chemical restraint options.

**Nets:** The net is the primary tool used to handle fractious cats or wildlife. It allows for the safe handling and transfer of even the most aggressive small mammal. Effective use of the net requires some training
and practice. If you need to handle a feral or fractious cat ask for assistance from a staff member.

**Muzzles:** Muzzles are used when a snappy or potentially aggressive dog must be handled. There are nylon muzzles and plastic basket available. A leash or strip of rolled gauze can be used as a temporary muzzle. Because dogs often try to remove a muzzle, it is important that the muzzle be placed securely.

A weak or poorly made muzzle may lead to a false sense of security and the possibility of being bitten. Even with a securely placed muzzle, appropriate handling must be used to prevent injury from an animal who resists.Muzzles designed for cats extend up to cover the eyes, reducing visual stimulation. For some cats these can be very useful for calming the animal and helping to protect the handler from injury,

**Drugs:** For animals who are too aggressive or stressed to handle safely for procedures, sedation and/or general anesthesia may be necessary to allow treatment. If you are unable to handle an animal, notify a staff member to determine whether sedation is appropriate. When receiving an animal for surgery who exhibits difficult or aggressive behavior consult the Anesthesia
Lead prior to kenneling the animal as we may opt to administer a pre-anesthetic sedative immediately
and expedite the surgery process to minimize the animal's time in the clinic.

**Credo: Never Let Go.**

The place where correct use of restraint is the most critical is when two people are handling the animal.This could be to perform a physical exam, administer anesthetic or to give medications. The "holder" is the person whose job it is to restrain the animal in such a way that the procedure can be

accomplished with the least amount of stress to both handlers and animal. The specific amount of restraint used to control the animal is the key to safety for the handlers and comfort for the animal. Too much restraint can cause the animal to fight back, too little restraint can result in the handler or others being injured or in the animal escaping.

### *Restraint and Handling of Animals*

#### General Principles

The use of proper restraint and handling techniques reduces stress to animals and also to the researcher. Handling stress represents an experimental variable and should be minimized whenever possible. Animals can inflict serious injuries to humans and to themselves as a result of improper handling.

- Animals experience stress as a result of shipping. All large animals must be allowed to acclimate to the facility for three days. During this time they may not be experimentally manipulated. Acclimation periods of up to one week are recommended for all animals.
- If a study will involve significant handling of animals it is recommended that the animals be acclimated to the handling. Prior to experimental manipulation, handle the animal on a regular basis in a non-threatening situation, e.g. weighing, petting, giving food treats. Most animals, even rodents will respond positively to handling and will learn to recognize individuals.
- Handle animals gently. Do not make loud noises or sudden movements that may startle them.
- Handle animals firmly. The animal will struggle more if it sees a chance to escape.
- Use an assistant whenever possible.
- Use restraint devices to assist when appropriate.
- Chemical restraint should be considered for any prolonged or potentially painful procedure.

#### Handling Methods

The methods described below will assist with performing basic manipulations. Alternate techniques may be needed for special procedures. Most of these methods are also demonstrated in video tapes available to investigator. For other information on animal handling or for individual training, contact RAR at 624-9100. An excellent website containing laboratory biomethodology for rodents and rabbits is also available with descriptions and pictures of drug administration, blood collection and sex determination.

#### Needle Re-Use Policy

The use of a new sterile needle and syringe for each animal when giving parenteral injections (intraperitoneal, subcutaneous, intravenous, intramuscular, etc.) is the recommended best practice to prevent the horizontal transfer of contamination between animals. However, the IACUC recognizes that there are some instances where it may be justified to use the same needle and syringe for multiple animals, usually in rodents. In those instances the Principal Investigator must provide justification to the IACUC and must adhere to the following guidelines. Use of the same needle and syringe may be permitted with justification on animals housed in the same cage. The needle must be assessed for continued sharpness and the presence of barbing or burring of the tip between animals. If dullness or needle deterioration is found, a new needle must be used.

**MICE**

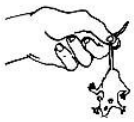
Tail restraint, as described below is adequate for examining animals and transfering them to another cage.

HOW TO PICK UP A MOUSE

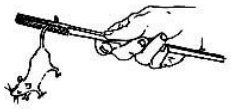
When you handle mice, all your movements should be slow and gentle. They are lively animals, but they will rarely bite unless they are afraid or mishandled.

Grasp the middle of the tail between your thumb and index finger, and lift the mouse.

NOTE: Frequently, the animal will hang quietly and can be carried in this manner. Occasionally, an individual will turn around and crawl onto the palm of your hand. It should be allowed to do this.



HOW TO PICK UP A MOUSE WITH THUMB FORCEPS

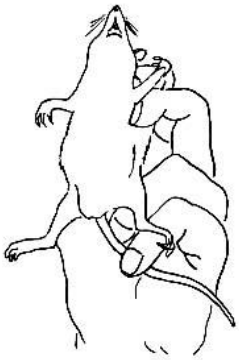


The thumb forceps are shaped somewhat like an eyebrow tweezer. The tips must be covered with rubber or polyethylene tubing to prevent damage to the mouse's tail.

Grasp the middle of the tail between the forceps and apply only enough pressure to lift the mouse.

CAUTION: Too much pressure may cause pain or even crush the tail.

These methods may be used to perform minor, non-painful procedures such as injections or ear tagging.



One-handed Method

Follow the above steps.

Then, place the mouse's tail between the last two fingers of the hand that is holding the nape.

Two-handed Method

Place the mouse on a rough surface while holding the tail firmly.

NOTE: Smooth surfaces will frighten the mouse because it cannot get a foothold. This may cause it to turn around and try to bite in its attempt to escape.

Grasp the nape gently and firmly with your free hand and lift the mouse.

**RATS** may be handled by the tail, with precautions similar to those used for mice, with emphasis on only grasping the tail base. Holding the tail distal to the base can result in a de-gloving injury to the tail that will require surgical repair or euthanasia.

This method should be used to restrain a rat for injections and other minor procedures.

HOW TO HOLD A RAT FOR TREATMENT

Pick up the rat. Rotate the wrist of your right hand to expose the mid-section.

Extend the rat's hind legs with your left hand, grasping one hind leg between your thumb and index finger and the other between your index and second fingers.

**HAMSTERS**

Because hamsters do not have tails, they must be grasped firmly by the loose skin of its back, or handled in a manner similar to the rat.

**GUINEA PIGS** rarely bite, but are very easily frightened and will vocalize and squirm to avoid restraint. The hind limbs must be supported at all times to prevent the animal from injuring its back.

HOW TO PICK UP AND CARRY A HAMSTER

There are two acceptable methods for picking up a hamster. You may use whichever is easier for you.

CUATION: Always handle hamsters over a bench or table, because they are liable to jump from your grasp and injure themselves.

Method 1

Form a cup with your hands and place it over the hamster. Gently press your palms against the animal as you pick it up.

Method 2

Grasp the loose fold of skin behind the neck with your thumb and index finger. Cup your other hand under the animal's rump and grasp the hind legs between your thumb and index finger.

Carry the hamster in the same position in which you picked him up.

For injecting the animal in the mid-section, merely stretch it out.

HOW TO PICK UP A GUINEA PIG

Approach the animal in a quiet, confident manner. Place one hand across the shoulders. Place your thumb behind the front legs and the fingers well forward on the opposite side.

NOTE: Do not apply excessive pressure to the animal's body. You can hurt the guinea pig and cause it to squirm needlessly.

Pick up the animal and hold it in an upright position.

HOW TO CARRY A GUINEA PIG

Pick up the guinea pig. Place your free hand palm uppermost under the hindquarters to support the animal's weight. If it struggles, hold the hind legs to get a more secure grip.

HOW TO HOLD A GUINEA PIG FOR INJECTIONS

Grasp and extend the hind limbs with your free hand. This will enable the investigator to inject the animal in the midsection.

**RABBITS** are very susceptible to lumbar spinal luxation, resulting in paralysis. It is necessary to support the animal's hindquarter at all times. Although rabbits seldom bite, they can inflict painful scratches with their hind legs. One way of lifting a rabbit is by grasping the skin over the shoulder with one hand and gently lifting it with the other arm cradling the body, the head nestled in the crook of your arm. Rabbits must never by lifted by the ears.

HOW TO REMOVE A RABBIT FROM A CAGE

Approach the cage and open the door slowly so that you will not startle the rabbit. Reach in and grip the loose skin over the shoulders firmly but gently with your left hand. Place your right hand under the rabbit's rump to support its weight.

NOTE: The right hand may be opened and placed flat under the rabbit's stomach instead of under the rump, but this requires more skill. NEVER use the flat-handed method for handling pregnant does. It might injure the unborn young.

Lift the rabbit out of the cage.

HOW TO CARRY A RABBIT

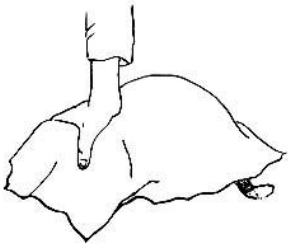
Grasp the loose skin over the neck with your left hand and place your right hand under the animal's rump, as you did in removing the rabbit from its cage.

Pick up the rabbit and hold it in an upright position.

CAUTION: Never lift or carry a rabbit by its ears. They aren't handles! Don't carry a rabbit by the neck skin alone. This is painful to the animal and will cause it to struggle. You run the chance of getting scratched by the sharp rear claws.

Carry the rabbit out in front of yourself.

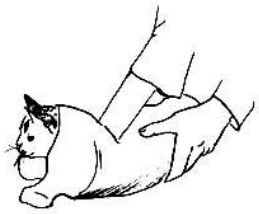
**CATS** are often cooperative enough to be restrained on a table by the loose skin at the back of the neck and hips, or with one hand restraining the body and the other restraining the head. A fractious cat may have to be wrapped in a heavy towel for restraint with any needed limbs carefully withdrawn for treatment.



**How to Use a Blanket (or Sack)**

Cover the cat with a blanket or sack. Grab it through the cloth and locate the head. Hold it firmly at the base of the skull.

Turn the blanket back to uncover the cat. Then pick up the animal in the usual manner.



HOW TO REMOVE A CAT FROM A CAGE

When you approach a cat's cage, all movements should be slow and deliberate. Cats are high-strung animals, and sudden movements might frighten them.

CAUTION: Look for warning signs that indicate a cat is on the defensive. These might include hissing or growling, flattening the ears against the head, and arching the back.

Open the cage door just wide enough to insert your left hand. Grasp the cat's neck behind the head to prevent the animal from escaping.

Rotate your left hand from behind the head to under the chin. Place your right hand under the cat to support its weight. Lift the cat from the cage and place it on your right hip.

Insert the first two fingers of your right hand between the front legs. Grip firmly over the paws with your thumb and other two fingers.

Secure the cat's body between the crook of your right arm and hip. Keep your left hand firmly under the chin to prevent the cat from biting.

## DOGS

A slip lead is highly recommended for working with dogs. A dog should always be carried with proper support. The dog can be restrained in lateral recumbancy or in a sitting position for injections and minor procedures. For venipuncture, the handler can restrain the dog on a table with one arm around its neck. The other hand is then free to restrain the body if necessary or to occlude the vein for the person with the syringe. A shy or fearful dog may need extra time spent with it to make it more comfortable. Moving slowly and speaking quietly will help to prevent alarming the animal.

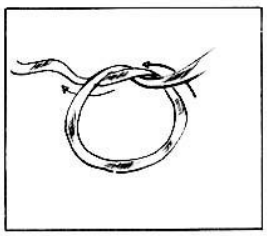
An intractable dog may need to be muzzled. A commercial muzzle may be purchased, or a gauze muzzle may used as described below.

HOW TO MAKE A TEMPORARY MUZZLE

Before you learn how to handle a dog, it's important that you know how to make and use a temporary muzzle, because you will need it in a few of the methods to follow.

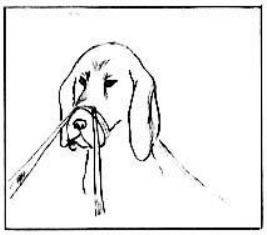
NOTE: Muzzle a short-nosed dog as you would a cat. The directions may be found on page 19.

Step 1



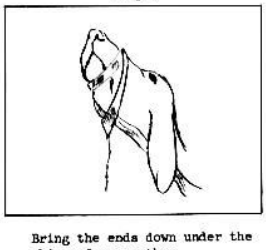
Make a loop in the center of a four-foot cord or double-thickness gauze bandage.

Step 2



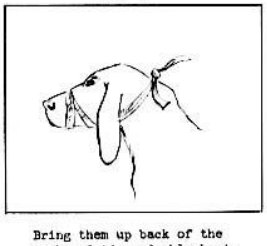
Slip the loop over the dog's muzzle and draw the ends snugly over the nose.

Step 3



Bring the ends down under the chin and cross them.

Step 4



Bring them up back of the neck and tie a double knot.

HOW TO LIFT A DOG

The size of the dog determines the method you will use to lift it. The beginning step is the same for all dogs.

Grasp the dog over the neck. Place your other arm around its body, with your hand supporting the dog's chest.

Large Dogs

Stand up straight, placing the weight of the dog on your hip, so you won't strain your back.

NOTE: Lift with your legs to prevent back strain.

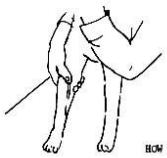
Carry the dog in the hip position, leaning away from it for balance.

Small Dogs

Carry the dog in the upright position, shifting it so its back is resting on the front of your hip. Lean slightly backwards for balance.

HOW TO HOLD A DOG FOR INJECTIONS

This method is the same as the one above, except that your right hand is used for a different purpose.



Reach over the dog's back with your right arm, but instead of grasping the dog's chest, hold the right forelimb firmly at the elbow.

Apply pressure to the top of the leg with your thumb, as shown in the drawing on the left. This will cause the vein to enlarge by stopping the blood flow, making it easier for your investigator to give the injection.

HOW TO RESTRAIN A DOG ON ITS SIDE

It is sometimes necessary to restrain a dog on its side to expose its legs, belly, or side. This position may be used for removing stitches, or for leg, foot, or midsection examinations.

Lay the dog on its side on the treatment table. Reach over its back and grasp both hind legs with one hand. Apply pressure with your forearm over the loin.

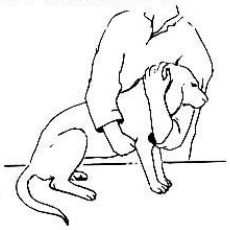
With your free hand, grasp both forelimbs. Apply pressure on the neck with your forearm. This will prevent the dog from twisting its head to bite you.

HOW TO RESTRAIN A DOG IN THE SITTING POSITION

This method of restraint is useful for many different types of treatment.

Seat the dog on a table and approach it from its left side. Place your left arm under the neck. Hook your hand over the top of the dog's head and pull the head close to you to prevent movement.

Reach over the dog's back with your right arm and grasp the chest. Pull the dog close to your body.

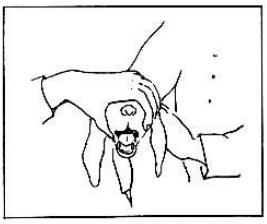


Pills are easily administered to most dogs if the proper technique is used.

HOW TO GIVE A DOG A PILL

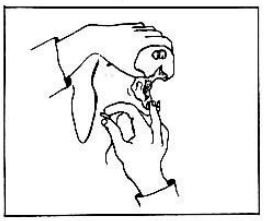
Pills are given to dogs for several reasons, for instance, to rid them of worms or to experiment with new drugs.

Step 1



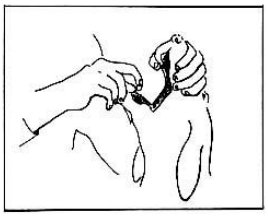
Have someone restrain the dog in the sitting position. Face the dog. Place one hand over the upper jaw, with thumb and fingers applying pressure to fold the lips inside the mouth. This will force the dog's mouth open part way.

Step 2



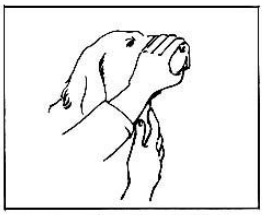
Pick up the pill between your index finger and thumb. Place the middle finger of your free hand on the lower incisors. These are the short teeth in the front end of the lower jaw. Press down, forcing the dog to open its mouth wider.

Step 3



Push the dog's head back, so that its muzzle points up. This will give you a clear view down its throat. Drop the pill on the back of the tongue.

Step 4



Close the dog's mouth and hold it shut. Stroke the throat until the dog swallows the pill. You will be able to feel the throat move in swallowing.

**NONHUMAN PRIMATES**, no matter how small, can be dangerous. Chemical immobilization with ketamine is nolrmally used. Injections can be given to a confined animal with the help of a squeeze cage.

***Safety:***

Absolute requirements for handling of nonhuman primates include attending a **training** module

Physical restraint of a conscious animal should only be attempted by trained, experienced personnel. Animals may be pole and collar trained if they will be handled frequently. Tether systems are recommended if animals must be administered drugs or if blood must be collected frequently.

given by RAR (contact 624-9100 to schedule), and wearing appropriate protective clothing. In addition, nonhuman primate users should be familiar with procedures to follow in case of a bite or scratch and the location of bite kits.

If a nonhuman primate has **escaped**, close all doors and contact RAR at 624-9100. The animal may be recaptured using a net or a dart gun.

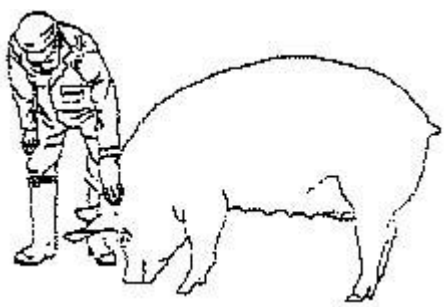
**GOATS, SHEEP and CALVES**

- Restrain against a wall or in a corner by placing a knee firmly in the flank.
- Restrain for blood collection by backing the animal into a corner and straddling them at the shoulder and firmly restraining the head and neck.
- Use a halter over their head and face.
- A sheep can be held for bleeding, shearing or hoof trimming by sitting the animal up on its hind end, leaning back against the restrainer.
- For long term restraint of sheep in the laboratory, a canvas sling and rack is available from several commercial suppliers. Animals are easily acclimated to such slings, and can be comfortable and relaxed enough to fall asleep in them.
- Additional references on handling of agricultural animals is available from the USDA.
- Temple Grandin's Website on **Low-Stress Handling of Farm Animals**

**RESTRAINT AND HANDLING OF SWINE**
**By Dr. Jack Risdahl**

Additional information on restraint of, and blood collection from, swine may be found on the UCDavis website. http://ehs.ucdavis.edu/animal/vet_care/training/Pig.cfm

Pigs in general are friendly and docile but will react severely to poor handling or a stressful environment. Pigs can be very vocal. If pigs are chronically stressed they will become skittish and fearful. Handling and restraint in pigs relies greatly on treating the pigs in a humane manner. The benefits of treating pigs well include reducing apprehension, fear and stress in the pigs. There are several levels of restraint and handling, from touching and coaxing a pig to restraining a pig for chronic procedures.

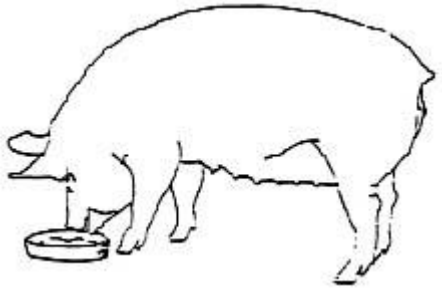


*Touch is a very important aid to good husbandry.*

**Animal-Human Contact**

When approaching a pig be sure it is made aware of your presence. If pigs are startled they may cause injury to themselves or others in the

pen. The best way to make pigs aware of your presence is to use your voice. It is important to use a soft soothing voice and not angry, loud, high pitched tone of voice which might startle or stress the animal. Pigs quickly learn to recognize voices, especially if they are associated with food. As pigs become familiar with handlers, the sound of a familiar voice is often calming to the animal. It is important to use touch when developing a rapport with pigs. This applies especially to the researcher who must collect frequent samples or data from pigs. As with voice, gentle petting and hand contact should be associated with feeding time or treats and the pig will become aware of the person in the vicinity and become adjusted to that persons presence. Probably one of the best forms of restraint in pigs is the use of food. Pigs are highly oriented to food and if they are comfortable with the handler will most often stand and eat while minor procedures and examinations are being performed on them. One can often flush catheters, give injections, treat minor wounds and take temperatures while pigs eat. The use of all three procedures - voice, touch, and food, will be the best investment in reducing stress among research swine and will ultimately reward the researcher with a happy stress free subject.



*The giving of food is one of the most effective forms of basic restraint in the pig.*

**Picking Up Pigs**

Pigs best tolerate being picked up in a "horizontal" fashion oriented to the ground. Pigs should not be picked up by the legs or held upside down as this will stress the animal and you will loose their trust. Usually only smaller animals may be picked up while larger animals (>35-40 kg) must be moved by alternative means. Smaller pigs may be easily picked up with their body supported while their legs hang. To perform the procedure in larger pigs place one arm under the chest cranial to the thoracic limbs and the other arm cranial to the pelvic limbs under the abdomen picking up the pig in a "scooping" fashion. Alternatively the arm may be placed caudally just above the pig's hock, hence supporting the animal by the pelvis rather than the abdomen. All handlers must beware to lift with legs and not back as injury can easily result - pigs are usually heavier than they appear! Always avoid picking pigs up by one leg or by the ears as injury may result!

**Moving Pigs**

*The small board used to apply pressure to the side of a pig.*

Pigs are best moved in a metal (box style) transport designed for use with large animals. At times this is not possible and pigs must be walked to their destination. When moving a pig always remember pigs will move away from walls toward openings. This is an advantage since one can use a "hog board" to simulate walls. The board is fashioned with a handle so that one can place it to the side, rear or front of the pig to direct them. Excessive force should not be needed to move a pig and is mostly counterproductive as pigs will become excited and belligerent. It should be remembered pigs will refuse to move if the place you wish them to go is dark (e.g. from daylight into a dark room). Sometimes pigs may be coaxed with food along with the use of the board. When pigs are unruly and where control is needed, pigs may be tethered in a harness and controlled by "holder" so that the pig does not run away. Often the use of the hog board may be used to stop pig and slow them down if they are moving too rapidly. The board may also be used to restrain a pig in a corner while minor procedures are performed. The size of the board varies depending on the size of pigs used and application. In general if the board is at least as tall as the pig and 2/3 to about as long as the pig it will usually suffice.

**Sling**

Several designs for slings to restrain pigs have been described. The most commonly used is that described by Panepinto et al 1983. Here the pig is placed in a hammock with four holes for the limbs. The hammock is supported by a metal frame. These are available in free standing or winch styles (so larger pigs may be raised by winch). The pig is placed in ventral recumbency in the sling with its limbs tied loosely to the frame. It has been our experience that this form of restraint requires some degree of training for pigs to acclimate to. In general most pigs will become stressed the first several times they are placed in the sling. Positive reinforcement (treats, petting) and

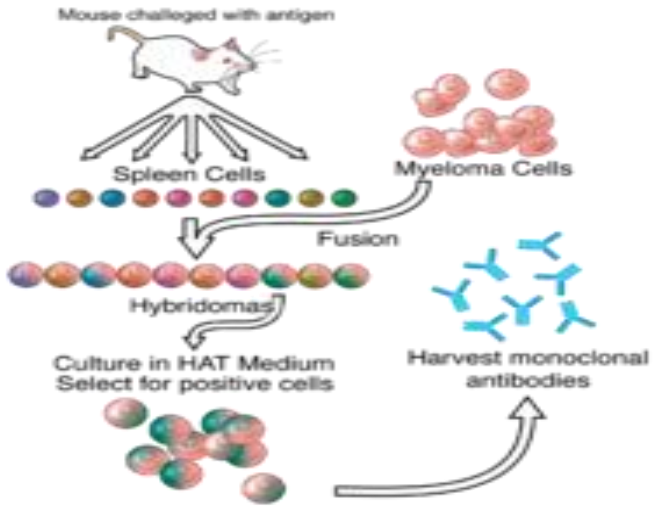
***Restraint Devices***

Restraint devices such as rabbit or rodent restrainers, swine slings or monkey chairs are useful for certain non-painful procedures. However, certain guidelines should be followed when using these devices.

- Animals should be adapted to the restraint devices. This means that for long-term restraint (i.e. more than an hour), it is advisable to "train" the animal to the device by placing it into the device for successively longer intervals until the maximum time of restraint can be achieved without causing distress to the animal.
- Animals in a restraint device be regularly monitored. This means not leaving the area for long intervals unless someone else is available to monitor the animal. Animals have an uncanny ability to attempt escape from devices, if they don't succeed completely, they may end up with a limb or their head entrapped. This could result in ischemia or hypoxia.
- Animals should have access to food or water at appropriate intervals, even when restrained, unless doing so would interfere with the goals of the experiment. Food or water should be offered twice daily. For rabbits and rodents, water should be offered more frequently.
- Animals should be released from restraint devices at least daily and allowed unrestrained activity to prevent muscle atrophy and skin necrosis, unless this interferes with achieving the experimental goals and is documented in an approved IACUC protocol.

**UNIT II ANTIBODIES & IMMUNODIAGNOSIS**

**Monoclonal antibodies**




**A general representation of the methods used to produce monoclonal antibodies.**

**Monoclonal antibodies** (**mAb** or **moAb**) are monospecific antibodies that are the same because they are made by identical immune cells that are all clones of a unique parent cell.

Given almost any substance, it is possible to create monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance. This has become an important tool in biochemistry, molecular biology and medicine. When used as medications, the non-proprietary drug name ends in *-mab*.

**Discovery**

The idea of a "magic bullet" was first proposed by Paul Ehrlich, who, at the beginning of the 20th century, postulated that, if a compound could be made that selectively targeted a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity. He and Élie Metchnikoff received the 1908 Nobel Prize for Physiology or Medicine for this work, which led to an effective syphilis treatment by 1910.

In the 1970s, the B-cell cancer multiple myeloma was known, and it was understood that these cancerous B-cells all produce a single type of antibody (a paraprotein). This was used to study the structure of antibodies, but it was not yet possible to produce identical antibodies specific to a given antigen.

Production of monoclonal antibodies involving human–mouse hybrid cells was described by Jerrold Schwaber in 1973and remains widely cited among those using human-derived hybridomas, but claims to priority have been controversial. A science history paper on the subject gave some credit to Schwaber for inventing a technique that was widely cited, but stopped short of suggesting that he had been cheated. The invention was conceived by George Pieczenik, with John Sedat, Elizabeth Blackburn's husband, as a witness and reduced to practice by Cotton and Milstein, and then by Kohler and Milstein. Georges Köhler, César Milstein, and Niels Kaj Jerne in 1975; who shared the Nobel Prize in Physiology or Medicine in 1984 for the discovery. The key idea was to use a line of myeloma cells that had lost their ability to secrete antibodies, come up with a technique to fuse these cells with healthy antibody-producing B-cells, and be able to select for the successfully fused cells.

In 1988, Greg Winter and his team pioneered the techniques to humanize monoclonal antibodies, removing the reactions that many monoclonal antibodies caused in some patients.

**Production**

Monoclonal antibodies can be grown in unlimited quantities in the bottles shown in this picture.

Technician hand-filling wells with a liquid for a research test. This test involves preparation of cultures in which hybrids are grown in large quantities to produce desired antibody. This is effected by fusing myeloma cell and mouse lymphocyte to form a hybrid cell (hybridoma).

**Hybridoma cell production**

Monoclonal antibodies are typically made by fusing myeloma cells with the spleen cells from a mouse that has been immunized with the desired antigen. However, recent advances have allowed the use of rabbit B-cells. Polyethylene glycol is used to fuse adjacent plasma membranes, but the success rate is low so a selective medium in which only fused cells can grow is used. This is because myeloma cells have lost the ability to synthesize hypoxanthine-guanine-phosphoribosyl

transferase (HGPRT), an enzyme necessary for the salvage synthesis of nucleic acids. The absence of HGPRT is not a problem for these cells unless the de novo purine synthesis pathway is also disrupted. By exposing cells to aminopterin (a folic acid analogue, which inhibits dihydrofolate reductase, DHFR), they are unable to use the de novo pathway and become fully auxotrophic for nucleic acids requiring supplementation to survive.

The selective culture medium is called HAT medium because it contains hypoxanthine, aminopterin, and thymidine. This medium is selective for fused (hybridoma) cells. Unfused myeloma cells cannot grow because they lack HGPRT, and thus cannot replicate their DNA. Unfused spleen cells cannot grow indefinitely because of their limited life span. Only fused hybrid cells, referred to as hybridomas, are able to grow indefinitely in the media because the spleen cell partner supplies HGPRT and the myeloma partner has traits that make it immortal (as it is a cancer cell).This mixture of cells is then diluted and clones are grown from single parent cells on microtitre wells. The antibodies secreted by the different clones are then assayed for their ability to bind to the antigen (with a test such as ELISA or Antigen Microarray Assay) or immuno-dot blot. The most productive and stable clone is then selected for future use.

The hybridomas can be grown indefinitely in a suitable cell culture media, or they can be injected in mice (in the peritoneal cavity, the gut), they produce tumors containing an antibody-rich fluid called ascites fluid. The medium must be enriched during selection to further favour hybridoma growth. This can be achieved by the use of a layer of feeder fibrocyte cells or supplement medium such as briclone. Culture-medium conditioned by macrophages can also be used. Production in cell culture is usually preferred as the ascites technique is painful to the animal and if replacement techniques exist, this method is considered unethical.

**Purification of monoclonal antibodies**

After obtaining either a media sample of cultured hybridomas or a sample of ascites fluid, the desired antibodies must be extracted. The contaminants in the cell culture sample would consist primarily of media components such as growth factors, hormones, and transferrins. In contrast, the in vivo sample is likely to have host antibodies, proteases, nucleases, nucleic acids, and viruses. In both cases, other secretions by the hybridomas such as cytokines may be present. There may also be bacterial contamination and, as a result, endotoxins that are secreted by the bacteria. Depending on the complexity of the media required in cell culture, and thus the contaminants in question, one method (in vivo or in vitro) may be preferable to the other.

The sample is first conditioned, or prepared for purification. Cells, cell debris, lipids, and clotted material are first removed, typically by centrifugation followed by filtration with a 0.45 μm filter. These large particles can cause a phenomenon called membrane fouling in later purification steps. In addition, the concentration of product in the sample may not be sufficient, especially in cases where the desired antibody is one produced by a low-secreting cell line. The sample is therefore condensed by ultrafiltration or dialysis.

Most of the charged impurities are usually anions such as nucleic acids and endotoxins. These are often separated by ion exchange chromatography, using columns such as the ProPac WCX-10, now regarded as the gold standard for antibody analysis. Either cation exchange chromatography is used at a low enough pH that the desired antibody binds to the column while anions flow through, or anion exchange chromatography is used at a high enough pH that the desired antibody flows through the column while anions bind to it. Various proteins can also be separated out along with the anions based on their isoelectric point (pI). For example, albumin has a pI of 4.8, which is significantly lower than that of most monoclonal antibodies, which have a pI of 6.1. In other words, at a given pH, the average charge of albumin molecules is likely to be more negative. Transferrin, on the other hand, has a pI of 5.9, so it cannot easily be separated out by this method. A difference in pI of at least 1 is necessary for a good separation.

Transferrin can instead be removed by size exclusion chromatography. The advantage of this purification method is that it is one of the more reliable chromatography techniques. Since we are dealing with proteins, properties such as charge and affinity are not consistent and vary with pH as molecules are protonated and deprotonated, while size stays relatively constant. Nonetheless, it has drawbacks such as low resolution, low capacity and low elution times.

A much quicker, single-step method of separation is Protein A/G affinity chromatography. The antibody selectively binds to Protein A/G, so a high level of purity (generally >80%) is obtained. However, this method may be problematic for antibodies that are easily damaged, as harsh conditions are generally used. A low pH can break the bonds to remove the antibody from the column. In addition to possibly affecting the product, low pH can cause Protein A/G itself to leak off the column and appear in the eluted sample. Gentle elution buffer systems that employ high salt concentrations are also available to avoid exposing sensitive antibodies to low pH. Cost is also an important consideration with this method because immobilized Protein A/G is a more expensive resin.

To achieve maximum purity in a single step, affinity purification can be performed, using the antigen to provide exquisite specificity for the antibody. In this method, the antigen used to generate the antibody is covalently attached to an agarose support. If the antigen is a peptide, it is commonly synthesized with a terminal cysteine, which allows selective attachment to a carrier protein, such as KLH during development and to the support for purification. The antibody-containing media is then incubated with the immobilized antigen, either in batch or as the antibody is passed through a column, where it selectively binds and can be retained while impurities are washed away. An elution with a low pH buffer or a more gentle, high salt elution buffer is then used to recover purified antibody from the support.

To further select for antibodies, the antibodies can be precipitated out using sodium sulfate or ammonium sulfate. Antibodies precipitate at low concentrations of the salt, while most other proteins precipitate at higher concentrations. The appropriate level of salt is added in order to achieve the best separation. Excess salt must then be removed by a desalting method such as dialysis.

The final purity can be analyzed using a chromatogram. Any impurities will produce peaks, and the volume under the peak indicates the amount of the impurity. Alternatively, gel electrophoresis and capillary electrophoresis can be carried out. Impurities will produce bands of varying intensity, depending on how much of the impurity is present.

**Recombinant**

The production of recombinant monoclonal antibodies involves technologies, referred to as *repertoire cloning* or *phage display/yeast display*. Recombinant antibody engineering involves the use of viruses or yeast to create antibodies, rather than mice. These techniques rely on rapid cloning of immunoglobulin gene segments to create libraries of antibodies with slightly different amino acid sequences from which antibodies with desired specificities can be selected. The phage antibody libraries are a variant of the phage antigen libraries first invented by George Pieczenik These techniques can be used to enhance the specificity with which antibodies recognize antigens, their stability in various environmental conditions, their therapeutic efficacy, and their detectability in diagnostic applications. Fermentation chambers have been used to produce these antibodies on a large scale.

**Chimeric antibodies**

Early on, a major problem for the therapeutic use of monoclonal antibodies in medicine was that initial methods used to produce them yielded mouse, not human antibodies. While structurally similardifferences between the two sufficient to invoke an immune response occurred when murine monoclonal antibodies were injected into humans and resulted in their rapid removal from

the blood, systemic inflammatory effects, and the production of human anti-mouse antibodies (HAMA).

In an effort to overcome this obstacle, approaches using recombinant DNA have been explored since the late 1980s. In one approach, mouse DNA encoding the binding portion of a monoclonal antibody was merged with human antibody-producing DNA in living cells, and the expression of this chimeric DNA through cell culture yielded half-mouse, half-human monoclonal antibody. For this product, the descriptive terms "chimeric" and "humanised" monoclonal antibody have been used to reflect the amount of human DNA used in the recombinant process.

**'Fully' human monoclonal antibodies**

Ever since the discovery that monoclonal antibodies could be generated in-vitro, scientists have targeted the creation of 'fully' human antibodies to avoid some of the side effects of humanised and chimeric antibodies. Two successful approaches were identified - phage display-generated antibodies and mice genetically engineered to produce more human-like antibodies.

One of the most successful commercial organisations behind therapeutic monoclonal antibodies was Cambridge Antibody Technology (CAT). Scientists at CAT demonstrated that phage display could be used such that variable antibody domains could be expressed on filamentous phage antibodies. This was reported in a key Nature publication.

CAT developed their display technologies further into several, patented antibody discovery/functional genomics tools, which were named Proximol™ and ProAb™. ProAb was announced in December 1997 and involved highthroughput screening of antibody libraries against diseased and non-diseased tissue, whilst Proximol used a free radical enzymatic reaction to label molecules in proximity to a given protein

Genetically engineered mice, so called transgenic mice, can be modified to produce human antibodies, and this has been exploited by a number of commercial organisations:

- Medarex - who market their UltiMab platform
- Abgenix - who marketed their Xenomouse technology. Abgenix were acquired in April 2006 by Amgen.
- Regeneron's VelocImmune technology.

Monoclonal antibodies have been generated and approved to treat: cancer, cardiovascular disease, inflammatory diseases, macular degeneration, transplant rejection, multiple sclerosis, and viral infection (see monoclonal antibody therapy).

In August 2006 the Pharmaceutical Research and Manufacturers of America reported that U.S. companies had 160 different monoclonal antibodies in clinical trials or awaiting approval by the Food and Drug Administration.

**Applications**

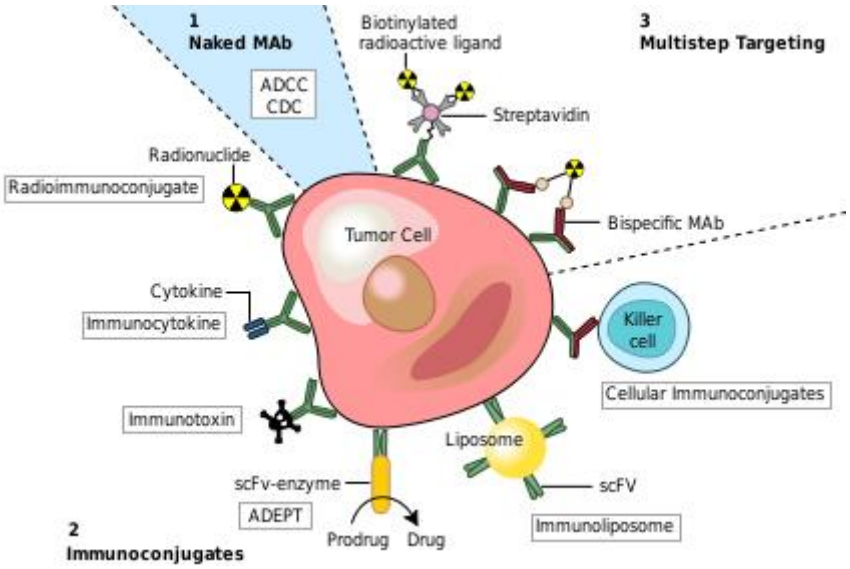
**Diagnostic tests**

Once monoclonal antibodies for a given substance have been produced, they can be used to detect the presence of this substance. The Western blot test and immuno dot blot tests detect the protein on a membrane. They are also very useful in immunohistochemistry, which detect antigen in fixed tissue sections and immunofluorescence test, which detect the substance in a frozen tissue section or in live cells.

**Therapeutic treatment**

Monoclonal antibody therapy for **Cancer treatment**

One possible treatment for cancer involves monoclonal antibodies that bind only to cancer cell-specific antigens and induce an immunological response against the target cancer cell. Such mAb could also be modified for delivery of a toxin, radioisotope, cytokine or other active conjugate; it is also possible to design bispecific antibodies that can bind with their Fab regions both to target antigen and to a conjugate or effector cell. In fact, every intact antibody can bind to cell receptors or other proteins with its Fc region.




**Monoclonal antibodies for cancer.** ADEPT, antibody directed enzyme prodrug therapy; ADCC, antibody dependent cell-mediated cytotoxicity; CDC, complement dependent cytotoxicity; MAb, monoclonal antibody; scFv, single-chain Fv fragment.

The illustration below shows all these possibilities:

MAbs approved by the FDA include

- Bevacizumab
- Cetuximab
- Panitumumab
- Traztuzumab
- Pertuzumab

**Autoimmune diseases**

Monoclonal antibodies used for autoimmune diseases include infliximab and adalimumab, which are effective in rheumatoid arthritis, Crohn's disease and ulcerative Colitis by their ability to bind to and inhibit TNF-α.[23] Basiliximab and daclizumab inhibit IL-2 on activated T cells and thereby help preventing acute rejection of kidney transplants.[23] Omalizumab inhibits human immunoglobulin E (IgE) and is useful in moderate-to-severe allergic asthma.

**Examples**

Below are examples of clinically important monoclonal antibodies.

| Main | Type | Application | Mechanism/Target | Mode |
|---|---|---|---|---|

| category | | | | |
|---|---|---|---|---|
| Anti-inflammatory | **infliximab** | • rheumatoid arthritis<br>• Crohn's disease<br>• Ulcerative Colitis | inhibits TNF-α | chimeric |
| | **adalimumab** | • rheumatoid arthritis<br>• Crohn's disease<br>• Ulcerative Colitis | inhibits TNF-α | human |
| | **etanercept** | • rheumatoid arthritis | Contains decoy TNF receptor | fusion protein |
| | **basiliximab** | • Acute rejection of kidney transplants | inhibits IL-2 on activated T cells | chimeric |
| | **daclizumab** | • Acute rejection of kidney transplants | inhibits IL-2 on activated T cells | humanized |
| | **omalizumab** | • moderate-to-severe allergic asthma | inhibits human immunoglobulin E (IgE) | humanized |
| Anti-cancer | **gemtuzumab** | • relapsed acute myeloid leukaemia | targets myeloid cell surface antigen CD33 on leukemia cells | humanized |
| | **alemtuzumab** | • B cell leukemia | targets an antigen CD52 on T- and B-lymphocytes | humanized |
| | **rituximab** | • non-Hodgkin's lymphoma | targets phosphoprotein CD20 on B lymphocytes | chimeric |
| | **trastuzumab** | • breast cancer with HER2/neu overexpression | targets the HER2/neu (erbB2) receptor | humanized |
| | **nimotuzumab** | • Approved in squamous cell carcinomas, Glioma<br>• Clinical trials for other indications underway | EGFR inhibitor | Humanized |
| | **cetuximab** | • Approved in squamous cell carcinomas, | EGFR inhibitor | Chimeric |

| | | | | |
|---|---|---|---|---|
| | | colorectal carcinoma | | |
| | **bevacizumab** | • Anti-angiogenic cancer therapy | inhibits VEGF | humanized |
| | **palivizumab**[ | • RSV infections in children | inhibits an RSV fusion (F) protein | humanized |
| **Other** | **abciximab** | • Prevent coagulation in coronary angioplasty | inhibits the receptor GpIIb/IIIa on platelets | chimeric |

**Rapid Characterization of Monoclonal Antibodies using the Piezoelectric Immunosensor**
Monoclonal antibodies with specificity against the *Francisella tularensis* outer lipopolysaccharide (LPS) membrane were prepared and characterized using the piezoelectric immunosensor with immobilized LPS antigen from *F. tularensis*. Signals obtained by the immunosensor were compared with ELISA and similar sensitivity was noticed. Signal of negative controls obtained using the biosensor was below 0.5% of the signal obtained for the selected specific antibody clone 4H3B9D3. Testing of cross reactivity based on the sensors with immobilized LPS from *Escherichia coli* and *Bacillus subtilis* confirmed selectivity of this antibody. Furthermore, the 4H3B9D3 antibody was successfully isotypized as IgM using the piezoelectric sensors with secondary antibodies. Kinetics parameters of antibody were evaluated in the flow-through arrangement. The kinetic rate constants for the antibody 4H3B9D3 were *k*a = (2.31 ± 0.20)·105 l mol-1s-1 (association) and *k*d = (0.0010 ±0.00062) s-1(dissociation) indicating very good affinity to the LPS antigen.

**Characterization of Monoclonal Antibody Products**

Characterization tests
– Provide detailed information on the molecule/product
– Requirement for Reference Standard characterization
– Required for comparability studies
– Often technically challenging for routine use

**Characterization tests for Mab products**
**Primary Structure**
– LC/MS Peptide Maps
– N-terminal Sequencing
– Verification of C-terminus
– Disulfide Bond Determination
– Glycan Map
– Intact Mass Determination

**Secondary and Tertiary Structure**
– FTIR
– Far UV CD
– Fluorescence
– Near UV CD

**Others**

– Non-reduced CE-SDS
– CEX-HPLC, low pH
– DSC
– AUC
– SE-MALS
– Extinction Coefficient
– Excipients
– Process impurities

**Functional characterization**

– Antigen binding
– Additional cell-based assays
– Epitope mapping
– Fcγ RI, RIII binding
– ADCC
– CDC
– FcRn binding

**Production Polyclonal antibodies Seen in detail under the topic of antigen preparation Unit I**

**Characterisation of Polyclonal antibodies**

***Basis of polyclonality***

Responses are polyclonal in nature as each clone somewhat specializes in producing antibodies against a given epitope, and because, each antigen contains multiple epitopes, each of which in turn can be recognized by more than one clone of B cells. But, to be able to react to innumerable antigens, as well as, multiple constituent epitopes, the immune system requires the ability to recognize a very great number of epitopes in all, i.e., there should be a great diversity of B cell clones.

**Clonality of B cells**

Memory and naïve B cells normally exist in relatively small numbers. As the body needs to be able to respond to a large number of potential pathogens, it maintains a pool of B cells with a wide range of specificities. Consequently, while there is almost always at least one B (naive or memory) cell capable of responding to any given epitope (of all that the immune system can react against), there are very few exact duplicates. However, when a single B cell encounters an antigen to which it can bind, it can proliferate very rapidly. Such a group of cells with identical specificity towards the epitope is known as a *clone*, and is derived from a common "mother" cell. All the "daughter" B cells match the original "mother" cell in their epitope specificity, and they secrete antibodies with identical paratopes. So, in this context, a polyclonal response is one in which *multiple clones of B cells react to the same antigen.*

**Single antigen contains multiple overlapping epitopes**

Blind Monks Examining an Elephant: An allegory for the polyclonal response: Each clone or antibody recognizes different parts of a single, larger antigen

A single antigen can be thought of as a sequence of multiple overlapping epitopes. Many unique B cell clones may be able to bind to the individual epitopes. This imparts even greater multiplicity to the overall response. All of these B cells can become activated and produce large colonies of plasma cell clones, each of which can secrete up to 1000 antibody molecules against each epitope per second.

**Multiple clones recognize single epitope**

In addition to different B cells reacting to *different* epitopes on the same antigen, B cells belonging to different clones may also be able to react to the *same* epitope. An epitope that can be attacked by many different B cells is said to be highly *immunogenic*. In these cases, the *binding affinities* for

respective epitope-paratope pairs vary, with some B cell clones producing antibodies that bind strongly to the epitope, and others producing antibodies that bind weakly.

**Clonal selection**

For more details on lymph nodes, germinal centers of lymph nodes and clonal selection of B cells, see Lymph node, Germinal center, Clonal selection.

The clones that bind to a particular epitope with greater strength are more likely to be *selected* for further proliferation in the germinal centers of the follicles in various lymphoid tissues like the lymph nodes. This is not unlike natural selection: clones are selected for their fitness to attack the epitopes (strength of binding) on the encountered pathogen. What makes the analogy even stronger is that the B lymphocytes have to compete with each other for signals that promote their survival in the germinal centers.

**Diversity of B cell clones**

Although there are many diverse pathogens, many of which are constantly mutating, it is a surprise that a majority of individuals remain free of infections. Thus, maintenance of health requires the body to recognize all pathogens (antigens they present or produce) likely to exist. This is achieved by maintaining a pool of immensely large (about $10^9$) clones of B cells, each of which reacts against a specific epitope by recognizing and producing antibodies against it. However, at any given time very few clones actually remain receptive to their specific epitope. Thus, approximately $10^7$ different epitopes can be recognized by all the B cell clones combined. Moreover, in a lifetime, an individual usually requires the generation of antibodies against very few antigens in comparison with the number that the body can recognize and respond against.

***Significance of the phenomenon***

**Increased probability of recognizing any antigen**

If an antigen can be recognized by more than one component of its structure, it is less likely to be "missed" by the immune system. Mutation of pathogenic organisms can result in modification of antigen—and, hence, epitope—structure. If the immune system "remembers" what the other epitopes look like, the antigen, and the organism, will still be recognized and subjected to the body's immune response. Thus, the polyclonal response widens the range of pathogens that can be recognized.

**Limitation of immune system against rapidly mutating viruses**

The clone 1 that got stimulated by first antigen gets stimulated by second antigen, too, which best binds with naive cell of clone 2. However, antibodies produced by plasma cells of clone 1 inhibit the proliferation of clone 2.

Many viruses undergo frequent mutations that result in changes in amino acid composition of their important proteins. Epitopes located on the protein may also undergo alterations in the process. Such an altered epitope binds less strongly with the antibodies specific to the unaltered epitope that would have stimulated the immune system. This is unfortunate because somatic hypermutation does give rise to clones capable of producing soluble antibodies that would have bound the altered epitope avidly enough to neutralize it. But these clones would consist of naive cells which are not allowed to proliferate by the weakly binding antibodies produced by the priorly stimulated clone. This doctrine is known as the *original antigenic sin*. This phenomenon comes into play particularly in immune responses against influenza, dengue and HIV viruses. This limitation, however, is not imposed *by* the phenomenon of polyclonal response, but rather, *against it* by an immune response that is biased in favor of experienced memory cells against the "novice" naive cells.

**Increased chances of autoimmune reactions**

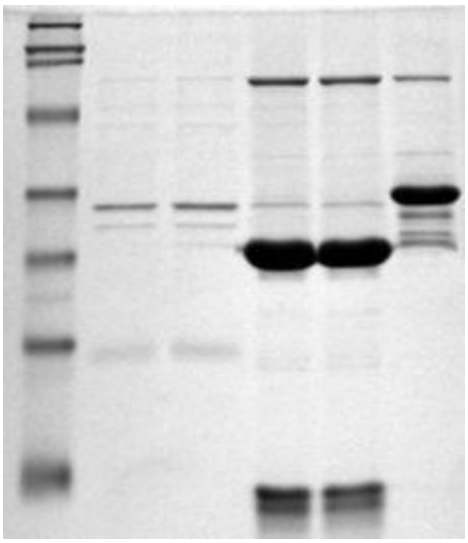
In autoimmunity the immune system wrongly recognizes certain native molecules in the body as foreign (*self-antigen*), and mounts an immune response against them. Since these native molecules, as normal parts of the body, will naturally always exist in the body, the attacks against them can get stronger over time (akin to secondary immune response). Moreover, many organisms exhibit

molecular mimicry, which involves showing those antigens on their surface that are antigenically similar to the host proteins. This has two possible consequences: first, either the organism will be spared as a self antigen; or secondly, that the antibodies produced against it will also bind to the mimicked native proteins. The antibodies will attack the self-antigens and the tissues harboring them by activating various mechanisms like the complement activation and antibody-dependent cell-mediated cytotoxicity. Hence, wider the range of antibody-specificities, greater the chance that one or the other will react against self-antigens (native molecules of the body).[26][27]

**Difficulty in producing monoclonal antibodies**

Monoclonal antibodies are structurally identical immunoglobulin molecules with identical epitope-specificity (all of them bind with the same epitope with same affinity) as against their polyclonal counterparts which have varying affinities for the same epitope. They are usually not produced in a natural immune response, but only in diseased states like multiple myeloma, or through specialized laboratory techniques. Because of their specificity, monoclonal antibodies are used in certain applications to quantify or detect the presence of substances (which act as antigen for the monoclonal antibodies), and for targeting individual cells (e.g. cancer cells). Monoclonal antibodies find use in various diagnostic modalities (see: western blot and immunofluorescence) and therapies—particularly of cancer and diseases with autoimmune component. But, since virtually all responses in nature are polyclonal, it makes production of immensely useful monoclonal antibodies less straightforward.

**SDS-PAGE**



Picture of an SDS-PAGE. The molecular marker is in the left lane

**SDS-PAGE**, **sodium dodecyl sulfate polyacrylamide gel electrophoresis**, is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). SDS gel electrophoresis of samples have identical charge per unit mass due to binding of SDS results in fractionation by size.

***Procedure***

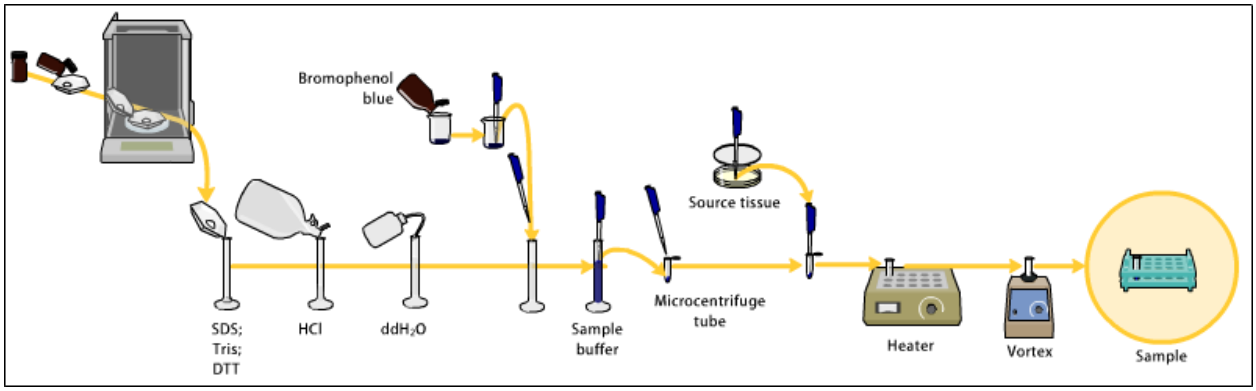
**Tissue preparation**

Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonicator. Cells may also be broken open by one of the above mechanical methods. However, it should be noted that bacteria, virus or environmental samples can be the source of protein and thus Western blotting is not restricted to cellular studies only.

A combination of biochemical and mechanical techniques – including various types of filtration and centrifugation – can be used to separate different cell compartments and organelles.

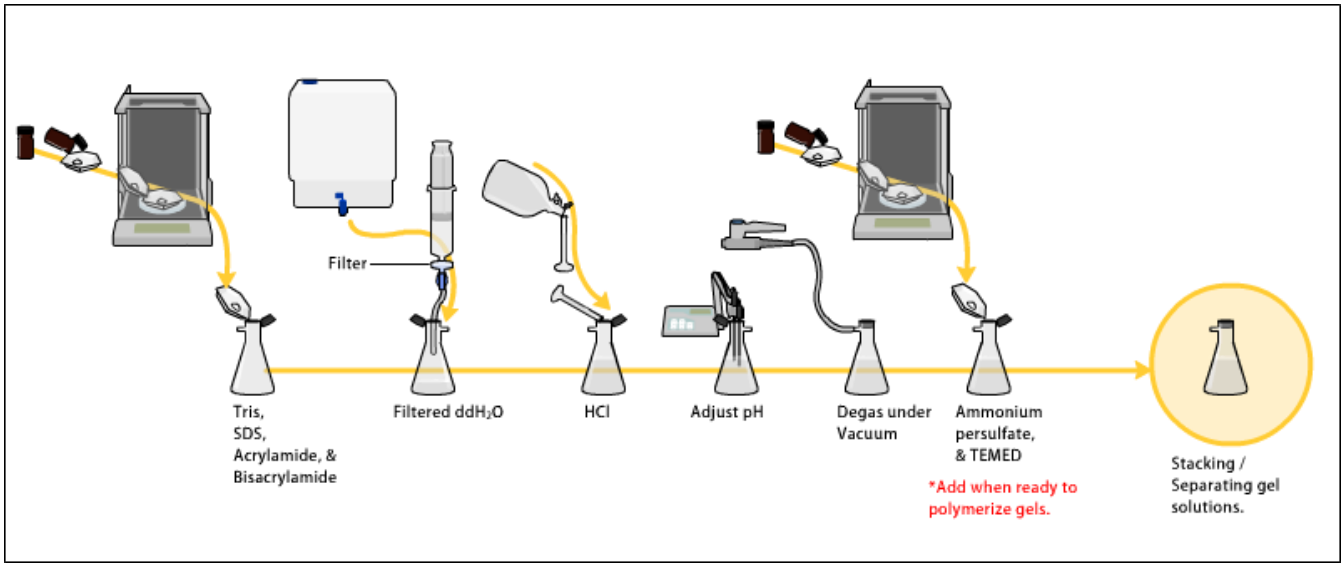
The solution of proteins to be analyzed is mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Heating the samples to at least 60 degrees C shakes up the molecules, helping SDS to bind.

A tracking dye may be added to the protein solution (of a size smaller than protein) to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.

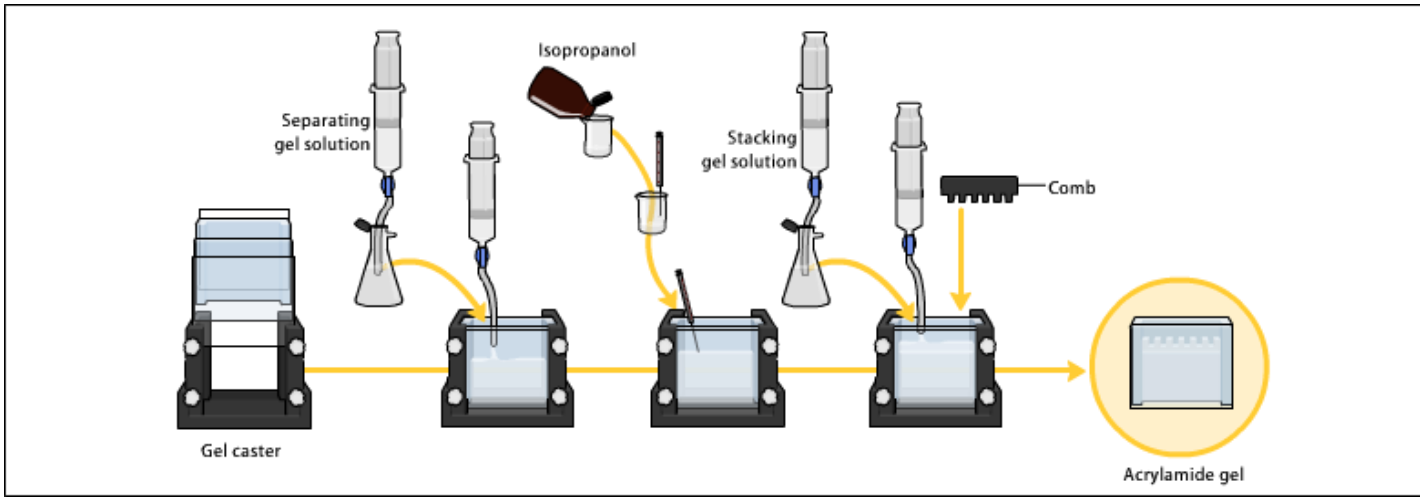



**Preparing acrylamide gels**

The gels generally consist of acrylamide, bisacrylamide, SDS, and a Tris-Cl buffer with adjusted pH. The solution is degassed under a vacuum to prevent air bubbles during polymerization. Ammonium persulfate and TEMED are added when the gel is ready to be polymerized. The separating or resolving gel is usually more basic and has a higher polyacrylamide content than the loading gel.

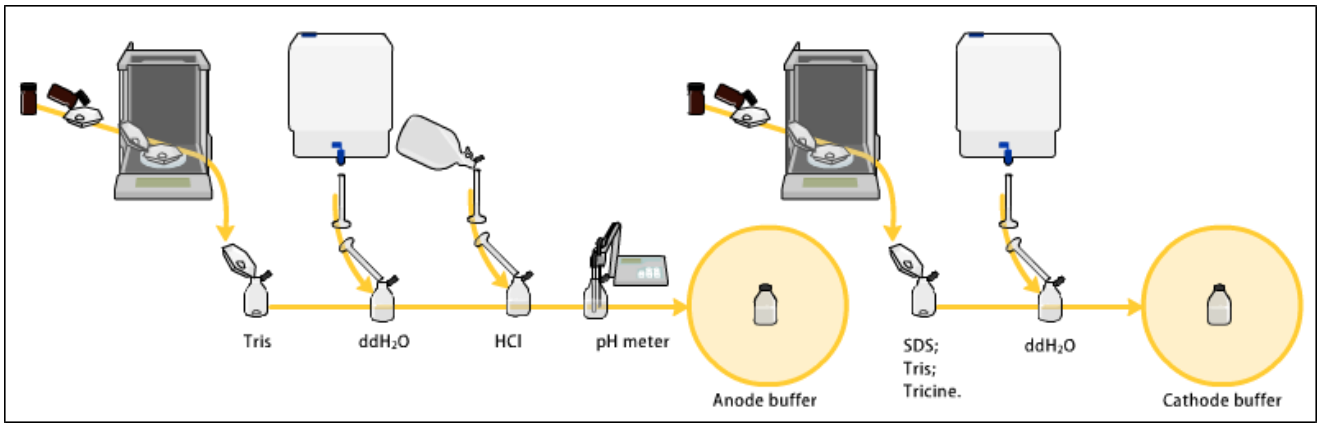



Gels are polymerized in a gel caster. First the separating gel is poured and allowed to polymerize. Next a thin layer of isopropanol is added. Next the loading gel is poured and a comb is placed to create the wells. After the loading gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

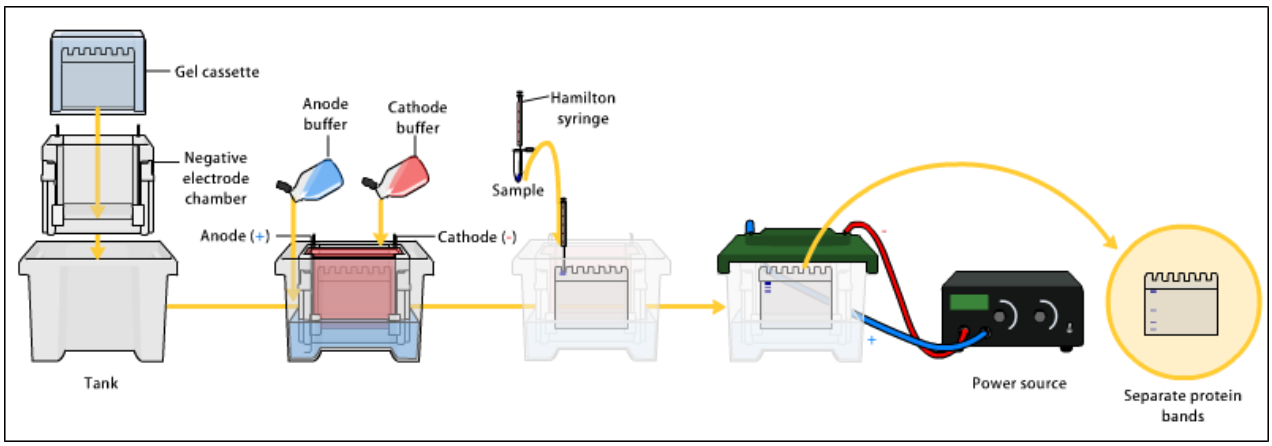



**Electrophoresis**

First the anode and cathode buffers are prepared. The anode buffer usually contains Tris-Cl, distilled deionized water and is adjusted to a higher pH than the cathode buffer. The cathode buffer contains SDS, Tris, Tricine, and distilled deionized water.[7] [8]




The electrophoresis apparatus is set up with cathode buffer covering the gel in the negative electrode chamber, and anode buffer in the lower positive electrode chamber. Next, the denatured sample proteins are added to the wells one end of the gel with a syringe or pipette. Finally, the apparatus is hooked up to a power source under appropriate running conditions to separate the protein bands.




An electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive (+) electrode (anode). Depending on their size, each protein will move

differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty (they encounter more resistance). After a set amount of time (usually a few hours- though this depends on the voltage applied across the gel; higher voltages run faster but tend to produce somewhat poorer resolution), the proteins will have differentially migrated based on their size; smaller proteins will have traveled farther down the gel, while larger ones will have remained closer to the point of origin. Therefore, proteins may be separated roughly according to size (and therefore, molecular weight), certain glycoproteins behave anomalously on SDS gels.

**Staining**

Two SDS-PAGE-gels after a completed run

Following electrophoresis, the gel may be stained (most commonly with Coomassie Brilliant Blue R-250 or silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot). After staining, different proteins will appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel, in order to calibrate the gel and determine the weight of unknown proteins by comparing the distance traveled relative to the marker. The gel is actually formed because the acrylamide solution contains a small amount, generally about 1 part in 35 of bisacrylamide, which can form cross-links between two polyacrylamide molecules. The ratio of acrylamide to bisacrylamide can be varied for special purposes. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight proteins, while much higher percentages are needed to resolve smaller proteins. Determining how much of the various solutions to mix together to make gels of particular acrylamide concentration can be done on line

Gel electrophoresis is usually the first choice as an assay of protein purity due to its reliability and ease. The presence of SDS and the denaturing step causes proteins to be separated solely based on size. False negatives and positives are possible. A comigrating contaminant can appear as the same band as the desired protein. This comigration could also cause a protein to run at a different position or to not be able to penetrate the gel. This is why it is important to stain the entire gel including the stacking section. Coomassie Brilliant Blue will also bind with less affinity to glycoproteins and fibrous proteins, which interferes with quantification.

### *Chemical ingredients and their roles*

***Polyacrylamide gel (PAG)*** had been known as a potential embedding medium for sectioning tissues as early as 1964. Two independent groups, Davis and Raymond, employed PAG in electrophoresis in 1959. It possesses several electrophoretically desirable features that make it a versatile medium. PAGE separates protein molecules according to both size and charge. It is a synthetic gel, thermo-stable, transparent, strong, relatively chemically inert, can be prepared with a wide range of average pore sizes. The pore size of a gel is determined by two factors, the total amount of acrylamide present (%T) (T = Total acrylamide-bisacrylamide monomer concentration) and the amount of cross-linker (%C) (C = Crosslinker concentration). Pore size decreases with increasing %T; with cross-linking, 5%C gives the smallest pore size. Any increase or decrease in %C increases the pore size, as pore size with respect to %C is a parabolic function with vertex as 5%C. This appears to be because of nonhomogeneous bundling of strands in the gel.

This gel material can also withstand high voltage gradients, feasible for various staining and destaining procedures, and can be digested to extract separated fractions or dried for autoradiography and permanent recording. DISC electrophoresis utilizes gels of different pore sizes. The name DISC was derived from the discontinuities in the electrophoretic matrix and coincidentally from the discoid shape of the separated zones of ions. There are two layers of gel, namely stacking or spacer gel, and resolving or separating gel.

**Stacking gel**

The stacking gel is a large pore PAG (4%T). This gel is prepared with Tris/HCl buffer pH 6.8 of about 2 pH units lower than that of electrophoresis buffer (Tris/Glycine). These conditions provide an environment for Kohlrausch reactions determining molar conductivity, as a result, SDS-coated proteins are concentrated to several fold and a thin starting zone of the order of 19 μm is achieved in a few minutes. This gel is cast over the resolving gel. The height of the stacking gel region is always maintained more than double the height and the volume of the sample to be applied. This is based on isotachophoresis.

**Chemical ingredients**

- **Tris (tris (hydroxy methyl) aminomethane) ($C_4H_{11}NO_3$; mW: 121.14).** It has been used as a buffer because it is an innocuous substance to most proteins. Its pKa is 8.3 at 20 °C, making it a very satisfactory buffer in the pH range from roughly 7 to 9.

- **Glycine (Amino Acetic Acid) ($C_2H_5NO_2$; mW: 75.07).** Glycine has been used as the source of trailing ion or slow ion because its pKa is 9.69 and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range. The minimum pH of this range is approximately 8.0.

- **Acrylamide ($C_3H_5NO$; mW: 71.08).** It is a white crystalline powder. While dissolving in water, autopolymerization of acrylamide takes place. It is a slow spontaneous process by which acrylamide molecules join together by head on tail fashion. But in presence of free radicals generating system, acrylamide monomers are activated into a free-radical state. These activated monomers polymerise quickly and form long chain polymers. This kind of reaction is known as Vinyl addition polymerisation. A solution of these polymer chains becomes viscous but does not form a gel, because the chains simply slide over one another. Gel formation requires hooking various chains together. Acrylamide is a neurotoxin. It is also essential to store acrylamide in a cool dark and dry place to reduce autopolymerisation and hydrolysis.

- **Bisacrylamide (N,N'-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17).** Bisacrylamide is the most frequently used cross linking agent for poly acrylamide gels. Chemically it is thought of having two-acrylamide molecules coupled head to head at their non-reactive ends.

- **Sodium Dodecyl Sulfate (SDS) ($C_{12}H_{25}NaO_4S$; mW: 288.38).** SDS is the most common dissociating agent used to denature native proteins to individual polypeptides. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g/g of polypeptide. In this process, the intrinsic charges of polypeptides becomes negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment becomes a rod like structure possessing a uniform charge density, that is same net negative charge per unit length. Mobilities of these proteins will be a linear function of the logarithms of their molecular weights.

  Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

- **Ammonium persulfate (APS)** ($N_2H_8S_2O_8$; mW: 228.2). APS is an initiator for gel formation.

- **TEMED (N, N, N', N'-tetramethylethylenediamine**) ($C_6H_{16}N_2$; mW: 116.21). Chemical polymerisation of acrylamide gel is used for SDS-PAGE. It can be initiated by ammonium persulfate and the quaternary amine, N,N,N',N'-tetramethylethylenediamine (TEMED). The rate of polymerisation and the properties of the resulting gel depends on the concentration

of APS and TEMED. Increasing the amount of APS and TEMED results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount of initiators shows the reverse effect. The lowest catalytic concentrations that will allow polymerisation in the optimal period of time should be used. APS and TEMED are used, approximately in equimolar concentrations in the range of 1 to 10 mM.

**Chemicals for processing and visualization**

The following chemicals are used for processing of the gel and the protein samples visualized in it:

- **Bromophenol blue (BPB**) (3',3",5',5" tetrabromophenolsulfonphthalein) **($C_{19}H_{10}Br_4O_5S$; mW: 669.99)**. BPB is the universal marker dye. Proteins and nucleic acids are mostly colourless. When they are subjected to electrophoresis, it is important to stop the run before they run off the gel. BPB is the most commonly employed tracking dye, because it is viable in alkali and neutral pH, it is a small molecule, it is ionisable and it is negatively charged above pH 4.6 and hence moves towards the anode. Being a small molecule it moves ahead of most proteins and nucleic acids. As it reaches the anodic end of the electrophoresis medium electrophoresis is stopped. It can bind with proteins weakly and give blue colour.

- **Glycerol ($C_3H_8O_3$; mW: 92.09).** It is a preservative and a weighing agent. Addition of glycerol (20-30 or 50%) is often recommended for the storage of enzymes. Glycerol maintains the protein solution at very low temperature, without freezing. It also helps to weigh down the sample into the wells without being spread while loading.

- **Coomassie Brilliant Blue R-250 (CBB)($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97)**. CBB is the most popular protein stain. It is an anionic dye, which binds with proteins non-specifically. The structure of CBB is predominantly non-polar. So is usually used (0.025%) in methanolic solution (40%) and acetic acid (7%). Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated in the gel can be removed by destaining with the same solution but without the dye. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution is recommended, approximately ten times the volume of the gel.

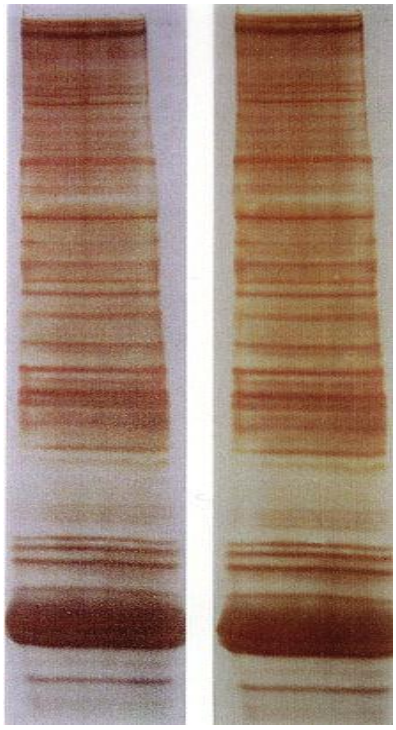
- **n-Butanol ($C_4H_{10}O$; mW: 74.12).** Water saturated butanol is used as an overlay solution on the resolving gel.

- **Dithiothreitol (DTT; $C_4H_{10}O_2S_2$; mW: 154.25).** DTT is a reducing agent used to disrupt disulfide bonds to ensure the protein is fully denatured before loading on the gel; ensuring the protein runs uniformly. Traditionally the toxic and less potent 2-mercaptoethanol was used.

***Reducing SDS-PAGE***

Besides the addition of SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as dithiothreitol (DTT) or traditionally 2-mercaptoethanol (beta-mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). This is known as reducing SDS-PAGE, and is most commonly used. Non-reducing SDS-PAGE (no boiling and no reducing agent) may be used when native structure is important in further analysis (e.g. enzyme activity, shown by the use of zymograms). For example, **q**uantitative **p**reparative **n**ative **c**ontinuous **p**oly**a**crylamide **g**el **e**lectrophoresis (QPNC-PAGE) is a new method for separating native metalloproteins in complex biological matrices.

### *Silver staining*



**Silver stained SDS Polyacrylamide gels**

In the 14th century the silver staining technique was developed for colouring the surface of glass. It has been used extensively for this purpose since the 16th century. The colour produced by the early silver stains ranged between light yellow and an orange-red. Camillo Golgi perfected the silver staining for the study of the nervous system. Golgi's method stains a limited number of cells at random in their entirety.[14] The exact chemical mechanism by which this happens is still largely unknown.[15] Silver staining was introduced by Kerenyi and Gallyas as a sensitive procedure to detect trace amounts of proteins in gels.[16] The technique has been extended to the study of other biological macromolecules that have been separated in a variety of supports.[17] Classical Coomassie Brilliant Blue staining can usually detect a 50 ng protein band, Silver staining increases the sensitivity typically 50 times. Many variables can influence the colour intensity and every protein has its own staining characteristics; clean glassware, pure reagents and water of highest purity are the key points to successful staining.[18]

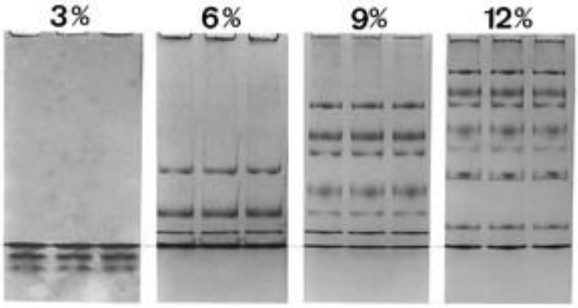
### *Buffer systems*

Most protein separations are performed using a "discontinuous" buffer system that significantly enhances the sharpness of the bands within the gel. During electrophoresis in a discontinuous gel system, an ion gradient is formed in the early stage of electrophoresis that causes all of the proteins to focus into a single sharp band. This occurs in a region of the gel that has larger pores so that the gel matrix does not retard the migration during the focusing or "stacking" event. Negative ions from the buffer in the tank then "outrun" the SDS-covered protein "stack" and eliminate the ion gradient so that the proteins subsequently separate by the sieving action in the lower, "resolving" region of the gel.

Many people continue to use a tris-glycine or "Laemmli" buffering system that stacks at a pH of 6.8 and resolves at a pH of ~8.3-9.0. These pHs promote disulfide bond formation between cysteine residues in the proteins, especially when they are present at high concentrations because the pKa of cysteine ranges from 8-9 and because reducing agent present in the loading buffer doesn't co-migrate with the proteins. Recent advances in buffering technology alleviate this problem by resolving the proteins at a pH well below the pKa of cysteine (e.g., bis-tris, pH 6.5) and include

reducing agents (e.g. sodium bisulfite) that move into the gel ahead of the proteins to maintain a reducing environment. An additional benefit of using buffers with lower pHs is that the acrylamide gel is more stable so the gels can be stored for long periods of time before use.[19][20]

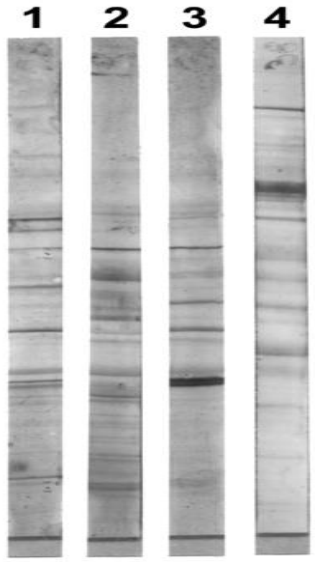
***SDS gradient gel electrophoresis of proteins***




Migration of proteins in SDS gels of varying acrylamide concentrations (%T). The migration of nine proteins ranging from 94 kDa to 14.4 kDa is shown. Stacking and unstacking occurs continuously in the gel, for every protein at a different gel concentration. The dotted line indicates the discontinuity at the $Gly^-/Cl^-$ moving boundary. Proteins between the fast leading electrolyte and the slow trailing electrolyte are not diluted by diffusion.

As voltage is applied, the anions (and negatively charged sample molecules) migrate toward the positive electrode (anode) in the lower chamber, the leading ion is $Cl^-$ ( high mobility and high concentration); glycinate is the trailing ion (low mobility and low concentration). SDS-protein particles do not migrate freely at the border between the $Cl^-$ of the gel buffer and the $Gly^-$ of the cathode buffer. Friedrich Kohlrausch found that Ohm's law also applies to dissolved electrolytes. Because of the voltage drop between the Cl- and Glycine-buffers, proteins are compressed (stacked) into micrometer thin layers. The boundary moves through a pore gradient and the protein stack gradually disperses due to an frictional resistance increase of the gel matrix. Stacking and unstacking occurs continuously in the gradient gel, for every protein at a different position. For a complete protein unstacking the polyacrylamide-gel concentration must exceed 16% T. The two-gel system of "Laemmli" is a simple gradient gel. The pH discontinuity of the buffers is of no significance for the separation quality, and a "stacking-gel" with a different pH is not needed.

**Western blot**




Western blot analysis of proteins separated by SDS-PAGE.

The **Western blot** (alternatively, **protein immunoblot**) is an extremely useful analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.

There are now many reagent companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against tens of thousands of different proteins. Commercial antibodies can be expensive, although the unbound antibody can be reused between experiments. This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines.

Other related techniques include using antibodies to detect proteins in tissues and cells by immunostaining and enzyme-linked immunosorbent assay (ELISA).

The method originated from the laboratory of George Stark at Stanford. The name *Western blot* was given to the technique by W. Neal Burnette and is a play on the name Southern blot, a technique for DNA detection developed earlier by Edwin Southern. Detection of RNA is termed northern blotting and the detection of post-translational modification of protein is termed eastern blotting.

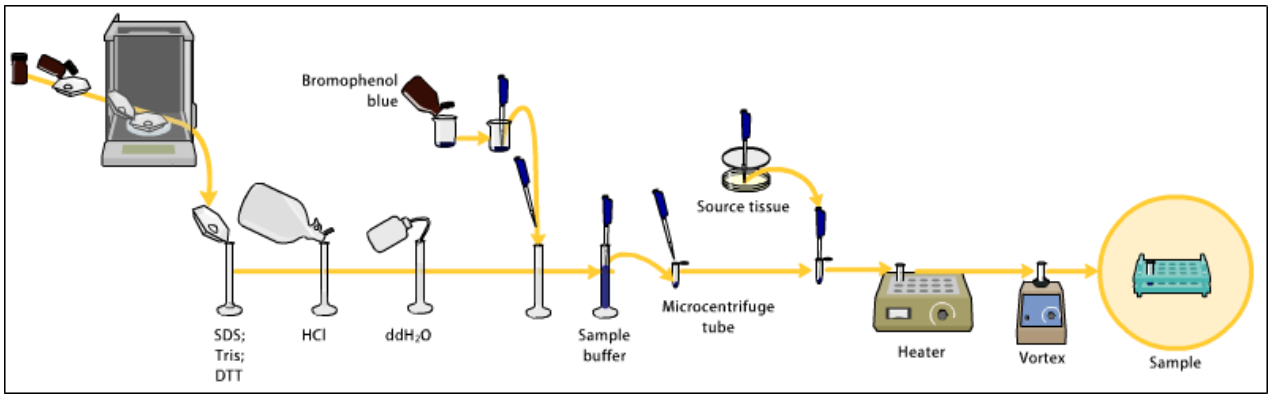
### *Steps in a Western blot*

#### Tissue preparation

Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication. Cells may also be broken open by one of the above mechanical methods. However, it should be noted that bacteria, virus or environmental samples can be the source of protein and thus Western blotting is not restricted to cellular studies only.

Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturing and degradation.

A combination of biochemical and mechanical techniques – including various types of filtration and centrifugation – can be used to separate different cell compartments and organelles.

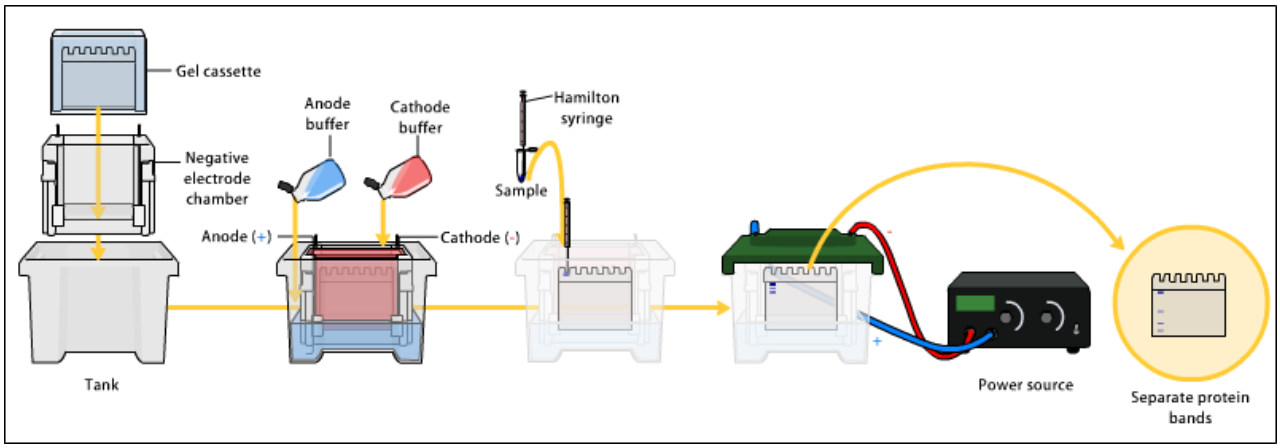



#### Gel electrophoresis

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel. This is a very useful way to determine a protein.

By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

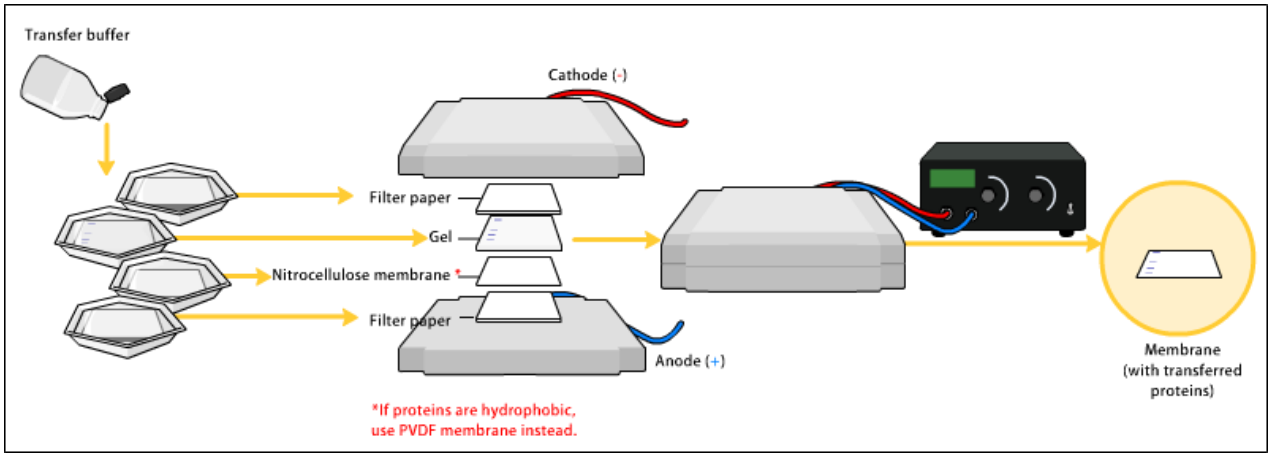
Samples are loaded into *wells* in the gel. One lane is usually reserved for a *marker* or *ladder*, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different *electrophoretic mobilities*) separate into *bands* within each *lane*.




It is also possible to use a two-dimensional (2-D) gel which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

**Transfer**

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of *nitrocellulose or polyvinylidene difluoride (PVDF*). The membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The protein move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection (see below). Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings.




The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie Brilliant Blue or Ponceau S dyes. Ponceau S is the more common of the two, due to Ponceau S's higher sensitivity and its water solubility makes it easier to subsequently destain and probe the membrane as described below.

**Blocking**

Since the membrane has been chosen for its ability to bind protein and as both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive) in Tris-Buffered Saline (TBS), with a minute percentage of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

**Detection**

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

**Two steps**

- Primary antibody

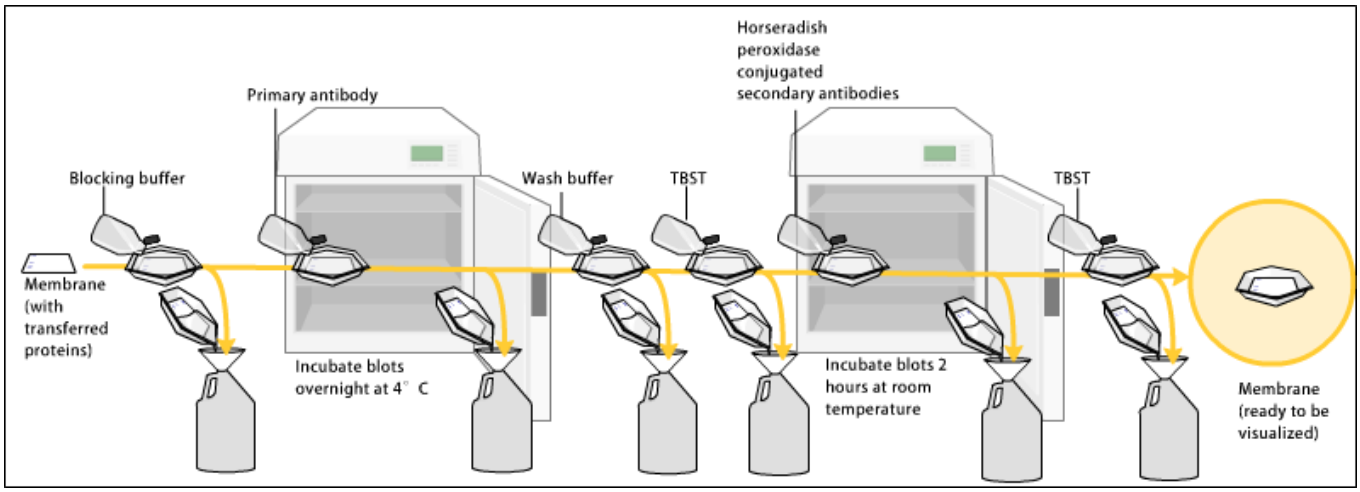
Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof). Normally, this is part of the immune response, whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly.

After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

- Secondary antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to almost any mouse-sourced primary antibody, which allows some cost savings by allowing an entire lab to share a single source of mass-produced antibody, and provides far more consistent results. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% hydrogen peroxide; reaction of peroxide radicals with 4-chloronaphthol produces a dark brown stain that can be photographed without using specialized photographic film.




As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane (see the figure below with blue bands).

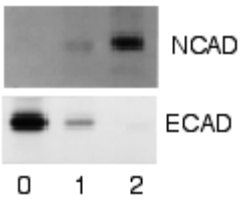
Another method of secondary antibody detection utilizes a near-infrared (NIR) fluorophore-linked antibody. Light produced from the excitation of a fluorescent dye is static, making fluorescent detection a more precise and accurate measure of the difference in signal produced by labeled antibodies bound to proteins on a Western blot. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as compared to chemiluminescence, in which light is measured in a dynamic state.

A third alternative is to use a radioactive label rather than an enzyme coupled to the secondary antibody, such as labeling an antibody-binding protein like *Staphylococcus* Protein A or Streptavidin with a radioactive isotope of iodine. Since other methods are safer, quicker, and cheaper, this method is now rarely used; however, an advantage of this approach is the sensitivity of auto-radiography based imaging, which enables highly accurate protein quantification when combined with optical software (e.g. Optiquant).

**One step**

Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This gives researchers and corporations huge advantages in terms of flexibility, and adds an amplification step to the detection

process. Given the advent of high-throughput protein analysis and lower limits of detection, however, there has been interest in developing one-step probing systems that would allow the process to occur faster and with less consumables. This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known protein tags. The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps.



Western blot using radioactive detection system

**Analysis**

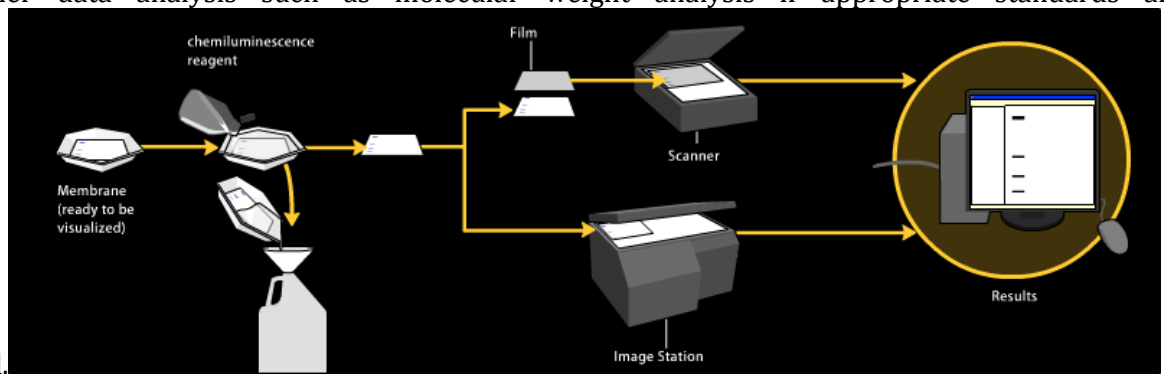
After the unbound probes are washed away, the Western blot is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all Westerns reveal protein only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

### Colorimetric detection

The colorimetric detection method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

### Chemiluminescent detection

Chemiluminescent detection methods depend on incubation of the Western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which capture a digital image of the Western blot. The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used.



**Radioactive detection**

Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the Western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest (see image to the right). The importance of radioactive detections methods is declining, because it is very expensive, health and safety risks are high, and ECL (enhanced chemiluminescence) provides a useful alternative.

**Fluorescent detection**

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as CCD camera equipped with appropriate emission filters which captures a digital image of the Western blot and allows further data analysis such as molecular weight analysis and a quantitative Western blot analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

**Secondary probing**

One major difference between nitrocellulose and PVDF membranes relates to the ability of each to support "stripping" antibodies off and reusing the membrane for subsequent antibody probes. While there are well-established protocols available for stripping nitrocellulose membranes, the sturdier PVDF allows for easier stripping, and for more reuse before background noise limits experiments. Another difference is that, unlike nitrocellulose, PVDF must be soaked in 95% ethanol, isopropanol or methanol before use. PVDF membranes also tend to be thicker and more resistant to damage during use.

***2-D gel electrophoresis***

2-dimensional SDS-PAGE uses the principles and techniques outlined above. 2-D SDS-PAGE, as the name suggests, involves the migration of polypeptides in 2 dimensions. For example, in the first dimension polypeptides are separated according to isoelectric point, while in the second dimension polypeptides are separated according to their molecular weight. The isoelectric point of a given protein is determined by the relative number of positively (e.g. lysine and arginine) and negatively (e.g. glutamate and aspartate) charged amino acids, with negatively charged amino acids contributing to a high isoelectric point and positively charged amino acids contributing to a low isoelectric point. Samples could also be separated first under nonreducing conditions using SDS-PAGE and under reducing conditions in the second dimension, which breaks apart disulfide bonds that hold subunits together. SDS-PAGE might also be coupled with urea-PAGE for a 2-dimensional gel.

In principle, this method allows for the separation of all cellular proteins on a single large gel. A major advantage of this method is that it often distinguishes between different isoforms of a particular protein - e.g. a protein that has been phosphorylated (by addition of a negatively charged group). Proteins that have been separated can be cut out of the gel and then analysed by mass spectrometry, which identifies the protein.

Please refer to reference articles for examples of the application of 2-D SDS PAGE.

***Medical diagnostic applications***

- The confirmatory HIV test employs a Western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody.

- A Western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- Some forms of Lyme disease testing employ Western blotting.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.
- In veterinary medicine, Western blot is sometimes used to confirm FIV+ status in cats.

**Immunoelectrophoresis**

**Immunoelectrophoresis** is a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies reacting with the proteins to be separated or characterized. The methods were developed and used extensively during the second half of the 20th century. In somewhat chronological order: Immunoelectrophoretic analysis (one-dimensional immunoelectrophoresis *ad modum* Grabar), crossed immunoelectrophoresis (two-dimensional quantitative immunoelectrophoresis *ad modum* Clarke and Freeman or *ad modum* Laurell), rocket-immunoelectrophoresis (one-dimensional quantitative immunoelectrophoresis *ad modum* Laurell), fused rocket immunoelectrophoresis *ad modum* Svendsen and Harboe, affinity immunoelectrophoresis *ad modum* Bøg-Hansen.

Agarose as 1 % gel slabs of about 1 mm thickness buffered at high pH (around 8.6) is traditionally preferred for the electrophoresis as well as the reaction with antibodies. The agarose was chosen as the gel matrix because it has large pores allowing free passage and separation of proteins, but provides an anchor for the immunoprecipitates of protein and specific antibodies. The high pH was chosen because antibodies are practically immobile at high pH.

Immunoprecipitates may be seen in the wet agarose gel, but are stained with protein stains like Coomassie Brilliant Blue in the dried gel. In contrast to SDS-gel electrophoresis, the electrophoresis in agarose allows native conditions, preserving the native structure and activities of the proteins under investigation, therefore immunoelectrophoresis allows characterization of enzyme activities and ligand binding etc in addition to electrophoretic separation.

The **immunoelectrophoretic analysis *ad modum* Grabar** is the classical method of immunoelectrophoresis. Proteins are separated by electrophoresis, then antibodies are applied in a trough next to the separated proteins and immunoprecipitates are formed after a period of diffusion of the separated proteins and antibodies against each other. The introduction of the immunoelectrophoretic analysis gave a great boost to protein chemistry, some of the very first results were the resolution of proteins in biological fluids and biological extracts. Among the important observations made were the great number of different proteins in serum, the existence of several immunoglobulin classes and their electrophoretic heterogeneity.

**Crossed immunoelectrophoresis** is also called two-dimensional quantitative immunoelectrophoresis *ad modum* Clarke and Freeman or *ad modum* Laurell. In this method the proteins are first separated during the first dimension electrophoresis, then instead of the diffusion towards the antibodies, the proteins are electrophoresed into an antibody-containing gel in the second dimension. Immunoprecipitation will take place during the second dimension electrophorsis and the immunoprecipitates have a characteristic bell-shape, each precipitate representing one antigen, the position of the precipitate being dependent on the amount of protein as well as the amount of specific antibody in the gel, so relative quantification can be performed. The sensitivity and resolving power of crossed immunoelectrophoresis is than that of the classical immunoelectrophoretic analysis and there are multiple variations of the technique useful for various purposes. Crossed immunoelectrophoresis has been used for studies of proteins in biological fluids, particularly human serum, and biological extracts.

**Rocket immunoelectrophoresis** is one-dimensional quantitative immunoelectrophoresis. The methods has been used for quantitation of human serum proteins before automated methods became available.

**Fused rocket immunoelectrophoresis** is a modification of one-dimensional quantitative immunoelectrophorsis used for detailed measurement of proteins in fractions from protein separation experiments.

**Affinity immunoelectrophoresis** is based on changes in the electrophoretic pattern of proteins through biospecific interaction or complex formation with other macromolecules or ligands. Affinity immunoelectrophoresis has been used for estimation of binding constants, as for instance with lectins or for characterization of proteins with specific features like glycan content or ligand binding. Some variants of affinity immunoelectrophoresis are similar to affinity chromatography by use of immobilized ligands.

The open structure of the immunoprecipitate in the agarose gel will allow additional binding of radioactively labeled antibodies to reveal specific proteins. This variation has been used for identification of allergens through reaction with IgE.

Two factors determine that immunoelectrophoretic methods are not widely used. First they are rather work intensive and require some manual expertise. Second they require rather large amounts of polyclonal antibodies. Today gel electrophoresis followed by electroblotting is the preferred method for protein characterization because its ease of operation, its high sensitivity, and its low requirement for specific antibodies. In addition proteins are separated by gel electrophoresis on the basis of their apparent molecular weight, which is not accomplished by immunoelectrophoresis, but nevertheless immunoelectrophoretic methods are still useful when non-reducing conditions are needed.

## Protein purification

**Protein purification** is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterisation of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps exploit differences in protein size, physico-chemical properties and binding affinity.

### *Purpose*

Purification may be *preparative* or *analytical*. Preparative purifications aim to produce a relatively large quantity of purified proteins for subsequent use. Examples include the preparation of commercial products such as enzymes (e.g. lactase), nutritional proteins (e.g. soy protein isolate), and certain biopharmaceuticals (e.g. insulin). Analytical purification produces a relatively small amount of a protein for a variety of research or analytical purposes, including identification, quantification, and studies of the protein's structure, post-translational modifications and function. Among the first purified proteins were urease and Concanavalin A.

### *Strategies*

Choice of a starting material is key to the design of a purification process. In a plant or animal, a particular protein usually isn't distributed homogeneously throughout the body; different organs or tissues have higher or lower concentrations of the protein. Use of only the tissues or organs with the highest concentration decreases the volumes needed to produce a given amount of purified

protein. If the protein is present in low abundance, or if it has a high value, scientists may use recombinant DNA technology to develop cells that will produce large quantities of the desired protein (this is known as an expression system). Recombinant expression allows the protein to be tagged, e.g. by a His-tag, to facilitate purification, which means that the purification can be done in fewer steps. In addition, recombinant expression usually starts with a higher fraction of the desired protein than is present in a natural source.

An analytical purification generally utilizes three properties to separate proteins. First, proteins may be purified according to their isoelectric points by running them through a pH graded gel or an ion exchange column. Second, proteins can be separated according to their size or molecular weight via size exclusion chromatography or by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis. Proteins are often purified by using 2D-PAGE and are then analysed by peptide mass fingerprinting to establish the protein identity. This is very useful for scientific purposes and the detection limits for protein are nowadays very low and nanogram amounts of protein are sufficient for their analysis. Thirdly, proteins may be separated by polarity/hydrophobicity via high performance liquid chromatography or reversed-phase chromatography.

### *Evaluating purification yield*

The most general method to monitor the purification process is by running a SDS-PAGE of the different steps. This method only gives a rough measure of the amounts of different proteins in the mixture, and it is not able to distinguish between proteins with similar apparent molecular weight.

If the protein has a distinguishing spectroscopic feature or an enzymatic activity, this property can be used to detect and quantify the specific protein, and thus to select the fractions of the separation, that contains the protein. If antibodies against the protein are available then western blotting and ELISA can specifically detect and quantify the amount of desired protein. Some proteins function as receptors and can be detected during purification steps by a ligand binding assay, often using a radioactive ligand.

In order to evaluate the process of multistep purification, the amount of the specific protein has to be compared to the amount of total protein. The latter can be determined by the Bradford total protein assay or by absorbance of light at 280 nm, however some reagents used during the purification process may interfere with the quantification. For example, imidazole (commonly used for purification of polyhistidine-tagged recombinant proteins) is an amino acid analogue and at low concentrations will interfere with the bicinchoninic acid (BCA) assay for total protein quantification. Impurities in low-grade imidazole will also absorb at 280 nm, resulting in an inaccurate reading of protein concentration from UV absorbance.

Another method to be considered is Surface Plasmon Resonance (SPR). SPR can detect binding of label free molecules on the surface of a chip. If the desired protein is an antibody, binding can be translated to directly to the activity of the protein. One can express the active concentration of the protein as the percent of the total protein. SPR can be a powerful method for quickly determining protein activity and overall yield. It is a powerful technology that requires an instrument to perform.

### *Methods of protein purification*

The methods used in protein purification can roughly be divided into analytical and preparative methods. The distinction is not exact, but the deciding factor is the amount of protein that can practically be purified with that method. Analytical methods aim to detect and identify a protein in a mixture, whereas preparative methods aim to produce large quantities of the protein for other purposes, such as structural biology or industrial use. In general, the preparative methods can be used in analytical applications, but not the other way around.

### *1. Extraction*

Depending on the source, the protein has to be brought into solution by breaking the tissue or cells containing it. There are several methods to achieve this: Repeated freezing and thawing, sonication, homogenization by high pressure, filtration (either via cellulose-based depth filters or cross-flow filtration[1]), or permeabilization by organic solvents. The method of choice depends on how fragile the protein is and how sturdy the cells are. After this extraction process soluble proteins will be in the solvent, and can be separated from cell membranes, DNA etc. by centrifugation. The extraction process also extracts proteases, which will start digesting the proteins in the solution. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly, and keep the extract cooled, to slow down proteolysis.

### *2. Precipitation and differential solubilization*

In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulfate $(NH_4)_2SO_4$. This is performed by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipitate protein. One advantage of this method is that it can be performed inexpensively with very large volumes.

The first proteins to be purified are water-soluble proteins. Purification of integral membrane proteins requires disruption of the cell membrane in order to isolate any one particular protein from others that are in the same membrane compartment. Sometimes a particular membrane fraction can be isolated first, such as isolating mitochondria from cells before purifying a protein located in a mitochondrial membrane. A detergent such as sodium dodecyl sulfate (SDS) can be used to dissolve cell membranes and keep membrane proteins in solution during purification; however, because SDS causes denaturation, milder detergents such as Triton X-100 or CHAPS can be used to retain the protein's native conformation during complete purification.

### *3. Ultracentrifugation*

**Centrifugation** is a process that uses centrifugal force to separate mixtures of particles of varying masses or densities suspended in a liquid. When a vessel (typically a tube or bottle) containing a mixture of proteins or other particulate matter, such as bacterial cells, is rotated at high speeds, the angular momentum yields an outward force to each particle that is proportional to its mass. The tendency of a given particle to move through the liquid because of this force is offset by the resistance the liquid exerts on the particle. The net effect of "spinning" the sample in a centrifuge is that massive, small, and dense particles move outward faster than less massive particles or particles with more "drag" in the liquid. When suspensions of particles are "spun" in a centrifuge, a "pellet" may form at the bottom of the vessel that is enriched for the most massive particles with low drag in the liquid. Non-compacted particles still remaining mostly in the liquid are called the "supernatant" and can be removed from the vessel to separate the supernatant from the pellet. The rate of centrifugation is specified by the angular acceleration applied to the sample, typically measured in comparison to the *g*. If samples are centrifuged long enough, the particles in the vessel will reach equilibrium wherein the particles accumulate specifically at a point in the vessel where their buoyant density is balanced with centrifugal force. Such an "equilibrium" centrifugation can allow extensive purification of a given particle.

Sucrose gradient centrifugation — a linear concentration gradient of sugar (typically sucrose, glycerol, or a silica based density gradient media, like Percoll) is generated in a tube such that the highest concentration is on the bottom and lowest on top. Percoll is a trademark owned by GE Healthcare companies. A protein sample is then layered on top of the gradient and spun at high speeds in an ultracentrifuge. This causes heavy macromolecules to migrate towards the bottom of the tube faster than lighter material. During centrifugation in the absence of sucrose, as particles move farther and farther from the center of rotation, they experience more and more centrifugal force (the further they move, the faster they move). The problem with this is that the useful separation range of within the vessel is restricted to a small observable window. Spinning a sample

twice as long doesn't mean the particle of interest will go twice as far, in fact, it will go significantly further. However, when the proteins are moving through a sucrose gradient, they encounter liquid of increasing density and viscosity. A properly designed sucrose gradient will counteract the increasing centrifugal force so the particles move in close proportion to the time they have been in the centrifugal field. Samples separated by these gradients are referred to as "rate zonal" centrifugations. After separating the protein/particles, the gradient is then fractionated and collected.

### *4. Chromatographic methods*

Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column. Usually proteins are detected as they are coming off the column by their absorbance at 280 nm. Many different chromatographic methods exist:

**a. Size exclusion chromatography**
**Gel permeation chromatography**

Chromatography can be used to separate protein in solution or denaturing conditions by using porous gels. This technique is known as size exclusion chromatography. The principle is that smaller molecules have to traverse a larger volume in a porous matrix. Consequentially, proteins of a certain range in size will require a variable volume of eluent (solvent) before being collected at the other end of the column of gel.

In the context of protein purification, the eluant is usually pooled in different test tubes. All test tubes containing no measurable trace of the protein to purify are discarded. The remaining solution is thus made of the protein to purify and any other similarly-sized proteins.

**b. Separation based on charge or hydrophobicity**

Hydrophobic Interaction Chromatography

**c. Ion exchange chromatography**

Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of charge. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds, while cation exchange resins have a negative charge and are used to separate positively charged molecules.

Before the separation begins a buffer is pumped through the column to equilibrate the opposing charged ions. Upon injection of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Becauses of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation.

Ion exchange chromatography is a very powerful tool for use in protein purification and is frequently used in both analytical and preparative separations.

Nickel-affinity column. The resin is blue since it has bound nickel.

### d. Affinity chromatography

Affinity Chromatography is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This "lock and key" fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is unretained.

Many membrane proteins are glycoproteins and can be purified by lectin affinity chromatography. Detergent-solubilized proteins can be allowed to bind to a chromatography resin that has been modified to have a covalently attached lectin. Proteins that do not bind to the lectin are washed away and then specifically bound glycoproteins can be eluted by adding a high concentration of a sugar that competes with the bound glycoproteins at the lectin binding site. Some lectins have high affinity binding to oligosaccharides of glycoproteins that is hard to compete with sugars, and bound glycoproteins need to be released by denaturing the lectin.

### e. Metal binding

A common technique involves engineering a sequence of 6 to 8 histidines into the N- or C-terminal of the protein. The polyhistidine binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis tag or Clontech's HAT tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

### f. Immunoaffinity chromatography

Immunoaffinity chromatography uses the specific binding of an antibody to the target protein to selectively purify the protein. The procedure involves immobilizing an antibody to a column material, which then selectively binds the protein, while everything else flows through. The protein can be eluted by changing the pH or the salinity. Because this method does not involve engineering in a tag, it can be used for proteins from natural sources.

## 5. Purification of a tagged protein

Adding a tag to the protein such as RuBPS gives the protein a binding affinity it would not otherwise have. Usually the recombinant protein is the only protein in the mixture with this affinity, which aids in separation. The most common tag is the Histidine-tag (His-tag), that has affinity towards nickel or cobalt ions. Thus by immobilizing nickel or cobalt ions on a resin, an affinity support that specifically binds to histidine-tagged proteins can be created. Since the protein is the only component with a His-tag, all other proteins will pass through the column, and leave the His-tagged protein bound to the resin. The protein is released from the column in a process called elution, which in this case involves adding imidazole, to compete with the His-tags for nickel binding, as it has a ring structure similar to histidine. The protein of interest is now the major protein component in the eluted mixture, and can easily be separated from any minor unwanted contaminants by a second step of purification, such as size exclusion chromatography or RP-HPLC.

Another way to tag proteins is to engineer an antigen peptide tag onto the protein, and then purify the protein on a column or by incubating with a loose resin that is coated with an immobilized antibody. This particular procedure is known as immunoprecipitation. Immunoprecipitation is quite capable of generating an extremely specific interaction which usually results in binding only

the desired protein. The purified tagged proteins can then easily be separated from the other proteins in solution and later eluted back into clean solution.

When the tags are not needed anymore, they can be cleaved off by a protease. This often involves engineering a protease cleavage site between the tag and the protein.

### *Concentration of the purified protein*

A selectively permeable membrane can be mounted in a centrifuge tube. The buffer is forced through the membrane by centrifugation, leaving the protein in the upper chamber.

At the end of a protein purification, the protein often has to be concentrated. Different methods exist.

#### Lyophilization

If the solution doesn't contain any other soluble component than the protein in question the protein can be lyophilized (dried). This is commonly done after an HPLC run. This simply removes all volatile component leaving the proteins behind.

#### Ultrafiltration

Ultrafiltration concentrates a protein solution using selective permeable membranes. The function of the membrane is to let the water and small molecules pass through while retaining the protein. The solution is forced against the membrane by mechanical pump or gas pressure or centrifugation.

### *Analytical*

#### Denaturing-Condition Electrophoresis

Gel electrophoresis is a common laboratory technique that can be used both as preparative and analytical method. The principle of electrophoresis relies on the movement of a charged ion in an electric field. In practice, the proteins are denatured in a solution containing a detergent (SDS). In these conditions, the proteins are unfolded and coated with negatively charged detergent molecules. The proteins in SDS-PAGE are separated on the sole basis of their size.

In analytical methods, the protein migrate as bands based on size. Each band can be detected using stains such as Coomassie blue dye or silver stain. Preparative methods to purify large amounts of protein, require the extraction of the protein from the electrophoretic gel. This extraction may involve excision of the gel containing a band, or eluting the band directly off the gel as it runs off the end of the gel.

In the context of a purification strategy, denaturing condition electrophoresis provides an improved resolution over size exclusion chromatography, but does not scale to large quantity of proteins in a sample as well as the late chromatography columns.

#### Non-Denaturing-Condition Electrophoresis

An important non-denaturing electrophoretic procedure for isolating bioactive metalloproteins in complex protein mixtures is termed 'quantitative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE).

#### Method for concentration and purification of antigens and antibodies

In carrying out a purification and/or concentration step of antigens or antibodies, a substrate is immersed in a first aqueous medium containing a specifically reacting antigen to coat said substrate with a monomolecular layer of said specifically reacting antigen. The resulting coated substrate is then immersed in a second aqueous medium containing immunologically reactive antibody specific to the antigen in the first aqueous medium to complex said immunologically

reactive antibody with said specifically reacting antigen. The resulting substrate is then immersed in a reagent capable of cleaving the immunological bond between said immunologically reactive antibody and said specifically reactive antigen and forming a solution of said immunologically reactive antibody in the immunological bond-cleaving solution and leaving said specifically reacting antigen coated on said substrate. The method can be reversed for preparing a purified concentration of an immunologically reactive antigen whereby a specifically reacting antibody is substituted for the antigen and the corresponding immunologically reactive antigen is substituted for the antibody.

**Synthesis of Antigens Through Cloned Genes** - Hundreds of genes in eukaryotes have been cloned either from genomic DNA or from cDNA. These cloned genes included a number of genes for specific antigens, and in some cases have been used for the synthesis of antigens leading to the preparation of vaccines.
Following two examples can be used to illustrate the use of cloned genes for vaccine preparation

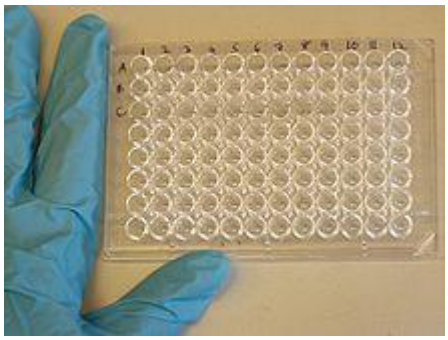
(i) **Cloning of Hepatitis B virus (HBY) genom**e. The HBV genome has been cloned in the plasmid pBR322 and propagated in E. coli. From this clone, antigen could be produced in good quantity, which reacted with hepatitis B core antibody (HBAb). This has therefore been used to produce hepatitis B vaccine, which was later approved for mass vaccination in several countries.

**(ii) Cloning of human malarial gene**. Despite the great menace and threat to human health due to malarial parasite Plasmodium falciparum, no anti-malaria vaccine, could be developed so far. Recently with cloning of a gene coding for surface protein of the sporozoite of P. falciparum, there is a hope for developing a vaccine.

In human host, malarial parasite passes. through several antigenically distinct phases, namely;
**(i) sporozoite**: the form in which the parasite is injected with mosquito bite; sporozoites enter the liver and multiply and develop into
**(ii) merozoites**, which in turn invade and multiply in red blood cells; small fraction of these merozoites in red blood cells form
**(iii) gametocytes**, which may be picked up by a mosquito to start another cycle.

Recent advances in the research of artificial antigen have shown that artificial antigens can be valuable approach for the treatment of some diseases as well as the detection of pesticide residues.By directly/indirectly coupling hapten to an appropriated carrier (macromolecule), artificial antigen can induce animals to produce hapten-specific antibody. Based on this principle, various vaccines have been developed. More impotently, new analytical method, immunological analysis has also been established. Comparing the conventional technologies, such as chromatographic methods, this promising method offers an alternative with high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period of time.

**ELISA**



A 96-well microtiter plate being used for ELISA.

**Enzyme-linked immunosorbent assay** (**ELISA**), also known as an **enzyme immunoassay** (**EIA**), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries. In simple terms, in ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal, most commonly a colour change in a chemical substrate.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bioconjugation. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

Traditional ELISA typically involves chromogenic reporters and substrates which produce some kind of observable color change to indicate the presence of antigen or analyte. Newer ELISA-like techniques utilize fluorogenic, electrochemiluminescent, and real-time PCR reporters to create quantifiable signals. These new reporters can have various advantages including higher sensitivities and multiplexing. Technically, newer assays of this type are not strictly ELISAs as they are not "enzyme-linked" but are instead linked to some non-enzymatic reporter. However, given that the general principles in these assays are largely similar, they are often grouped in the same category as ELISAs.

### *Applications*

ELISA results using S-OIV A neuraminidase antibody at 1 µg/ml to probe the immunogenic and the corresponding seasonal influenza A neuraminidase peptides at 50, 10, 2 and 0 ng/ml.

Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool for determining serum antibody concentrations (such as with the HIV test[3] or West Nile Virus). It has also found applications in the food industry in detecting potential food allergens such as milk, peanuts, walnuts, almonds, and eggs. ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs.

The ELISA was the first screening test widely used for HIV because of its high sensitivity. In an ELISA, a person's serum is diluted 400-fold and applied to a plate to which HIV antigens are attached. If antibodies to HIV are present in the serum, they may bind to these HIV antigens. The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" — an antibody that binds to other antibodies — is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme. Thus, the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence. ELISA results are reported as a number; the most controversial aspect of this test is determining the "cut-off" point between a positive and negative result.

A cut-off point may be determined by comparing it with a known standard. If an ELISA test is used for drug screening at workplace, a cut-off concentration, 50 ng/mL, for example, is established, and a sample will be prepared which contains the standard concentration of analyte. Unknowns that generate a signal that is stronger than the known sample are "positive". Those that generate weaker signal are "negative."

### *History*

Before the development of the ELISA, the only option for conducting an immunoassay was radioimmunoassay, a technique using radioactively-labeled antigens or antibodies. In radioimmunoassay, the radioactivity provides the signal which indicates whether a specific antigen or antibody is present in the sample. Radioimmunoassay was first described in a paper by Rosalyn Sussman Yalow and Solomon Berson published in 1960.

Because radioactivity poses a potential health threat, a safer alternative was sought. A suitable alternative to radioimmunoassay would substitute a non-radioactive signal in place of the radioactive signal. When enzymes (such as peroxidase) react with appropriate substrates (such as ABTS or 3,3’,5,5’-Tetramethylbenzidine), this causes a change in color, which is used as a signal. However, the signal has to be associated with the presence of antibody or antigen, which is why the enzyme has to be linked to an appropriate antibody. This linking process was independently developed by Stratis Avrameas and G.B. Pierce. Since it is necessary to remove any unbound antibody or antigen by washing, the antibody or antigen has to be fixed to the surface of the container, i.e. the *immunosorbent* has to be prepared. A technique to accomplish this was published by Wide and Jerker Porath in 1966.

In 1971, Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in The Netherlands, independently published papers which synthesized this knowledge into methods to perform EIA/ELISA.

### *Types*

#### "Indirect" ELISA

The steps of "indirect" ELISA follows the mechanism below:-

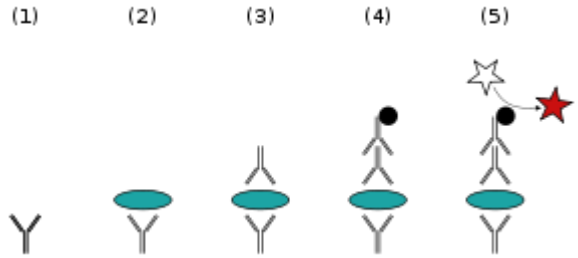
- A buffered solution of the antigen to be tested for is added to each well of a microtiter plate, where it is given time to adhere to the plastic through charge interactions.
- A solution of non-reacting protein, such as bovine serum albumin, or casein is added to block any plastic surface in the well that remains uncoated by the antigen.
- Next the primary antibody, generally in the form of serum is added, which contains a mixture of the serum donor's antibodies, of unknown concentration, some of which may bind specifically to the test antigen that is coating the well.

- Afterwards, a secondary antibody is added, which will bind any antibody produced by a member of the donor's species (for example, an antibody produced in a mouse that will bind any rabbit antibody). This secondary antibody often has an enzyme attached to it, which has a negligible effect on the binding properties of the antibody.
- A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme. The color change shows that secondary antibody has bound to primary antibody, which strongly implies that the donor has had an immune reaction to the test antigen. This can be helpful in a clinical setting, and in R&D.
- The higher the concentration of the primary antibody that was present in the serum, the stronger the color change. Often a spectrometer is used to give quantitative values for color strength.

The enzyme acts as an amplifier; even if only few enzyme-linked antibodies remain bound, the enzyme molecules will produce many signal molecules. Within common-sense limitations the enzyme can go on producing color indefinitely, but the more primary antibody is present in the donor serum, the more secondary antibody + enzyme will bind, and the faster color will develop. A major disadvantage of the indirect ELISA is that the method of antigen immobilization is non-specific; when serum is used as the source of test antigen, all proteins in the sample may stick to the microtiter plate well, so small concentrations of analyte in serum must compete with other serum proteins when binding to the well surface. The sandwich or direct ELISA provides a solution to this problem, by using a "capture" antibody specific for the test antigen to pull it out of the serum's molecular mixture.

ELISA may be run in a qualitative or quantitative format. Qualitative results provide a simple positive or negative result (yes or no) for a sample. The cutoff between positive and negative is determined by the analyst and may be statistical. Two or three times the standard deviation (error inherent in a test) is often used to distinguish positive from negative samples. In quantitative ELISA, the optical density (OD) of the sample is compared to a standard curve, which is typically a serial dilution of a known-concentration solution of the target molecule. For example if your test sample returns an OD of 1.0, the point on your standard curve that gave OD = 1.0 must be of the same analyte concentration as your sample.

**Sandwich ELISA**




**A sandwich *ELISA***. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.

A less-common variant of this technique, called "sandwich" ELISA, is used to detect sample antigen. The steps are as follows:

1. Prepare a surface to which a known quantity of capture antibody is bound.
2. Block any non specific binding sites on the surface.
3. Apply the antigen-containing sample to the plate.

4. Wash the plate, so that unbound antigen is removed.
5. Apply enzyme linked primary antibodies as detection antibodies which also bind specifically to the antigen.
6. Wash the plate, so that the unbound antibody-enzyme conjugates are removed.
7. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
8. Measure the absorbency or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

The image to the right includes the use of a secondary antibody conjugated to an enzyme, though technically this is not necessary if the primary antibody is conjugated to an enzyme. However, use of a secondary-antibody conjugate avoids the expensive process of creating enzyme-linked antibodies for every antigen one might want to detect. By using an enzyme-linked antibody that binds the Fc region of other antibodies, this same enzyme-linked antibody can be used in a variety of situations. Without the first layer of "capture" antibody, any proteins in the sample (including serum proteins) may competitively adsorb to the plate surface, lowering the quantity of antigen immobilized.Use of the purified specific antibody to attach the antigen to the plastic eliminates a need to purify the antigen from complicated mixtures before the measurement, simplifying the assay, and increasing the specificity and the sensitivity of the assay.

**Application of sandwich ELISA to home pregnancy testing using hCG hormone antibody.**

**Competitive ELISA**

A third use of ELISA is through competitive binding. The steps for this ELISA are somewhat different than the first two examples:

1. Unlabeled antibody is incubated in the presence of its antigen (Sample)
2. These bound antibody/antigen complexes are then added to an antigen coated well.
3. The plate is washed, so that unbound antibody is removed. (The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence "competition.")
4. The secondary antibody, specific to the primary antibody is added. This second antibody is coupled to the enzyme.
5. A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.

For competitive ELISA, the higher the sample antigen concentration, the weaker the eventual signal. The major advantage of a competitive ELISA is the ability to use crude or impure samples and still selectively bind any antigen that may be present.

(Note that some competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody. The labeled antigen competes for primary antibody binding sites with your sample antigen (unlabeled). The more antigen in the sample, the less labeled antigen is retained in the well and the weaker the signal).Commonly the antigen is not first positioned in the well.

**Multiple and Portable ELISA (M&P ELISA)**

A new technique (EP 1 499 894 B1 in EPO Bulletin 25.02.209 N. 2009/09; USPTO 7510687 in USPTO Bulletin 31.03.2009; ZL 03810029.0 in SIPO PRC Bulletin 08.04.2009) uses a solid phase made up of an immunosorbent polystyrene rod with 8-12 protruding ogives. The entire device is

immersed in a test tube containing the collected sample and the following steps (washing, incubation in conjugate and incubation in chromogenous) are carried out by dipping the ogives in microwells of standard microplates pre-filled with reagents.

The advantages of this technique are as follows:

1. The ogives can each be sensitized to a different reagent, allowing the simultaneous detection of different antibodies and / or different antigens for multi-target assays;
2. The sample volume can be increased to improve the test sensitivity in clinical (saliva, urine), food (bulk milk, pooled eggs) and environmental (water) samples;
3. One ogive is left unsensitized to measure the non-specific reactions of the sample;
4. The use of laboratory supplies for dispensing sample aliquots, washing solution and reagents in microwells is not required, facilitating the deveopment of ready-to-use lab-kits and on-site kits.

**Radioimmunoassay**

**Radioimmunoassay** (RIA) is an in vitro nuclear medicine very sensitive technique used to measure concentrations of antigens (for example, hormone levels in the blood) without the need to use a bioassay.

Although the RIA technique is extremely sensitive and extremely specific, it requires specialized equipment, but remains the least expensive method to perform such tests. It requires special precautions and licensing, since radioactive substances are used. Today it has been supplanted by the ELISA method, where the antigen-antibody reaction is measured using colorimetric signals instead of a radioactive signal. However, because of its robustness, consistent results and low price per test , RIA methods are again becoming popular.

The RAST test (radioallergosorbent test) is an example of radioimmunoassay. It is used to detect the causative allergen for an allergy.

***Method***

To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine attached to tyrosine. This radiolabeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two chemically bind to one another. Then, a sample of serum from a patient containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radiolabeled antigen ("hot") for antibody binding sites. As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radiolabeled variant, and reducing the ratio of antibody-bound radiolabeled antigen to free radiolabeled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigen remaining in the supernatant is measured using a gamma counter. Using known standards, a binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived.

***History***

It was developed by Rosalyn Yalow and Solomon Aaron Berson in the 1950s. In 1977, Rosalyn Sussman Yalow received the Nobel Prize in Medicine for the development of the RIA for insulin: the precise measurement of minute amounts of such a hormone was considered a breakthrough in endocrinology.

With this technique, separating bound from unbound antigen is crucial. Initially, the method of separation employed was the use of a second "anti-antibody" directed against the first for precipitation and centrifugation. The use of charcoal suspension for precipitation was extended but replaced later by Drs. Werner and Acebedo at Columbia University for RIA of T3 and T4. An ultramicro RIA for human TSH was published in BBRC (1975) by Drs. Acebedo, Hayek et al.[3]

**Applications of Radioimmunoassays**

- **Endocrinology**
    - **Insulin, HCG, Vasopressin**
    - **Detects Endocrine Disorders**
    - **Physiology of Endocrine Function**
- **Pharmacology**
    - **Morphine**
    - **Detect Drug Abuse or Drug Poisoning**
    - **Study Drug Kinetics**

**Non isotopic methods of detection of antigens**

**Sensitive non-isotopic DNA hybridisation assay or immediate-early antigen detection for rapid identification of human cytomegalovirus in urine.**

A sensitive non-radioactive DNA hybridisation assay employing digoxigenin-labelled probes was compared with immediate-early antigen detection and conventional virus isolation for the identification of human cytomegalovirus (HCMV) in 249 urine samples. Of 44 specimens yielding HCMV by virus isolation, more were positive by DNA hybridisation (32; 73%) than by immediate-early antigen detection (25; 52%) ($P = 0.05$). The specificity of the hybridisation assay in 45 apparently falsely positive specimens was supported by detection of HCMV DNA in 40 of these specimens using the polymerase chain reaction. Many urine specimens may thus contain large amounts of non-viable virus or free viral DNA. Evaluation of various protocols for the extraction and denaturation of virus DNA prior to hybridisation showed that proteinase K digestion with phenol/chloroform extraction was the most sensitive and reliable procedure. We conclude that the non-radioactive DNA hybridisation assay described is a potentially valuable routine diagnostic test.

**Cytomegalovirus detection by nonisotopic in situ DNA hybridization and viral antigen immunostaining using a two-color technique.**

Rapid methods of specific viral diagnosis in formalin fixed, paraffin embedded tissues include identification of viral incusions in routinely stained histologic sections, immunologic staining of viral antigens, and in situ nucleic acid hybridization. To correlate in situ hybridization with immunologic detection methods, sequential two-color staining was used on tissues from 12 patients, each containing characteristic cytomegalovirus (CMV) inclusions, using a biotinylated CMV DNA probe in an avidin-alkaline phosphatase-linked reaction followed by avidin-biotin complex immunoperoxidase staining of CMV antigen. CMV genetic material was seen in all 17 tissues. CMV antigen was detected in 11 of 17 tissues (65%). The DNA hybridization technique provided more intense staining, detected greater numbers of inclusions, and had less background staining than the immunoperoxidase technique. The alkaline phosphatase reaction product was stable through subsequent immunostaining steps, and immunologic reactivity of CMV antigen was not significantly reduced by prior hybridization steps. CMV DNA probe was localized predominantly within cell nuclei, while CMV antigen immunostaining was predominantly cytoplasmic. It was concluded that sequential in situ hybridization and immunocytochemistry can be performed on standard histologic sections. Furthermore, it is likely that the majority of CMV nucleic acid detected by this tissue hybridization technique is unencapsidated, intranuclear viral DNA and not DNA contained within complete CMV nucleocapsids.

**Chemiluminescence**

**Chemiluminescence** (sometimes "**chemoluminescence**") is the emission of light with limited emission of heat (luminescence), as the result of a chemical reaction. Given reactants **A** and **B**, with an excited intermediate ◊,

$$[\mathbf{A}] + [\mathbf{B}] \rightarrow [\lozenge] \rightarrow [\textit{Products}] + \text{light}$$

For example, if [A] is luminol and [B] is hydrogen peroxide in the presence of a suitable catalyst we have:

$$\text{luminol} + H_2O_2 \rightarrow \text{3-APA}[\lozenge] \rightarrow \text{3-APA} + \text{light}$$

where:

- where 3-APA is 3-aminophthalate
- 3-APA[◊] is the excited state fluorescing as it decays to a lower energy level.

The decay of the excited state[◊] to a lower energy level causes the emission of light. In theory, one photon of light should be given off for each molecule of reactant, or Avogadro's number of photons per mole. In actual practice, non-enzymatic reactions seldom exceed 1% $Q_C$, quantum efficiency.

In a chemical reaction, reactants collide to form a transition state, the enthalpic maximum in a reaction coordinate diagram, which proceeds to the product. Normally, reactants form products of lesser chemical energy. The difference in energy between reactants and products, represented as $\Delta H_{rxn}$, is turned into heat, physically realized as excitations in the vibrational state of the normal modes of the product. Since vibrational energy is generally much greater than the thermal agitation, it is rapidly dispersed into the solvent through solvent molecules' rotation and translation. This is how exothermic reactions make their solutions hotter. In a chemiluminescent reaction, the direct product of a reaction is delivered in an excited electronic state, which then decays into an electronic ground state through either fluorescence or phosphorescence, depending on the spin state of the electronic excited state formed. This is possible because chemical bond formation can occur on a timescale faster than electronic transitions, and therefore can result in discrete products in excited electronic states.

Chemiluminescence differs from fluorescence in that the electronic excited state is derived from the product of a chemical reaction rather than the more typical way of creating electronic excited states, namely absorption. It is the antithesis of a photochemical reaction, in which light is used to drive an endothermic chemical reaction. Here, light is *generated* from a chemically exothermic reaction.

A standard example of chemiluminescence in the laboratory setting is found in the luminol test, where evidence of blood is taken when the sample glows upon contact with iron. When chemiluminescence takes place in living organisms, the phenomenon is called bioluminescence. A lightstick emits light by chemiluminescence.

### *Liquid-phase reactions*

- Luminol in an alkaline solution with hydrogen peroxide in the presence of iron or copper, or an auxiliary oxidant, produces chemiluminescence. The luminol reaction is

  $$\text{luminol} + H_2O_2 \rightarrow \text{3-APA}[\lozenge] \rightarrow \text{3-APA} + \text{light}$$

The quantum efficiency, $Q_C$ is 1%. For the laboratory experiment see references.

- Cyalume, as used in a lightstick, emits light by chemiluminescence of a fluorescent dye (also called fluorescor) activated by cyalume reacting with hydrogen peroxide in the presence of a catalyst,such as sodium salicylate. It is the most efficient chemiluminescent reaction known. up to 15% quantum efficiency.

  cyalume + $H_2O_2$ + dye → phenol + $2CO_2$ + dye[◊]

When the activated fluorescent dye decays to a lower energy level, light is given off. The color depends upon the dye.

| **Color** | **Sensitiser** |
|---|---|
| Blue | 9,10-Diphenylanthracene |
| Green | 9,10-Bis(phenylethynyl)anthracene |
| Yellow-green | Tetracene |
| Yellow | 1-Chloro-9,10-bis(phenylethynyl)anthracene |
| Orange | 5,12-Bis(phenylethynyl)naphthacene, Rubrene, Rhodamine 6G |
| Red | Rhodamine B |

- Oxalyl chloride ($C_2O_2Cl_2$) produces light when oxidized - but only in the presence of a sensitiser, similar to the above examples. If oxalyl chloride is treated with $H_2O_2$ in *non-aqueous* media (e.g. $CH_2Cl_2$) in the presence of a sensitiser, emission of light is obtained. The colour, intensity and duration of light emission depend on the sensitiser used. Rodamin 6 G gives bright orange light with moderate duration of emission.

- $Ru(bipy)_3^{2+}$ is a ruthenium(II) complex which undergoes oxidation to ruthenium(III) if certain oxidizing agents are introduced. If ruthenium(III) complex is then reduced in alkaline medium, emission of light occurs. First, there is a reaction:

  $2Ru(bipy)_3^{2+} + PbO_2 + 4H^+ \rightarrow 2Ru(bipy)_3^{3+} + Pb^{2+} + 2H_2O$
  Here, Ru(III) is obtained. Further reaction includes use of solution of sodium tetrahydroborate(III), $NaBH_4$ in alkaline medium. When the solution is added, Ru(III) is reduced to Ru(II) and orange light is emitted.

- TMAE (tetrakis(dimethylamino)ethylene) emits clear blue-green light upon oxidation by air.

- Pyrogallol (1,2,3-trihydroxibenzene) is also capable of light emission. If an aqueous solution of pyrogallol, NaOH and $K_2CO_3$ is mixed with formaldehyde, short-lived red emission occurs.

- Singlet oxygen ($O_2$) can also emit light. If solutions of 30% hydrogen peroxide and 5% alkaline sodium hypochlorite (NaClO) are mixed, red light is emitted. It is barely visible, though - for this reason a sensitiser is often included to boost light emission in terms of

brightness and intensity. Again, both colour and intensity of light depend on the sensitiser used.

- Lucigenin oxidation is also very well known among chemiluminescence reactions. If an aqueous lucigenin solution is mixed with highly alkaline aqueous solution containing ethanol or acetone and hydrogen peroxide, very bright green emission is produced that decays to greenish blue and finally blue emission. The duration of the emission can be up to a couple of minutes under the right circumstances.
- Solutions containing ions of manganese (VII, IV, III) show chemiluminescence (690 nm) when reduced by sodium borohydride at low pH to Mn(II)
- Other liquid phase chemilumiscence reagents:
    - peroxyoxalates
    - Aryl oxalates
    - Acridiniumesters
    - dioxetanes

***Gas-phase reactions***

A green and blue glowstick.

- One of the oldest known chemoluminescent reactions is that of elemental white phosphorus oxidizing in moist air, producing a green glow. This is a gas-phase reaction of phosphorus vapor, above the solid, with oxygen producing the excited states $(PO)_2$ and HPO.
- Another gas phase reaction is the basis of nitric oxide detection in commercial analytic instruments applied to environmental air quality testing. Ozone is combined with nitric oxide to form nitrogen dioxide in an activated state.

  $NO+O_3 \rightarrow NO_2[◊]+ O_2$

  The activated $NO_2[◊]$ luminesces broadband visible to infrared light as it reverts to a lower energy state. A photomultiplier and associated electronics counts the photons which are proportional to the amount of NO present. To determine the amount of nitrogen dioxide, $NO_2$, in a sample (containing no NO) it must first be converted to nitric oxide, NO, by passing the sample through a converter before the above ozone activation reaction is applied. The ozone reaction produces a photon count proportional to NO which is proportional to $NO_2$ before it was converted to NO. In the case of a mixed sample containing both NO and $NO_2$, the above reaction yields the amount of NO and $NO_2$ combined in the air sample, assuming that the sample is passed through the converter. If the mixed sample is not passed through the converter, the ozone reaction produces activated $NO_2[◊]$ only in proportion to the NO in the sample. The $NO_2$ in the sample is not activated by the ozone reaction. Though unactivated $NO_2$ is present with the activated $NO_2[◊]$, photons are only emitted by the activated species which is proportional to original NO. Final step, subtract NO from $(NO + NO_2)$ to yield $NO_2$

***Enhanced chemiluminescence***

**Enhanced chemiluminescence** is a common technique for a variety of detection assays in biology. A horseradish peroxidase enzyme (HRP) is tethered to the molecule of interest (usually through labeling an immunoglobulin that specifically recognizes the molecule). This enzyme complex, then catalyzes the conversion of the enhanced chemiluminescent substrate into a sensitized reagent in the vicinity of the molecule of interest, which on further oxidation by hydrogen peroxide, produces a triplet (excited) carbonyl which emits light when it decays to the singlet carbonyl. Enhanced

chemiluminescence allows detection of minute quantities of a biomolecule. Proteins can be detected down to femtomole quantities, well below the detection limit for most assay systems.

### *Applications*

- gas analysis: for determining small amounts of impurities or poisons in air. Other compounds can also be determined by this method (ozone, N-oxides, S-compounds). A typical example is NO determination with detection limits down to 1 ppb
- analysis of inorganic species in liquid phase
- analysis of organic species: useful with enzymes, where the substrate isn't directly involved in chemiluminescence reaction, but the product is
- detection and assay of biomolecules in systems such as ELISA and Western blots
- DNA sequencing using pyrosequencing
- Lighting objects. Chemiluminescence kites, emergency lighting, glow sticks (party decorations).
- Combustion analysis: certain radical species (such as CH* and OH*) give off radiation at specific wavelengths. The heat release rate is calculated by measuring the amount of light radiated from a flame at those wavelengths.
- children's toys

**Electrochemiluminescence**

**Electrochemiluminescence** or **electrogenerated chemiluminescence** (**ECL**) is a kind of luminescence produced during electrochemical reactions in solutions. In electrogenerated chemiluminescence, electrochemically generated intermediates undergo a highly exergonic reaction to produce an electronically excited state that then emits light. ECL excitation is caused by energetic electron transfer (redox) reactions of electrogenerated species. Such luminescence excitation is a form of chemiluminescence where one/all reactants are produced electrochemically on the electrodes.

ECL is usually observed during application of potential (several volts) to electrodes of electrochemical cell that contains solution of luminescent species (polycyclic aromatic hydrocarbons, metal complexes) in aprotic organic solvent (ECL composition).

### *Application*

ECL proved to be very useful in analytical applications as a highly sensitive and selective method. It combines analytical advantages of chemiluminescent analysis (absence of background optical signal) with ease of reaction control by applying electrode potential. Enhanced selectivity of ECL analysis is reached by variation of electrode potential thus controlling species that are oxidized/reduced at the electrode and take part in ECL reaction.

It generally uses Ruthenium complexes, esp $[Ru(Bpy)3]^{2+}$ (which releases a photon at ~620 nm) regenerating with TPA (Tripropylamine) in liquid phase or liquid-solid interface. It can be used as monolayer immobilized on an electrode surface (made eg of nafion, or special thin films made by Langmuir-Blogett technique or self-assembly technique) or as a coreactant or more commonly as a tag and used in HPLC, Ru tagged antibody based immunoassays, Ru Tagged DNA probes for PCR etc, NADH or $H_2O_2$ generation based biosensors, oxalate and organic amine detection and many other applications and can be detected from picomolar sensitivity to dynamic range of more than six orders of magnitude. Photon detection is done with photomultiplier tubes (PMT) or silicon

photodiode or gold coated fiber-optic sensors. ECL is heavily used commercially for many clinical lab applications.

## UNIT III ASSESSMENT OF CELL MEDIATED IMMUNITY

### Identification of lymphocytes and their subsets in blood

To clarify the immune mechanism in myocarditis, immunofluorescence techniques with laser flow cytometry were used to examine serial changes in lymphocyte subsets in the heart, spleen, and peripheral blood of DBA/2 and BALB/c mice inoculated with encephalomyocarditis virus (Experiment I). B cells were identified by staining with fluorescein isothiocyanate-labelled rabbit anti-mouse immunoglobulin. T- cell subsets were identified with rat anti-Thy 1.2, and nonpolymorphic Lyt 1 and Lyt 2 monoclonal antibodies plus fluorescein isothiocyanate- labelled anti-mouse immunoglobulin. On days 7 and 14 postinfection, the percentage of Thy 1.2+ (pan T) cells in both strains had decreased in the peripheral blood; B cells showed no significant changes throughout the entire period. On the other hand, Thy 1.2+ (pan T) and Lyt 1+, 23+ (precursor and immature) T cells appeared to occupy the major portion of the myocardium on days 7 and 14 when congestive heart failure developed. To confirm this, serial immunohistologic studies (immunoperoxidase staining) of the hearts of DBA/2 and BALB/c mice with encephalomyocarditis virus-induced myocarditis were performed (Experiment II). In Experiment II, most of the stained cells in the hearts of both strains were Thy 1.2 positive and Lyt 1 and Lyt 2 positive on days 7 and 14. Thus, Experiments I and II demonstrated that lymphocytes at the site of inflammation in acute viral myocarditis carried antigenic markers that differed from those of peripheral lymphocytes and suggested that Thy 1.2+ (pan T) cells, especially the Lyt 1+, 23+ subset (immature T cells and T-cell subset precursors) were involved in the development of myocarditis in these animals.

### T cell activation parameters

**1. T lymphocyte activation gene identification by coregulated expression on DNA m croarrays.**

High-capacity methods for assessing gene function have become increasingly important because of the increasing number of newly identified genes emerging from large-scale genome sequencing and cDNA cloning efforts. We investigated the use of DNA microarrays to identify uncharacterized genes specifically involved in human T cell activation. Activation of human peripheral blood T lymphocytes induced significant changes in hundreds of transcripts, but most of these were not unique to T cell activation. Variation of experimental parameters and analysis techniques allowed better enrichment for gene expression changes unique to T cell activation. Best results were achieved by identification of genes that were most highly coregulated with the T-cell-specific transcript interleukin 2 (IL2) in a "compendium" of experiments involving both T cells and other cell types. Among the genes most highly coregulated with IL2 were many genes known to function during T cell activation, together with ESTs of unknown function. Four of these ESTs were extended to novel full-length clones encoding T-cell-regulated proteins with predicted functions in GTP metabolism, cell organization, and signal transduction.

**2. Determination of soluble CD21 as a parameter of B cell activation.**

In this study we established a novel solid-phase immunoassay for CD21 using the time-resolved fluorescence of lanthanide chelates. The capture assay was able to detect concentrations of as low as 100 pg of CD21 antigen per millilitre of sample and was used for quantitative determination of CD21 in lysates of different cell lines as well as in patient serum specimens. CD21 was measured in lysates of tonsils and cell lines of B, T cell and myelomonocyte lineage, and appeared to consist of monomeric antigen under the detergent conditions used. Elevated levels of soluble CD21 were observed in serum of patients with Epstein-Barr virus (EBV) infection, a disease known to be associated with polyclonal B cell activation, and in infection with the lymphotropic rubella virus.

Significantly increased levels were also found in malignancies which are associated with EBV. In patients with nasopharyngeal carcinoma (NPC), a correlation with the titre of EBV-specific IgA was observed, thus supporting a possible role of soluble CD21 as a marker for disease activity in certain malignancies. Our data suggest that measurement of soluble CD21 could serve as a marker for activation of the immune system and diseases involving the B cell lymphoid system. Possible mechanisms and functions of soluble CD21 are discussed.

**3. Multiple CD4 and CD8 T-cell activation parameters predict vaccine efficacy *in vivo* mediated by individual DC-activating agonists**

A systematic comparison of the immunostimulatory capacity of TLR 2, 3, 4, 5, 7 and 9 agonists and an agonistic CD40-specific antibody was performed in a single long peptide vaccination model. All adjuvants activated DC *in vitro* but not all induced a strong functional T-cell response *in vivo*. Optimal clonal CD8+ T-cell expansion depended on the capacity of agonists to mature pro-inflammatory DC and the duration of their *in vivo* stimulatory effect. Strong agonists promoted the induction of both antigen-specific IFNγ-producing CD4+ T-helper cells and high numbers of IFNγ producing CD8+ effector T-cells that killed target cells *in vivo.* Importantly, the capacity of an agonist to function as an adjuvant depended on the vaccine strategy used. Collectively, the multi-parameter system presented here can be used as a general road map to develop therapeutic vaccines.

**4. Anti-Qa-2-induced T cell activation. The parameters of activation, the definition of mitogenic and nonmitogenic antibodies, and the differential effects on CD4+ vs CD8+ T cells**

The MHC Ag Qa-2 is a glycolipid anchored class I molecule expressed at high levels on all peripheral T lymphocytes. In this study we found that anti-Qa-2 antibodies could stimulate the proliferation of murine T cells in vitro. Anti-Qa-2-induced proliferation required secondary cross-linking with anti-Ig antibody and the presence of PMA. Only Qa-2+ strains could be induced to proliferate by anti-Qa-2 antibody, but under the conditions employed, anti-CD3 could induce proliferation in Qa-2+ and Qa-2-strains. Interestingly, only anti-Qa-2 reagents directed against the alpha 3 domain of the Qa-2 class I molecule were effective in inducing proliferation. Furthermore, unlike purified CD4+ cells, purified CD8+ cells were unable to be stimulated by the anti-Qa-2 antibodies. These results lead to the inclusion of Qa-2 in a group of physiologically relevant, glycolipid-anchored, cell-surface molecules, mobilization of which can generate signals that initiate the proliferation of T cells. Such molecules may play a secondary role in cellular activation after the primary engagement of the TCR.

**5. Resistance to macrophage-mediated killing as a factor influencing the pathogenesis of chronic cutaneous leishmaniasis**

Cutaneous leishmaniasis can be either a spontaneously healing or chronic disease, depending upon the strain of parasite and the immunological status of the host. We have investigated parasite factors responsible for the variable pathogenesis observed in leishmanial infections by testing the sensitivity of several leishmanial strains to intracellular killing in lymphokine (LK) activated mouse macrophages. Significant microbicidal activity against Leishmania tropica, a strain which heals in C57BL/6 (B6) mice, was found. In contrast, a strain (Maria) which has previously been shown to induce chronic nonhealing cutaneous lesions in B6 mice was resistant to killing in activated macrophages. This resistance to killing was observed in macrophages activated by LK obtained from either Bacille Calmette-Guerin-, L. tropica, or the Maria strain infected mice. The inability of LK activated macrophages to kill the Maria strain was shown not to be due to parasite induced inhibition of killing mechanisms, since Maria strain infected, LK treated macrophages exhibited tumoricidial activity similar to uninfected macrophages. Furthermore, LK activated macrophages simultaneously infected with the Maria strain and another intracellular pathogen, Toxoplasma gondii, killed Toxoplasma, but not the Maria strain. Temperature was also found to significantly

influence the multiplication and killing of Leishmania parasites. As would be expected from their cutaneous nature, L. tropica and Maria strain parasites multiplied better at 35 degrees C than at 37 degrees C. Also consistent with the failure of cutaneous strains to visceralize in immunocompetent mice was the observation that the killing of leishmanial parasites was enhanced at the higher temperature. Thus, the temperature dependent growth capacity and sensitivity to killing of a given leishmanial strain in macrophages may be important factors influencing the pathogenesis of cutaneous leishmaniasis.

**6. Effect of patulin on microbicidal activity of mouse peritoneal macrophages**

Patulin, a fungal metabolite shown previously to exert immunosuppressive effects on the cellular and humoral immune systems, was examined for its *in vitro* effects on some functions of murine peritoneal macrophages. The cells were pre-incubated for 2hr with mycotoxin concentrations of 0.01–2 μg/ml. Phagocytosis and phagosome-lysosome fusion were diminished above 0.1 μg patulin/ml and lysosomal nzymes and microbicidal activity above 0.5 μg/ml,whereas $O_2^-$ production was inhibited only above 2 μg/ml. This indicated that the killing mechanism did not depend on products of the oxidative burst. The concentrations used did not decrease the cell viability. Under natural circumstances, patulin may constitute a health risk for animals.

**CYTOKINES**

Cytokines are believed to be involved in the pathogenesis and enhanced expression in patients with Hodgkin's and Non-Hodgkins lymphoma. Based on this phenomenon, a multicentric study was carried out in various lymphoma cases. The diagnosis of lymphoma was made on tissue biopsies and fine needle aspiration cytology (FNAC). Out of a total of 72 cases studied, 45 were of Hodgkin's lymphoma (62.5%) and 27 cases were of Non-Hodgkin's lymphoma (37.5%). Maximum cases of Hodgkin's disease occurred in the age group of 30-40 years and males outnumbered females. Hodgkin's lymphoma cases were predominantly of mixed cellularity histologic type (46.66%) whereas majority cases of Non-Hodgkin's lymphoma were of high grade histologic type (48.14%) with predominance in the age group 51-60 years. In both these type of lymphomas, the IL-2R and IL-6 levels were found to be increased more than four fold (as compared to healthy controls) ($p<0.05$). The cytokine levels decreased after chemotherapy in patients showing response to therapy. However, there were few conflicting and unreliable trends in the IL-6 levels after chemotherapy where elevated IL-6 levels persisted in patients in clinical remission. Overall, it was seen that both IL-2R and IL- 6 can be used as an indicator for assessing prognosis and drug therapy in lymphoma cases. IL-2R was found to be a better prognostic marker than IL-6 in assessing the response of lymphoma patient to chemotherapy, more so in Hodgkin's disease.

**Macrophage Activation**

Macrophage effector function significantly influences the quality, duration, and magnitude of most inflammatory reactions. Traditionally, macrophages have been described as antigen-presenting phagocytes that secrete pro-inflammatory and antimicrobial mediators. Mounting evidence, however, describes a more complex model involving multiple macrophage phenotypes carrying out differential functions and eliciting divergent effects on surrounding cells and tissues. Stein *et al.* were the first to describe "alternatively" activated macrophages as having a phenotype distinct from what are now called "classically" activated macrophages. From this seminal observation, a model of two major macrophage classes has developed. Classically activated macrophages exhibit a Th1-like phenotype, promoting inflammation, extracellular matrix (ECM) destruction, and apoptosis, while alternatively activated macrophages display a Th2-like phenotype, promoting ECM construction, cell proliferation, and angiogenesis. Although both phenotypes are important components of both the innate and adaptive immune systems, the classically activated macrophage tends to elicit chronic inflammation and tissue injury whereas the

alternatively activated macrophage tends to resolve inflammation and facilitate wound healing (Figure 1).

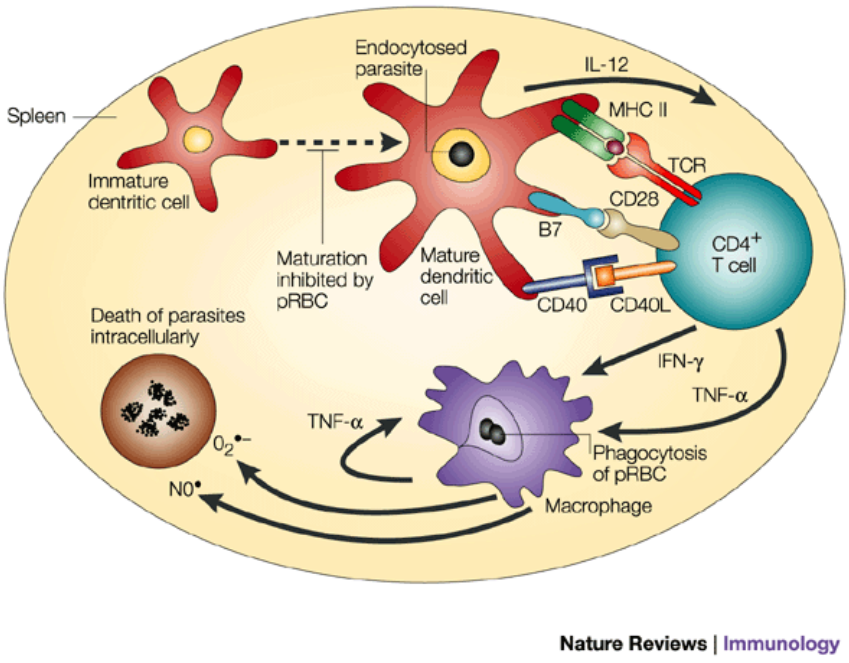



**Figure 1.** Schematic of classical (A) versus alternative (B) activation of macrophages depicting the priming signal (classical only) and stimuli, their effects on cellular function, and subsequent effects on surrounding tissue physiology. [Note: figure adapted from Gordon, S. (2003) Nat. Rev. Immunol. **3**:23.]

**Classically Activated Macrophages**

Differentiation of classically activated macrophages requires a priming signal in the form of IFN-gamma[7] via the IFN-gamma R. When the primed macrophage subsequently encounters an appropriate stimulus, such as bacterial LPS, it becomes classically activated. LPS is first bound by soluble LBP and then by either soluble or membrane-bound CD14. CD14 delivers LPS to the LPS recognition complex, which consists of at least TLR410 and MD-2. Pathogens and pathogen components are subsequently taken up by phagocytosisand delivered to lysosomes where they are exposed to a variety of degradation enzymes including several Cathepsin cysteine proteases. Suitable antigens are processed and loaded onto MHC class II molecules in late endocytic

compartments and antigen/MHCII complexes as well as co-stimulatory B7 family members are presented to T cells.

These events are followed closely by a significant change in cellular morph-ology and a dramatic alteration in the secretory profile of the cell. A variety of chemokines including IL-8/CXCL8, IP-10/CXCL10, MIP-1 alpha/CCL3, MIP-1 beta/CCL4, and RANTES/CCL5, are released as chemoattractants for neutrophils, immature dendritic cells, natural killer cells, and activated T cells. Further, several pro-inflammatory cytokines are released including IL-1 beta/IL-1F2, IL-6, and TNF-alpha/TNFSF1A. TNF-alpha also contributes to the pro-apoptotic activity of the classically activated macrophage. TNF-alpha is accompanied by Fas Ligand/TNFSF6 secretion[16] and NO release as a result of iNOS upregulation. In addition, the classically activated macrophage releases proteolytic enzymes including MMP-1, -2, -7, -9, and -12, which degrade Collagen, Elastin, Fibronectin, and other ECM components.

While the release of these molecules is important for host defense and direction of the adaptive immune system, when uncontrolled they can levy significant collateral damage on the microenvironment. By eliciting massive leukocyte infiltration and flooding the surrounding tissue with inflammatory mediators, pro-apoptotic factors, and matrix degrading proteases, the classically activated macrophage is capable of dismantling tissues to the point of inflicting serious injury. Tissue destruction perpetrated by chronic inflammation has been associated with the development of tumors, type 1 autoimmune diseases, and glomerulonephritis among other pathologies (Figure 1A).

**Alternatively Activated Macrophages**

Differentiation of alternatively activated macrophages does not require any priming. IL-4[2] and/or IL-13 can act as sufficient stimuli. The binding of these factors to their respective receptors is followed by fluid-phase pinocytosis of soluble antigen.Soluble antigen is then loaded onto MHC class II molecules and antigen/MHCII complexes and co-stimulatory B7 family members are subsequently displayed to T cells.

Similar to the classically activated macrophage, the alternatively activated macrophage changes its cellular morphology and secretory pattern as a result of appropriate stimulation. Leukocytes are attracted by the macrophage via its release of chemokines including MDC/CCL22PARC/CCL18and TARC/CCL17. Inflammation is counteracted by the release of factors such as IL-1ra/IL-1F3, Ym1, Ym2, RELMa,IL-10,and TGF-beta. TGF-beta also functions indirectly to promote ECM building by inducing nearby fibroblasts to produce ECM components. The alternatively activated macrophage itself secretes the ECM components, Fibronectin and bIG-H3, the ECM cross-linking enzyme, Trans-glutaminase, and Osteopontin, which is involved in cell adhesion to the ECM.

In addition, alternatively activated macrophages upregulate the enzyme Arginase I, which is involved in proline as well as polyamine biosynthesis. Proline promotes ECM construction while polyamines are involved in cell proliferation. Other factors secreted by the alternatively activated macrophage that promote cell proliferation include PDGF, IGF, and TGF-beta. These factors, along with FGF basic, TGF-alpha, and VEGF, also participate in angiogenesis.

The molecules secreted by the alternatively activated macrophage work toward resolution of inflammation and promotion of wound repair due to their anti-inflammatory, fibrotic, proliferative, and angiogenic activities. This macrophage is also especially efficient at combating parasitic infections such as Schistosomiasis. In addition to its beneficial activities, the alternatively activated macrophage has been implicated in several pathologies, the most prominent of which are allergy and asthma (Figure 1B).

**Macrophage Microbicidal Assays**

A main function of macrophages in the host defense against infection is the phagocytosis of nonopsonized or opsonized microorganisms, and the subsequent growth restriction or killing of ingested microorganisms. The antimicrobial activity of macrophages is mediated by oxidative and

non-oxidative mechanisms. The oxidative antimicrobial mechanisms, which include the action of reactive oxygen intermediates Interleukin 10 (IL-10) inhibits interferon rvinduced macrophage activation for cytotoxicity against larvae of the human parasite Schistosoma mansoni by suppressing production of the toxic effector molecule nitric oxide (NO). In this study, the mechanism of IL-10 action was identified as inhibition of endogenous tumor necrosis factor a (TNF-a) production by interferon y-activated macrophages. TNF-a appears to serve as a cofactor for interferon –meated activation, since both schismulum killing and NO production
were inhibited by anti-TNF-a antibody, whereas TNF-a alone was unable to stimulate these macrophage functions. IL-10 blocked TNF-a production by interferon vtreated macrophages
at the levels of both protein and mRNA synthesis. Addition of exogenous TNF-a reversed IL-10-mel ated suppression of macrophage cytotoxic activity as well as NO production. Likewise, addition of a macrophage-triggering agent (bacterial lipopolysaccharide or muramyl dipeptide), which induced the production ofTNF-a, also reversed the suppressive effect of IL-10 on cytotoxic function. In contrast to IL-10, two other cytokines, IL-4 and transforming growth factor 3, which also inhibit macrophage activation for shi n klg and NO production, did not substantially suppress endogenous TNF-a production. These results, therefore, describe a separate pathway by which macrophage microbicidal function is inhibited by the down-regulatory cytokine IL-10.

**Quantitation of microbicidal activity of mononuclear phagocytes: an in vitro technique.**

An in vitro assay technique was set up to determine the phagocytic and microbicidal activity of a monocyte-macrophage cell line using Candida species as test organisms. The norms were determined for the activity of peritoneal macrophages of rats (24.69 +/- 2.6% phagocytosis and 35.4 +/- 5.22% ICK) and human (27.89 +/- 3.63% phagocytosis and 50.91 +/- 6.3% ICK). The assay technique was used to test the degree of activation of macrophages induced by metronidazole, Tinospora cordifolia and Asparaqus racemousus and to compare their effects with a standard immunomodulator muramyl-dipeptide. All the three test agents increased the phagocytic and killing capacity of macrophages in a dose dependent manner upto a certain dose, beyond which either these activities were found to have plateaued or decreased. The optimal doses for MDP, Metronidazole, Asparagus racemosus and Tinospora cordifolia were found to be 100 micrograms, 300 mg/kg, 200 mg/kg and 100 mg/kg respectively. Patients with cirrhosis were screened for defects in monocyte function. The depressed monocyte function (20.58 +/- 5% phago and 41.24 +/- 12.19% ICK; $P < 0.05$) was observed indicating a compromised host defense. The utility of this candidicidal assay in experimental and clinical studies is discussed.

**In-vitro experimentation**

Macrophages treated with culture fluids from EL-4 cells, a continuous T cell line, were activated to kill mKSA-TU-5 fibrosarcoma cells, amastigotes of Leishmania tropica, and schistosomula of Schistosoma mansoni. Active EL-4 factors eluted from Sephadex G-100 in two distinct regions: molecular weight 45,000 (activities induced killing of unrelated intracellular and extracellular
targets) and molecular weight 23,000 (activities induced killing of extracellular targets only). These results confirm heterogeneity among activation signals for the induction of macrophage microbicidal and tumoricidal activities. Factors that induced cytocidal activity against extracellular tumor cells and shistosomula were distinct from those that induced destruction of intracellular amastigotes.

## UNIT IV IMMUNOPATHOLOGY

### Sampling and preservation of tissues

Carcasses or remnants of dead animals, faeces and other biological material found in the wild may be very useful for obtaining data about wild populations with little disturbance of live animals or their habitat. It can no longer be regarded as ethical to kill threatened wild animals for obtaining skins for collections; carcasses of animals found or confiscated from poachers may serve as a better source of material for reference collections. Therefore, careful consideration which parts of a specimen have to be damaged or destroyed for examination, and preservation of which parts in reference collections is more useful (one of us: A. Nekaris; see also Groves, 2002 in press)

**Specimens which may be useful in reference collections:**

Skins; study skins, mounted specimens of the study species (the possibility of colour changes caused by preservation should be considered)

Wet preserved specimens

Skeletal material, skulls; if no dead animals are available and killing of specimens is supposed to be avoided, for instance very detailed casts of dentition of anesthetized specimens with dentists´ materials are possible. Owl pellets may be an interesting source of bones (one of us: C. Groves)

Hair samples; reference hair collection of sympatric species

**Formalin preservation:**
After weighing and measuring the animal and attaching an adequate label (see labelling), very small specimens (up to 100 g) can be fixed whole by submerging them in 10 % buffered formalin (tissue - formalin solution ratio of at least 1 : 12). the body cavity can be filled with formalin solution by injection until it is turgid and firm; some formalin may also be injected under the skin, into the body cavity, larger muscles and organs. If hypodermic needles are not available, the body cavity can be opened ventrally by making a slit instead, allowing the formalin to enter. Keeping the mouth open with a piece of wood or cotton may later allow examination of teeth. Then the whole body can be immersed in formalin, in the posture in which it is supposed to stay permanently because it will harden. The ratio of formalin to carcass must be at least 12 to 1 to assure a good fixation. Tissues can be left in buffered neutralized formalin for several months, but formalin hardens specimens; therefore, after fixation, longterm storage in alcohol may be better. After preservation the carcass should therefore be washed in water and transferred into ethanol for permanent storage, see below: longterm liquid preservation (Nagorsen, Peterson, 1980; Munson, 2000; Rabinowitz et al., 2000).
Equipment necessary: formalin, buffer, water, scalpel and / or hypodermic syringes, material for permanent labels, containers (not metal containers unless they are acid-proof lined, because corrosion of the metal would discolour the specimen) (Nagorsen, Peterson 1980).Formalin, however, has some disadvantages; for instance it discolours the fur, after a longish immersion, softens the bones (one of us: C. Groves) and prevents further examination for microbiology.

**Preservation in alcohol:**
After weighing, a whole animal can be preserved in a container of alcohol (70-90%). Removal of the intestine prior to storage of the animal in alcohol is recommended (Rabinowitz et al., 2000).

**Preservation by cooling or freezing:**
Removal of the skin with insulating fur before cooling or freezing may help to cool the carcass down more quickly (Schoon, lecture manuscript).
Freezing is not recommended if histological examination is planned (Wobeser and Spraker, 1980).

**Laboratory preparation of skins earlier dried in the field** as described by Downing (1945):
1) Relaxation of the dry skin by soaking it in lukewarm tap water, usually over night.
2) Brief washing of the relaxed skin with soap and water.
3) Rinsing of the skin in a degreasing agent such as varsol or carbon-tetrachloride; if greasy, the skins were allowed to stand in it for half an hour or so.
4) Drying of the skin in sawdust, using compressed air to assist the drying and to blow the sawdust out of the hair.
Colour changes caused by this method, particularly by soaking, see below

**Problem of colour changes of hair during preservation of fur**
Hair colour of live animals may differ from the colour of preserved specimen for several reasons. Some dyes like plant juice may cause a reddish hair colour, algae may cause a greenish hair colour

in certain arboreal species, for instance in the sloth *Bradypus* or a bright green dorsal colour in *Galagoides demidoff*, which fades rapidly after the death of the animal (Sanderson 1940).
In addition, colour differences in series of mammal skins may be caused by preservation and storage methods rather than showing the occurrence of different colour types (red and grey varieties, random erythrism) in one species (Sanderson, 1940).
Use of **chemicals** may lead to colour changes. Formalin discolours the fur (one of us: C. Groves). Long immersion in solutions of alcohol, salt, alum or similar preservatives also alters colour (Downing, 1945). Sumner (1927) who cleaned fur with benzine or other chemical agents for better comparability mentions colour changes.
**Drying methods** in the field have some influence. Sanderson (1940) found that series of skins dried in bright sun in the field tended to turn reddish. Experiments with a maroon-coloured rat, *Malacomys longipes*, showed that furs became grey, dark brown or reddish-brown, according to whether they were dried in a closed container, in shade or bright sunlight. Series of *Praomys*, including examples of bright reddish and olive-grey varieties, could be evenly dried to a corresponding dull siena when submitted to reciprocal treatment. Drying over a fire by smoke may also change colours (Sanderson 1940).
**Soaking of dried skins** has a considerable effect. Downing, Cross and Prince at the Royal Ontario Museum of Zoology found a marked dichromatism in collectons of squirrel skins after different preservation: tawny olive skins had been made up in the field; skins which showed a dark tone and reddish coloration had been dried in the field and later relaxed by soaking in water and made up in the museum laboratory. Some tests with pieces of skin of a freshly killed squirrel showed that treatment with several dry preservatives (arsenic, alum, borax, salt) and subsequent drying in an electric oven at 60° Centigrade for 24 hours did not lead to colour changes, but soaking of further fresh and dried samples, with and without preserving chemicals added, in warm water led to color changes. After soaking originally tawny samples for one hour, a borax treated and salt treated sample had become hazel, an arsenic treated sample between tawny and russet, an alum treated sample between russet and hazel, and even a distilled water treated sample showed a perceptible change. Samples that had been soaked for longer periods showed further darkening and deepening of color. Arsenic treated and distilled water treated samples showed the least change, borax treated the most change; alum treated and salt treated samples were intermediate. Although the above preservatives increased the amount of change which took place, even soaking the skin in distilled water caused a marked alteration of the normal hair color. Examination of the museum collections of other species showed similar changes after soaking, although less severe than in sciuridae, besides reddening the yellow and buffy colors had become much deeper in tone and exhibited a cinnamon, pink or reddish cast. Changes were not evenly distributed over the body; certain parts changed more than others. Downing concludes that such changes in color may render specimens almost useless for taxonomic studies in which color is an essential character, that more adequate methods for cleaning and relaxing skins are necessary, possibly with minimizing of the time in which the skin is moist, and that such possibly colour-changing treatment must be noted on labels (Downing, 1945).
Later changes of colour of hair in museum specimens may occur due to fading because of exposal to light, old age, proximity of certain chemicals, radiators and other influences (Sanderson 1940).

**Hairs, hair reference collections**
Hair may have microscopically visible features allowing taxonomic identification. Hair may not only be collected from live animals or carcasses, but also for instance with hair tubes ( for smaller mammals: tubes of a width slightly larger than the study species with double-sided sticky tape stuck to the inside) or hair catchers (facilities with wire-brush-like structures; animals are encouraged to squeeze through), baited ore just attached where animals are likely to pass, left in the field for 1-2 weeks.
Comparison with taxonomically identified hair samples of sympatric species (either from an own hair reference collection or in museum collections) may allow identification of food / prey of the study species, of remnants of the study species in carnivore faeces or owl pellets or hairs for instance collected from nests or with hair catchers.

**Hair samples for DNA analysis**
Hair must be plucked (not cut) to include follicle cells. A minimum of 10-20 hairs should be obtained (AZA Prosimian Taxon Advisory Group, 2002). Bearder et al. (1996) recommend to collect especially the long guard hairs plucked from between the shoulder blades and hairs from scent glands. Loose hairs which can easily be removed by plugging may already be dead with little follicle cells with DNA left (one of us: Ch. Roos). To prevent contamination from human skin, use of clean gloves or an instrument for plugging is recommended. Samples should be stored in paper (not plastic) envelopes; no special preservation is necessary (AZA Prosimian Taxon Advisory Group, 2002).

***Egg, Ovarian Tissue, Embryo, and Sperm Freezing***

Our cryopreservation (sperm, egg, embryo, and ovarian tissue storage) program is one of the best in the world. We have a 55% pregnancy rate per cycle with frozen embryos, which is no different than for fresh, or unfrozen, embryos. Our frozen embryo survival is almost 99%. Therefore, if you have "extra" viable embryos resulting from your IVF procedure, you can feel comfortable allowing us to freeze them.

We can now also offer egg freezing for women who wish to delay childbearing, and who want to preserve their fertility for the future. Although the freezing of *embryos* has been quite reliable, the freezing of *eggs* was for a long time only experimental, and, until recently, not very successful. However, the Infertility Center of St. Louis has begun a partnership with the Kato Ladies Clinic in Tokyo to bring an exciting new technique, , to the United States. With this method, we can 'flash-freeze' unfertilized eggs with a remarkable effectiveness, and then thaw them at any later date for reimplantation. We can also offer ovarian tissue freezing for women who do not want to undergo IVF in the future but who just want to conceive naturally.

The classic problem with freezing eggs used to be that as one lowered the temperature below the freezing point, the egg's uncombined genetic material (ready for fertilization, but also in a complex and delicate state) would suffer damage due to ice crystals forming inside the cell. It was only possible to freeze embryos, in which the genetic material had already combined with that from the sperm, and stabilized. The classic freezing techniques (which have been known since 1983) were based on trying to extract water from the cell as the temperature drops, to minimize ice crystal damage. This has all changed now with the development of our new vitrification techniques. We no longer have to play a tenuous game of *minimizing* ice crystal formation, we can now *entirely avoid it* -- so that there is no internal damage to the egg whatsoever.

We also offer very excellent quality ovarian tissue freezing. This procedure can be used to preserve the possibility of future fertility for women who are about to undergo radiation or chemotherapy for cancer. It may also be used for any women who may not be able to plan for children until they are older. We have had many successful ovarian tissue transplants and pregnancies, and so ovarian tissue freezing has excellent promise for preserving your natural fertility.

Sperm freezing may be needed if the husband is about to undergo radiation or chemotherapy for cancer, or is being deployed to a war zone, but wants to father children later. In addition, sperm may be frozen if the husband has no sperm in his ejaculate and requires a onetime surgical sperm extraction procedure, or simply, if the husband is afraid that on the day of your IVF procedure he may be too nervous to provide a specimen. Again, you can be very confident in the reliability of our sperm freezing and storage system.

### *Freezing Eggs or Embryos by the Vitrification Process*

This new technique of freezing called "vitrification" avoids the damage caused by ice forming inside the cell by not trying to pull every last molecule of water out, because it is impossible to do this 100%. In fact, 70% of the cell is water, and at best you can reduce that to 30%. So with the conventional controlled rate slow-freezing technique, there is always going to be some intra-cellular ice crystal formation, causing some damage to embryos, and severely damaging most eggs. Vitrification uses a super high concentration of antifreeze (DMSO and ethylene glycol), and drops the temperature so rapidly that the water inside the cell never becomes ice. It just instantaneously super-cools into a solid with no ice crystal formation at all.

We can now freeze and thaw, and even refreeze and rethaw, with impunity, using this new protocol from Dr. Masashige Kuwayama from the Kato Clinic in Tokyo. With conventional "slow freezing," the temperature of the embryo goes down at precisely 0.3°C per minute. With vitrification (using four times the concentration of antifreeze, or cryoprotectant), the temperature is dropped at 23,000 degrees C° per minute, that is 70,000 times faster. At that speed of cooling, and at that concentration of antifreeze, ice crystals simply cannot form.

Of course, it is not quite as simple as it might sound. Such high concentrations of antifreeze, in a few minutes, could be toxic to cells. Therefore, the embryos (or eggs) must first be placed in lower concentrations of antifreeze (and sucrose to draw some water out), and then left in high concentrations only for less than a minute before instantaneous freezing. Then when the time comes to thaw the embryo, it must be instantaneously warmed, immediately taken out of the high concentration of antifreeze, and then placed into a solution with lower concentration, in order to avoid antifreeze toxicity. This requires more skill than conventional freezing, but it is faster, cheaper, and most importantly, avoids almost all freezing damage to either eggs or embryos. Such a reliable method of embryo freezing gives the IVF program much greater ability to avoid dangerous multiple pregnancy, and makes scheduling for procedures like egg donation simpler for the patient.

Using this vitrification technique for freezing, we can reliably preserve eggs as well as embryos so that the pregnancy rate is no different than if the eggs or embryos had never been frozen. This allows us to preserve the fertility of young women for the future if they wish to delay childbearing, but not lose their fertility as they age.

### **Tissue sample preservation techniques in microgravity and related implications for hardware design.**

Chemical fixation is an essential method of preserving biological specimens during space flight for detailed analyses after return to earth-based laboratories. In the design of the Gravitational Biology Facility (GBF), it was determined that a high percentage of GBF experiments in cell, developmental and plant biology will necessitate tissue preservation for a broad range of specimen types. Trade-offs between scientific requirements for sample preservation and hardware designs are impacted by space station safety requirements and logistics constraints. A review of chemical fixatives previously flown, and discussion of other specimen preservation techniques, increases our understanding of in-flight specimen fixation, as well as reveals strategies for improved performance in this area. GBF hardware design, cost, and schedule are impacted by these issues.

### **Identification Of Various Cell Types And Antigens In Tissues**

Identifying the antigens that have the potential to trigger endogenous antitumor responses in an individual cancer patient is likely to enhance the efficacy of cancer immunotherapy, but current methodologies do not efficiently identify such antigens. This study describes what we believe to be a new method of comprehensively identifying candidate tissue antigens that spontaneously cause T cell responses in disease situations. We used the newly developed automated, two-dimensional chromatography system PF2D to fractionate the proteome of human tumor tissues and tested

protein fractions for recognition by preexisting tumor-specific CD4+ The cells and CTLs. Applying this method using mice transgenic for a TCR that recognizes an OVA peptide presented by MHC class I, we demonstrated efficient separation, processing, and cross-presentation to CD8+ T cells by DCs of OVA expressed by the OVA-transfected mouse lymphoma RMA-OVA. Applying this method to human tumor tissues, we identified MUC1 and EGFR as tumor-associated antigens selectively recognized by T cells in patients with head and neck cancer. Finally, in an exemplary patient with a malignant brain tumor, we detected CD4+ and CD8+ T cell responses against two novel antigens, transthyretin and calgranulin B/S100A9, which were expressed in tumor and endothelial cells. The immunogenicity of these antigens was confirmed in 4 of 10 other brain tumor patients. This fast and inexpensive method therefore appears suitable for identifying candidate T cell antigens in various disease situations, such as autoimmune and malignant diseases, without being restricted to expression by a certain cell type or HLA allele.

**Identification of major cell types in paraffin sections of bovine tissues**

**Abstract**

Identification of cell types in bovine tissue sections is complicated by the limited availability of anti-bovine antibodies, and by antigen retrieval treatments required for formalin-fixed tissue samples. We have evaluated an antibody and lectin panel for identifying major cell types in paraffin-embedded bovine tissue sections, and report optimized pretreatments for these markers.
The panel of markers allows the identification of all major cell types in paraffin-embedded cattle tissue sections by immunohistochemistry or lectin histochemistry. Heat-induced epitope retrieval methods are required for most antibodies.

**Introduction**

Specific identification of cell types in bovine tissues is hindered by the limited availability of anti-bovine antibodies. The species cross-reactivity information of other commercially available antibodies is also often limited. Thus, suitable antibodies must be searched for by trial and error. This is further complicated by the fact that for many antibodies a successful immunostaining is **only accomplished after an optimized antigen retrieval treatment.**

**Method**

Tissue samples were obtained from slaughtered animals. The use of animals was approved by the animal ethics committee of the University of Helsinki. Tissue samples were fixed either in 4% phosphate-buffered paraformaldehyde (PFA) for 24 hours at +4°C or in 100 % ethanol for 2 hours at +4°C followed by 120 hours at -20°C, embedded in paraffin, and sectioned to 2–4 µm (PFA) or 4 µm (ethanol) sections.
Antibodies and lectins

**Immunohistochemistry** was performed using either the ABC method (avidin biotin complex) or tyramide amplification, using Shandon Coverplates (ThermoElectron). Paraffin-embedded sections were dewaxed, rehydrated, subjected to an antigen retrieval procedure (see below), and permeabilized with 0.1% to 1% Tween-20 in phosphate-buffered saline (PBS). The sections were then blocked for endogenous biotin, with 10% egg white powder in water (as an avidin solution) and 1 mg/ml D-biotin (Sigma-Aldrich, St. Louis, MO) in PBS, when necessary, and for nonspecific binding with 1% goat serum in PBS. They were incubated in the primary antibody overnight at +4°C, in PBS containing 1% bovine serum albumin, washed, and incubated with goat biotinylated anti-mouse or anti-rabbit secondary antibody (Dako, Glostrup, Denmark) for 2 hours in room temperature. The ABC detection was performed using the Vectastain Elite ABC kit and the DAB (diaminobenzidine) substrate kit (both Vector Laboratories, Burlingame, CA) according to manufacturer's instructions. For tyramide amplification, sections were incubated in avidin D - conjugated peroxidase (Vector), in biotinylated tyramide again in avidin-peroxidase, and in the DAB substrate. The amplification typically allows four to ten times more dilute antibody solutions than with the ABC method. All sections were counterstained with Mayer's hematoxylin and embedded with Faramount (Dako).

**Antigen retrieval**

Heat-induced antigen retrieval was performed in a standard kitchen microwave oven. The slides were heated in 500 ml of retrieval solution at 750 W power for 15 minutes (for the caspase antibody, 10 minutes), followed by a cooling period of 20 minutes (for the caspase antibody, 30 minutes). The following solutions were used: for acid retrieval, 50 mM glycine-HCl pH 3; for neutral retrieval, 2 × SSC pH 6 (sodium chloride, sodium citrate buffer); and for alkaline retrieval, 10 mM Tris-HCl pH 9.5, 1 mM EDTA.

Protease-induced antigen retrieval was performed in Coverplates, at 37°C for 30 minutes, with 10 to 50 μg/ml (ethanol-fixed samples) or 50 to 100 μg/ml (PFA-fixed samples) protease P6911 (Sigma-Adrich) in 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA.

**Microscopy and photography**

The stained sections were viewed with a Leica DM4000 microscope and photographed using a SIS Colorview 12 digital camera.

**Discussion**

As a by-product of a research project on stem cell fates, we have evaluated a selection of antibodies for identifying major bovine cell types in paraffin-embedded tissue sections. Some of these have been raised against bovine antigens, some are previously known to be bovine cross-reactive, and others we have tested without such prior knowledge. Optimal antigen retrieval methods for each antibody are reported. In addition to antibodies, two lectins are presented. The emphasis is on paraformaldehyde-fixed tissues, but as some antibodies are incompatible with such material, we have also used ethanol fixation.

**Epithelium**

Several anti-keratin antibodies were evaluated for epithelial markers. The AE1/AE3 monoclonal antibody cocktail, raised against human epidermal keratin, was the most useful pan-epithelial marker. With alkaline antigen retrieval (see Methods), most types of epithelia were strongly and specifically stained with this antibody. Neutral or protease-induced retrieval was sufficient for some but not all tissues.

Another pan-keratin antibody Lu-5also stained most epithelia with protease-induced retrieval, but did not cover all epithelia as comprehensively as AE1/AE3 in our hands.

The polyclonal pan-keratin antibody tested yielded nonspecific staining of cells. The high molecular weight cytokeratin antibody 34β E1provided very strong staining of some epithelia (notably epidermis and liver), but is obviously not as comprehensive as the pan-keratin markers.

Few bovine endothelial markers are available. The anti- von Willebrand antibodytested stains many but not all endothelia. Notably, new blood vessels in granulation tissue were strongly stained. The lectins ML-I and especially GSL I-B4yielded strong staining of endothelial cells, but they also stain some leukocyte populations. We failed to obtain a good staining with the anti- type IV collagen antibody M3F7or with the polyclonal anti- endothelial nitric oxide synthase antibody.

**Connective tissue**

Vimentin is a general marker for cells of the mesenchymal lineage. The monoclonal anti- porcine vimentin antibody V9 provided strong and specific staining even without antigen retrieval.

The type I procollagen antibody SP1.D8 stained active fibroblasts in various connective tissues. The best staining was obtained with the alkaline retrieval method. Some nonspecific staining was seen, in sebaceous glands for example.

**Muscle**

The desmin antibody D33 stained all muscle types, performing best after neutral antigen retrieval . The muscle actin antibody HHF35 recognizes the alpha and gamma isotypes present in all muscle types. The acid and alkaline retrieval methods yielded optimal results. The α-smooth muscle actin antibody 1A4 stains only a subset of smooth muscle tissues, due to the more restricted expression pattern of the antigen. The circular muscle layer in the intestine was not stained. The neutral antigen retrieval method produced the best results with this antibody. All these antibodies were specific for muscle tissues.

**Nervous tissue**

The monoclonal anti- NeuN antibody A60 was the most comprehensive neuronal marker tested. It stained most neurons, Purkinje cells being a notable exception. Best results were obtained with alkaline retrieval.

Tubulin and neurofilament antibodies are also useful as general neuronal markers. Majority of neurons were stained by both tubulin βIII antibodies and the pan-neurofilament cocktail, and more restricted subgroups by the neurofilament 160/200 kD antibody, as expected.
Astroglia were beautifully stained with the polyclonal anti-GFAP antibody, with most pretreatments. Oligodendroglia were successfully stained with the anti-CNPase antibody 11-5B, whereas the anti-O4 antibody 81failed to produce any specific staining in our hands. S100is a more general marker for glial cells. It is also expressed in several cell types outside the nervous system.
The microglial cells were very weakly stained with the pan-leukocyte and macrophage markers tested. The mistletoe lectin ML-Iyielded successful staining with all pre-treatments. It also stains most endothelial cells.

**Leukocytes**

Leukocyte markers are often species-specific, and most commonly used in flow cytometry. Thus, most of the antibodies tested were raised against bovine antigens, but information on histological applications was limited.
Of the pan-leukocyte markers, the anti- CD45 antibody CACTB51A, worked well on ethanol-fixed material with mild protease treatment, except for the microglia, which were weakly stained. The quality of the CC1 antibodyappeared to suffer from a change in the production method during the research project.
We tested two CD11a/18 antibodies, of which BAT75Awas useful with acid antigen retrieval, while MUC76Afailed with any pre-treatment.
For lymphocytes, the CD3ε (T cells) and CD79α (B cells) antibodies raised against synthetic cytoplasmic peptides worked well with antigen retrieval, providing a strong and specific staining. The anti- immunoglobulin antibodies successfully stained B cells with alkaline retrieval.
Myeloid cells were stained with the anti- CD11b antibody MM10Ausing acid retrieval or ethanol-fixed material. The macrophage marker CD68was also useful with protease-treated sections, but did not stain microglia. The CD14 antibody MM61Awas only useful with ethanol-fixed material. The polyclonal anti-lysozyme antibodystained a number of cells in the intestinal epithelium, for example, but we failed to confirm the specificity of the staining.

**Cell status**

The proliferation marker MIB-1yielded good staining with alkaline antigen retrieval. Apoptotic cells were successfully stained with the cleaved caspase 3 antibodyusing neutral retrieval, although the staining was not very strong.

**Table 1**

Evaluation of markers. ++: good staining (strong, specific), +: poor staining (weak or including non-specifically stained cells), -: unsuccesful staining. PFA: paraformaldehyde fixation, EtOH: ethanol fixation. N: no epitope retrieval, P: protease-induced epitope retrieval, H3: heat-induced epitope retrieval (HIER) at pH 3, H6: HIER at pH 6, H9: HIER at pH 9.5. Methods: A = avidin-biotin-complex, T = tyramide signal amplification. NF = neurofilament. Sources: DHSB = Developmental Studies Hybridoma Bank, BD = BD Biosciences, N/L = NeoMarkers/LabVision, Vector = Vector Laboratories, W/H = Witten/Herdecke University, Ch = Chemicon, SM = Sternberger Monoclonals, b/C = bAbco/Covance, VMRD: Veterinary Medicine Research Diagnostics, SC = Santa Cruz, BL = Bethyl Laboratories, Bm = Biomeda, CST = Cell Signaling Technology.

| **Marker** | **clone/type** | **Immunogen** | **Source** | **Ref.** | **Staining** | | | | | | | **Dilution (method)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **PFA** | | | | | **EtOH** | | |
| | | | | | **N** | **P** | **H3** | **H6** | **H9** | **N** | **P** | |
| EPITHELIUM, ENDOTHELIUM | | | | | | | | | | | | |
| collagenType IV | M3F7 | human | DSHB | [10] | - | - | - | - | - | - | - | 1:200T |

| Marker | clone/type | Immunogen | Source | Ref. | Staining | | | | | | | Dilution (method) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | PFA | | | | | EtOH | | |
| | | | | | N | P | H3 | H6 | H9 | N | P | |
| | | collagen | | | | | | | | | | |
| cytokeratin, HMW | 34betaE12 | human epid. keratin | Dako | [5] | | | | ++ | - | ++ | | 1:1000T |
| cytokeratin, pan | rabbit polycl. | bovine epid. keratin | Zymed | n.a. | + | + | | + | | + | | 1:1000T |
| eNos/NOSType III | rabbit polycl. | peptide (human) | BD | [11] | | | + | - | + | + | | 1:1000T |
| keratin, pan | AE1 + AE3 | human epid. keratin | N/L | [2,3] | - | ++ | - | ++ | ++ | ++ | | 1:100A, 1:1000T |
| keratin, pan | Lu-5 | lung cancer cell line | N/L | [4] | | ++ | | ++ | - | ++ | | 1:100A, 1:1000T |
| lectin GSL I-B4 | *G. simplicifolia* | n.a. | Vector | [8,9] | ++ | ++ | | ++ | | ++ | | 1:4000T |
| lectin ML-I | *Viscum album* | n.a. | W/H | [7] | ++ | ++ | ++ | ++ | ++ | | | 1:2000T |
| von Willebrand | rabbit polycl. | human vWF | Dako | [6] | + | | | | | | ++ | 1:400A, 1:800T |
| CONNECTIVE TISSUE | | | | | | | | | | | | |
| procollagenType I | SP1.D8 | ovine aminopropept. | DSHB | [14] | + | + | + | + | ++ | - | - | 1:2000T |
| vimentin | V9 | porcine vimentin | Dako | [12,13] | ++ | | | ++ | | | ++ | 1:100A, 1:500T |
| MUSCLE | | | | | | | | | | | | |
| actin, muscle | HHF35 | n.a. | Enzo | [17] | + | | ++ | + | ++ | ++ | ++ | 1:100A |
| actin, smooth muscle α | 1A4 | peptide | Dako | [18] | | | | ++ | + | | | 1:500T |
| desmin | D33 | human desmin | Dako | [15,16] | + | | | ++ | | ++ | | 1:400A, 1:2000T |
| NEURONAL TISSUE | | | | | | | | | | | | |
| CNPase | 11-5B | human brain CNPase | Ch | n.a. | | ++ | - | ++ | - | - | | 1:400A |
| GFAP | rabbit polycl. | human GFAP | Zymed | n.a. | ++ | ++ | + | ++ | ++ | ++ | | 1:200A |
| NeuN | A60 | mouse neuronal nuclei | Ch | [19,22] | + | + | - | + | ++ | ++ | + | 1:4000T |
| NF 160/200 kD | RMdO-20 | rat neurofilaments | Zymed | [22] | ++ | | - | - | ++ | ++ | | 1:400A |
| NF, pan | SMI311 | n.a. | SM | [21] | ++ | | + | ++ | ++ | + | | 1:1000A, 1:2000T |

| Marker | clone/type | Immunogen | Source | Ref. | Staining | | | | | | | Dilution (method) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | PFA | | | | | EtOH | | |
| | | | | | N | P | H3 | H6 | H9 | N | P | |
| | (cockt) | | | | | | | | | | | |
| O4 | 81 | bovine brain | Roche | [23] | | | - | - | - | | - | 1:2A |
| S100 | rabbit polycl. | bovine S100 | Dako | [24] | | | | ++ | | | | 1:800A |
| tubulin βIII | rabbit polycl. | peptide (rat) | b/C | n.a. | | | - | ++ | - | + | | 1:6000A,1:10000T |
| tubulin βIII | TU-20 | n.a. | Ch | [20] | ++ | | ++ | ++ | ++ | ++ | | 1:400A, 1:3000T |
| LEUKOCYTES | | | | | | | | | | | | |
| CD11a/18 | MUC76A | sheep, pig leukocytes | VMRD | [28] | - | | - | - | - | | | 1:40A |
| CD11a/18 | BAT75A | ruminant leukocytes | VMRD | [27] | - | | ++ | - | - | + | | 1:40A, 1:200T |
| CD11b | MM10A | n.a. | VMRD | [32] | | | ++ | | | ++ | | 1:500T |
| CD14 | MM61A | bovine mononucl. cells | VMRD | [25] | + | | | | | + | | 1:200 |
| CD34 | rabbit polycl. | peptide (human) | SC | n.a. | | | + | - | - | | | 1:200A, 1:200T |
| CD3ε | rabbit polycl. | peptide (human) | Dako | [29] | - | | | ++ | | + | ++ | 1:100A, 1:1000T |
| CD45 | CACTB51A | bovine act. lymphocytes | VMRD | [25] | | + | + | + | + | + | ++ | 1:800T |
| CD45 | CC1 | n.a. | Serotec | [26] | | | | + | + | | | 1:100A |
| CD68 | EBM11 | human macrophages | Dako | [33] | - | + | | - | | | ++ | 1:80A |
| CD79αcy | HM57 | peptide (human) | Dako | [30] | | | - | + | ++ | | | 1:500T |
| IgA | rabbit polycl. | bovine IgA | BL | n.a. | | ++ | | | ++ | | ++ | 1:100A |
| IgM | rabbit polycl. | bovine IgM | BL | n.a. | + | | | | ++ | | | 1:1500A |
| IgM | BIg73A | bovine Ig | VMRD | [31] | | | - | + | ++ | | | 1:5000A |
| lysozyme | rabbit polycl. | human lysozyme | Bm | [34] | | + | - | - | + | + | + | 1:500T |
| CELL STATUS | | | | | | | | | | | | |
| cleaved caspase 3 (Asp175) | rabbit polycl. | peptide (human) | CST | [37] | | | | ++ | + | | | 1:75A |

| Marker | clone/type | Immunogen | Source | Ref. | Staining | | | | | | | Dilution (method) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | PFA | | | | | EtOH | | |
| | | | | | N | P | H3 | H6 | H9 | N | P | |
| Ki67 antigen | MIB-1 | peptide (human) | Dako | [35,36] | | | | | | | | |

**Isolation And Characterization Of Cell Types From Inflammatory Sites And Infected Tissues**

***Cellular component***

The *cellular component* involves leukocytes, which normally reside in blood and must move into the inflamed tissue via *extravasation* to aid in inflammation. Some act as phagocytes, ingesting bacteria, viruses, and cellular debris. Others release enzymatic granules which damage pathogenic invaders. Leukocytes also release inflammatory mediators which develop and maintain the inflammatory response. Generally speaking, acute inflammation is mediated by granulocytes, while chronic inflammation is mediated by mononuclear cells such as monocytes and lymphocytes.

The process of acute inflammation is initiated by cells already present in all tissues, mainly resident macrophages, dendritic cells, histiocytes, Kupffer cells and mastocytes.

Comparison between acute and chronic inflammation:

| Acute | Chronic |
|---|---|
| Pathogens, injured tissues | Persistent acute inflammation due to non-degradable pathogens, persistent foreign bodies, or autoimmune reactions |
| Neutrophils, mononuclear cells (monocytes, macrophages) | Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts |
| Vasoactive amines, eicosanoids | IFN-γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes |
| Immediate | Delayed |
| Few days | Up to many months, or years |
| Resolution, abscess formation, chronic inflammation | Tissue destruction, fibrosis |

**1. The isolation and characterization of globule leucocytes: their derivation from mucosal mast cells in parasitized sheep**

Ovine mucosal mast cells and globule leucocytes have been isolated from the abomasum of normal sheep, and from animals challenged with Ostertagia circumcincta. The ultrastructural, morphological and histochemical properties of these cells have been investigated. The granules of

ovine mucosal immunoglobulin and a serine esterase. These cells also possess surface immunoglobulin. Cells morphologically intermediate between mucosal mast cells and bulin. Cells morphologically intermediate between mucosal mast cells and globule leucocytes have similar granule and surface properties. These observations, together with quantitative data, indicate that alterations in the granule structure of mucosal mast cells as a consequence of prolonged antigenic challenge give rise to mast cells in the epithelium which, in the past, have been commonly recognized as globule leucocytes.

**2. Isolation and characterization of granulocyte lysosomal proteins and study of their effects on the clotting system.**

Lysosomes (granules) of rabbit PMN leukocytes were extracted with either HCl or H2SO4, and the extracts were chromatographed over Sephadex to separate protein constituents. Some of the low molecular weight cationic proteins homogeneous on SDS PAGE (8% and 12.5% gels) were characterized by electrophoretic mobility in acid gels and by amino acid analysis. A 3,700 dalton polypeptide, rich in arginine and cysteine, prolonged the partial thromboplastin time of normal plasma. In low concentration, this protein shortened the clotting time of pure fibrinogen by thrombin. In high concentration this lysosomal cationic protein precipitated fibrinogen from solution; no fibrinopeptides were released to suggest cleavage of fibrinogen. Fibrinolytic protease activity was detected in crude H2SO4 extracts but not in crude HCl extracts. Two separate plasminogen activators, differing from kallikrein or prekallikrein, were isolated from the H2SO4 lysosomal extract and were partially characterized; neither exhibited proteolytic activity on fibrinogen free of plasminogen.

**3. Isolation and characterization of LMC, a novel lymphocyte and monocyte chemoattractant human CC chemokine, with myelosuppressive activity.**

By searching the Expressed Sequence Tag (EST) data base, we identified a partial cDNA sequence encoding a novel human CC chemokine. The entire cDNA sequence was determined and revealed a CC chemokine whose mature protein consisted of 100 amino acids with predicted molecular weight of 11 kd. The chemokine preferantially chemoattracted lymphocytes and monocytes but not neutrophils. It was, therefore, named LMC (Lymphocyte and Monocyte Chemoattractant). LMC exhibited potent myelosuppressive activity, which was comparable to that of MIP-1alpha. We identified several bacterial artificial clones (BAC) containing the LMC gene along with two human CC chemokine subfamily members; leukotactin-1 (Lkn-1) and CKbeta8-1/CKbeta8. This data suggests that the LMC gene is located at human chromosome 17q which encompasses a human CC chemokine gene cluster.

**4. Isolation and characterization of macrophages from rat embryonic muscle culture**.

We have previously described the wide distribution of resident macrophages in normal rat skeletal muscle. In this study, we investigated the characteristics of the macrophages that occur in rat embryo muscle cultures. We showed that cells of monocyte-macrophage lineage are present in primary muscle cultures of rat embryos (18 days in gestation) and that these cells form morphologically and phenotypically heterogeneous populations, based on their reaction with monoclonal antibodies ED1, ED2, ED3, and OX43. Constitutively Ia+ cells with dendritic appearance were also observed. Furthermore, we established the procedure for isolation of macrophages from the primary muscle cultures. The isolated cells, mostly ED1+, expressed class I and CD4 antigens and bore complement (C3) receptors on their surfaces. The fact that cell of monocyte-macrophage lineage occur in the embryonic muscle suggests that during embryogenesis these cells may enter the developing muscle and give rise to a population of tissue-associated macrophages.

**5. Isolation and characterization of dendritic cells from common marmosets for preclinical cell therapy studies**

Dendritic cells (DCs) have important functions as modulators of immune responses, and their ability to activate T cells is of great value in cancer immunotherapy. The isolation of DCs from the peripheral blood of rhesus and African green monkeys has been reported, but the immune system in the common marmoset remains poorly characterized, although it offers many potential advantages for preclinical studies. In the present study, we devised methods, based on techniques

developed for mouse and human DC preparation, for isolating DCs from three major tissue sources in the common marmoset: bone marrow (BM), spleen and peripheral blood. Each set of separated cells was analysed using the cell surface DC-associated markers CD11c, CD80, CD83, CD86 and human leucocyte antigen (HLA)-DR, all of which are antibodies against human antigens, and the cells were further characterized both functionally and morphologically as antigen-presenting cells. BM proved to be an excellent cell source for the isolation of DCs intended for preclinical studies on cell therapy, for which large quantities of cells are required. In the BM-derived $CD11c^{+}$ cell population, cells exhibiting the characteristic features of DCs were enriched, with the typical DC morphology and the abilities to undergo endocytosis, to secrete interleukin (IL)-12, and to stimulate Xenogenic T cells. Moreover, BM-derived DCs produced the neurotrophic factor NT-3, which is also found in murine splenic DCs. These results suggest that BM-derived DCs from the common marmoset may be useful for biological analysis and for preclinical studies on cell therapy for central nervous system diseases and cancer.

**6. Isolation and characterisation of a mast cell degranulating substance from *Ascaris suum***

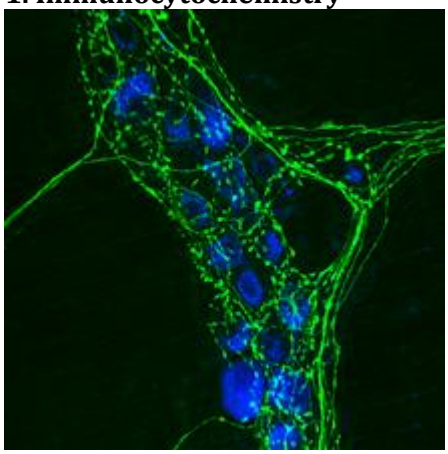
1. During investigations of allergic phenomena associated with parasitic infestations, a substance which induces degranulation of mast cells has been isolated from the body fluid of *Ascaris suum*. It has been assayed by its ability to cause degranulation of rat peritoneal mast cells *in vitro*.
2. The mast cell factor has reasonable stability to heat. To retain mast cell factor activity during isolation and storage of the various preparations, it was essential to prevent aggregation of the mast cell factor, presumably resulting from oxidation of sulphydryl groups. Aggregation was prevented by incorporation in the buffers of 2-mercaptoethanol and dithiothreitol. Precautions were also necessary to avoid loss of activity during concentration of fractions particularly because the molecular size of mast cell factor barely permitted its retention by the usual dialysis membranes.
3. The scheme adopted for purification of mast cell factor involved successive chromatographic fractionations on DEAE-cellulose, Sephadex G-75 and SE-Sephadex. The product gave a single band on polyacrylamide-gel electrophoresis in gradient and 7% gels, as well as in gels containing sodium dodecyl sulphate. In the last system proteins were first reduced and unfolded; by comparison in dodecyl sulfate gels of the mobility of mast cell factor with that of reference proteins, mast cell factor was estimated to have a molecular weight of 8800.
4. The sedimentation-velocity pattern showed a single peak with an *s* value of 1.07, a result corresponding to a molecular weight of the order of 8600. Amino acid analysis indicated a molecular weight of 8937. The above evidence collectively suggests that the isolated preparation of mast cell factor is substantially homogeneous.

**7. Isolation and Characterization of a Novel Inducible Mammalian Galectin***

A novel mammalian galectin cDNA (*ovgal11*) was isolated by representational difference analysis from sheep stomach (abomasal) tissue infected with the nematode parasite, *Haemonchus contortus*. The mRNA is greatly up-regulated in helminth larval infected gastrointestinal
tissue subject to inflammation and eosinophil infiltration. Immunohistological analysis indicates that the protein is localized in the cytoplasm and nucleus of upper epithelial cells of the gastrointestinal tract. The protein is also detected in mucus samples collected from infected abomasum but not from uninfected tissue. The restricted and inducible expression of *ovgal11* mRNA and limited secretion of the protein support the hypothesis that OVGAL11 may be involved in gastrointestinal immune/inflammatory responses and possibly protection
against infection.

**Functional Studies On Isolated Cells**

## 1. Immunocytochemistry



Immunocytochemistry labels individual proteins within cells, such as TH (green) in the axons of sympathetic autonomic neurons.

**Immunocytochemistry** (**ICC**) is a common laboratory technique that uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. These bound antibodies can then be detected using several different methods. ICC allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. In cases where an immunopositive signal is found, ICC also allows researchers to determine which sub-cellular compartments are expressing the antigen.

### *Immuno*cyto*chemistry vs. immuno*histo*chemistry*

Immunocytochemistry differs from immunohistochemistry in that the former is performed on samples of intact cells that have had most, if not all, of their surrounding extracellular matrix removed. This includes cells grown within a culture, deposited from suspension, or taken from a smear. In contrast, immunohistochemical samples are sections of biological tissue, where each cell is surrounded by tissue architecture and other cells normally found in the intact tissue. Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells (cultured cells, cell suspensions) by use of a specific antibody, which binds to it, thereby allowing visualization and examination under a microscope. It is a valuable tool for the determination of cellular contents from individual cells. Samples that can be analyzed include blood smears, aspirates, swabs, cultured cells, and cell suspensions.

There are many ways to prepare cell samples for immunocytochemical analysis. Each method has its own strengths and unique characteristics so the right method can be chosen for the desired sample and outcome.

Cells to be stained can be attached to a solid support to allow easy handling in subsequent procedures. This can be achieved by several methods: adherent cells may be grown on microscope slides, coverslips, or an optically suitable plastic support. Suspension cells can be centrifuged onto glass slides (cytospin), bound to solid support using chemical linkers, or in some cases handled in suspension.

Concentrated cellular suspensions that exist in a low-viscosity medium make good candidates for smear preparations. Dilute cell suspensions existing in a dilute medium are best suited for the preparation of cytospins through cytocentrifugation. Cell suspensions that exist in a high-viscosity medium, are best suited to be tested as swab preparations. The constant among these preparations is that the whole cell is present on the slide surface. For any intercellular reaction to take place, immunoglobulin must first traverse the cell membrane that is intact in these preparations. Reactions taking place in the nucleus can be more difficult, and the extracellular fluids can create unique obstacles in the performance of immunocytochemistry. In this situation, permeabilizing

cells using detergent (Triton X-100 or Tween-20) or choosing organic fixatives (acetone, methanol, or ethanol) becomes necessary.

Antibodies are an important tool for demonstrating both the presence and the subcellular localization of an antigen. Cell staining is a very versatile technique and, if the antigen is highly localized, can detect as few as a thousand antigen molecules in a cell. In some circumstances, cell staining may also be used to determine the approximate concentration of an antigen, especially by an image analyzer.

***Methods***

There are many methods to obtain immunological detection on tissues, including those tied directly to primary antibodies or antisera. A direct method involves the use of a detectable tag (e.g., fluorescent molecule, gold particles, etc., ) directly to the antibody that is then allowed to bind to the antigen (e.g., protein) in a cell.

Alternatively, there are many **indirect methods**. In one such method, the antigen is bound by a primary antibody which is then amplified by use of a secondary antibody which binds to the primary antibody. Next, a tertiary reagent containing an enzymatic moiety is applied and binds to the secondary antibody. When the quaternary reagent, or substrate, is applied, the enzymatic end of the tertiary reagent converts the substrate into a pigment reaction product, which produces a

color (many colors are possible; brown, black, red, etc.,) in the same location that the original primary antibody recognized that antigen of interest.

Some examples of **substrates** used (also known as chromogens) are AEC (3-Amino-9-EthylCarbazole), or DAB (3,3'-Diaminobenzidine). Use of one of these reagents after exposure to the necessary enzyme (e.g., horseradish peroxidase conjugated to an antibody reagent) produces a positive immunoreaction product. Immunocytochemical visualization of specific antigens of interest can be used when a less specific stain like H&E (Hematoxylin and Eosin) cannot be used for a diagnosis to be made or to provide additional predictive information regarding treatment (in some cancers, for example).

Alternatively the secondary antibody may be covalently linked to a fluorophore (FITC and Rhodamine are the most common) which is detected in a fluorescence or confocal microscope. The location of fluorescence will vary according to the target molecule, external for membrane proteins, and internal for cytoplasmic proteins. In this way immunofluorescence is a powerful technique when combined with confocal microscopy for studying the location of proteins and dynamic processes (exocytosis, endocytosis, etc.).

**Cell Array Immunocytochemistry Protocol**

*Note*: Do not let the tissues dry out once they are re-hydrate.
Use separate tubs for antibodies and negative control slides to avoid contamination.

**MATERIALS**

Cell Array Slide
Coverslips
Slide racks
Staining dishes with lids
Plastic slide tray
Orbital shaker
Transfer pipettes

Deionized water (DI $H_2O$)
PBS (Phosphate Buffered Saline)
Triton X-100
Hydrogen peroxide ($H_2O_2$)
Primary antibody
Biotinylated secondary antibody, HRP conjugated
Bovine Serum Albumin (BSA – for blocking)
Streptavidin-HRP
DAB
Hematoxylin (optional)
Acetic Acid (optional)
Glycerol

**Permeabilize Membrane** (Optional if detecting a membrane protein.)

1. Add one drop of PBS/0.1% Triton X-100 to each well to permeabilize the cells. Incubate slides for one (1) minute at room temperature.
2. Remove the liquid and wash the slides twice (2x) in PBS, 5 minutes each on the shaker.
3. Remove the liquid and place the slides onto a tray.

**Blocking**

4. Soak slides in 1.5% $H_2O_2$ /PBS solution for 15 minutes.
5. Wash twice (2x) in PBS for 5 minutes each on the shaker.
6. Incubate with 5% BSA into each well to block for overnight at 4°C in a humid chamber.

**Primary Antibody**

7. Dilute the primary antibody to the recommended concentration in 1% BSA diluent.
8. Remove BSA from the slides.
9. Add 35µL of primary antibody to each well. Incubate for one (1) hour at room temperature.
10. Remove the primary antibody solution and wash slides three (3) times in PBS, 5 minutes each on the shaker.

**Secondary Antibody and Detection**

11. Dilute the biotinylated secondary antibody to 1:200 in a solution of 1% BSA diluent.
12. Remove the excess fluid and add one drop secondary antibody solution into each well. Incubate for one (1) hour at room temperature.
13. Wash in PBS three (3) times 5 minutes each on an orbital shaker. Remove excess fluid.
14. Add one drop streptavidin-HRP to each well. Incubate for 30 minutes at room temperature.
15. Wash three (3) times 5 minutes in PBS on an orbital shaker. Remove excess fluid.
16. Add DAB solution to each cell well. Once the cells start turning brown (inexperienced technicians may wish to observe this under a microscope) wash twice (2x) in PBS for 5 minutes each time on the shaker.

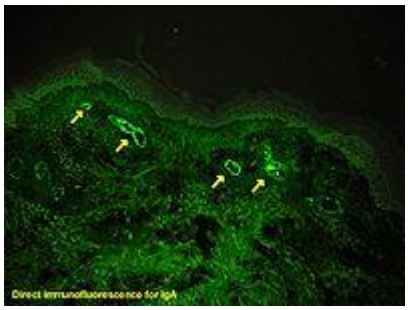
**Optional Counterstain**

17. Dip the slide rack with the slides into a staining dish of hematoxylin for 30 seconds.
18. Remove and place into an acid bath (200mL DI $H_2O$ and one to three drops of acetic acid). Rinse with DI $H_2O$.

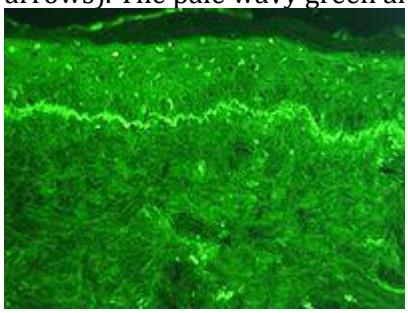
**Cover Slips**

19. Add several drops of coverslip solution (50% glycerol / DI $H_2O$) to the slide.
20. Place the coverslip on top of the slide.
21. Store slides at room temperature.

**Immunofluorescence**




Microphotograph of a histological section of human skin prepared for **direct immunofluorescence** using an anti-IgA antibody. The skin is from a patient with Henoch-Schonlein purpura: IgA deposits are found in the walls of small superficial capillaries (yellow arrows). The pale wavy green area on top is the epidermis, the bottom fibrous area is the dermis.



Microphotograph of a histological section of human skin prepared for **direct immunofluorescence** using an anti-IgG antibody. The skin is from a patient with systemic lupus erythematosus and shows IgG deposit at two different places: The first is a band-like deposit along the epidermal basement membrane ("lupus band test" is positive). The second is within the nuclei of the epidermal cells (anti-nuclear antibodies).

**Immunofluorescence** is a technique used for light microscopy with a fluorescence microscope and is used primarily on biological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualisation of the distribution of the target molecule through the sample. Immunofluorescence is a widely used example of immunostaining and is a specific example of immunohistochemistry that makes use of fluorophores to visualise the location of the antibodies. [1]

Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells, and may be used to analyse the distribution of proteins, glycans, and small biological and non-biological molecules. Immunofluoresence can be used in combination with other, non-antibody methods of fluorescent staining, for example, use of DAPI to label DNA. Several microscope designs can be used for analysis of immunofluorescence samples; the simplest is the epifluorescence microscope, and the confocal microscope is also widely used. Various super-resolution microscope designs that are capable of much higher resolution can also be used.[2]

### *Types of immunofluorescence*

There are two classes of immunofluorescence techniques, primary (or direct) and secondary (or indirect).

**Primary (direct)**

Primary, or direct, immunofluorescence uses a single antibody that is chemically linked to a fluorophore. The antibody recognises the target molecule and binds to it, and the fluorophore it carries can be detected via microscope. This technique has several advantages over the secondary (or indirect) protocol below because of the direct conjugation of the antibody to the fluorophore. This reduces the number of steps in the staining procedure, is therefore faster, and can avoid some issues with antibody cross-reactivity or non-specificity, which can lead to increased background signal.

**Secondary (indirect)**

Secondary, or indirect, immunofluorescence uses two antibodies; the first (the primary antibody) recognises the target molecule and binds to it, and the second (the secondary antibody), which carries the fluorophore, recognises the primary antibody and binds to it. This protocol is more complex than the primary (or direct) protocol above and takes more time but allows more flexibility.

This protocol is possible because an antibody consists of two parts, a variable region (which recognizes the antigen) and an invariant region (which makes up the structure of the antibody molecule). A researcher can generate several primary antibodies that recognize various antigens (have different variable regions), but all share the same invariant region. All these antibodies may therefore be recognized by a single secondary antibody. This saves the cost of modifying the primary antibodies to directly carry a fluorophore.

Different primary antibodies with different invariant regions are typically generated by raising the antibody in different species. For example, a researcher might create primary antibodies in a goat that recognize several antigens, and then employ dye-coupled rabbit secondary antibodies that recognize the goat antibody invariant region ("rabbit anti-goat" antibodies). The researcher may then create a second set of primary antibodies in a mouse that could be recognised by a separate "donkey anti-mouse" secondary antibody. This allows re-use of the difficult-to-make dye-coupled antibodies in multiple experiments.

### *Limitations*

As with most fluorescence techniques, a significant problem with immunofluorescence is photobleaching. Loss of activity caused by photobleaching can be controlled by reducing the intensity or time-span of light exposure, by increasing the concentration of fluorophores, or by employing more robust fluorophores that are less prone to bleaching (e.g., Alexa Fluors, Seta Fluors, or DyLight Fluors).

In general, immunofluorescence is limited to fixed (i.e., dead) samples. Analysis of structures within live cells by immunofluorescence is not possible, as antibodies cannot cross the cell membrane. As such some uses of immunofluorescence have been outmoded by the development of recombinant proteins containing fluorescent protein domains, e.g., green fluorescent protein (GFP). Use of such "tagged" proteins allows determination of their localisation in live cells.

### *Immunofluorescence Method*

The purpose of immunofluorescence is to detect the location and relative abundance of any protein for which you have an antibody. Once you have antibodies to your favorite protein, you can use them to indicate where the protein is located. In this example, we will use antibodies for the **calcium ATPase**, or pump, that is located in the **endoplasmic reticulum** (ER) of very cell. The

antibody used here only recognized the chicken calcium ATPase but immunofluorescence can be used on any protein.

The key to this entire process is the ability to visualize the antibody when looking through a microscope. Since antibodies are smaller than calcium ATPases, you cannot see the antibody directly. Therefore, you have to use a fluorescent dye that is covalently attached to the antibody. When a light illuminates the fluorescent dye, it absorbs the light an emits a different color light which is visible to the investigator and can be photographed.

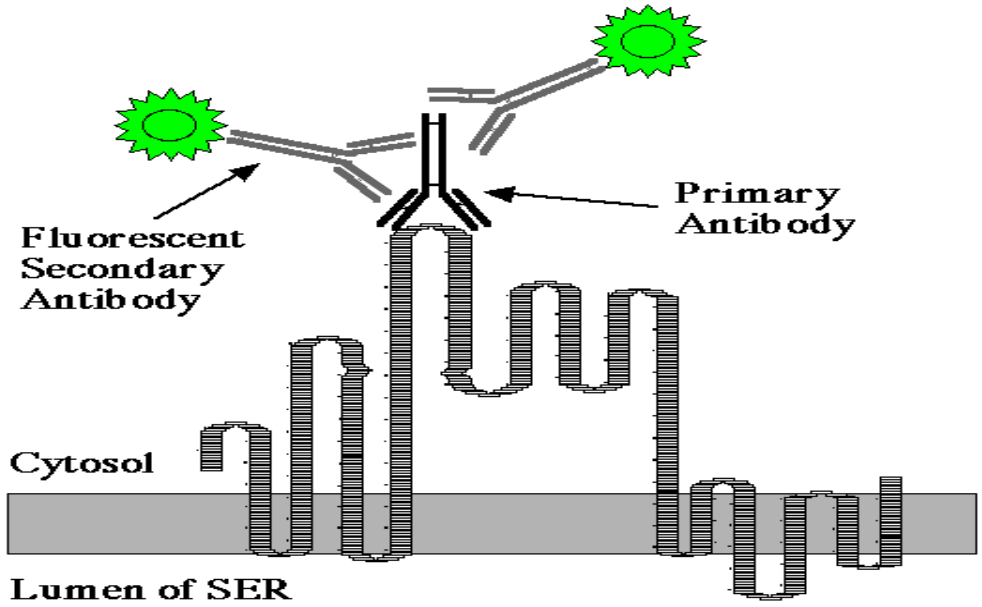



**Figure 1.** In most immunofluorescence experiments, two antibodies are employed. The first one, called the **primary antibody**, is typically generated in a mouse and binds to your favorite protein, which in this case is the chicken calcium ATPase (shown as a series undulating striped line that zigzags through teh ER membrane 10 times). The **secondary antibody** was purchased from a company that sells antibodies that bind to mouse antibodies and have a fluorescent dye covalently attached to it. As illustrated here, the secondary antibodies can bind to multiple sites on the primary antibody and thus produce a brighter signal since more dyes are brought to a single location. The first step is to choose your cells of interest. In this case, we will look at a chicken fibroblast, or skin cell. It was grown in tissue culture and so it appears as an isolated cell witn no visible neighbors. The cell was fixed with formaldehyde to retain the shape and location of all cellular proteins. The cell was treated with a mild detergent to disolve small holes in the membranes so the antibodies could have access to the cytoplasm. Because the calcium ATPase is located in the ER, the antibodies must have access to the cytoplasm or they could not bind to the target protein.

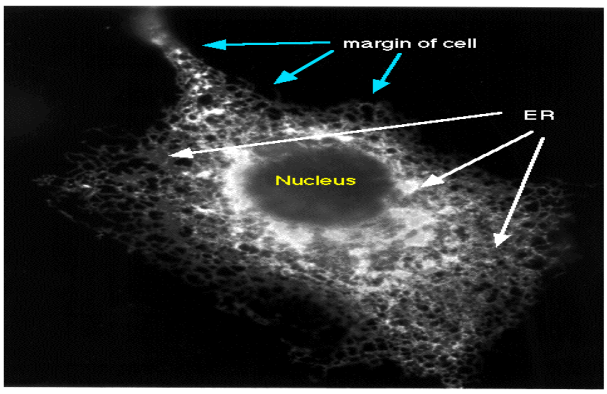


**Figure 2.** This immunofluorescence micrograph shows the ER being labeled with a monoclonal antibody against the the chicken calcium ATPase. This chicken cell was fixed, permeablilized, and processed for immunofluorescence. White indicates the location of the fluorescent antibody and thus the calcium ATPase to which the antibody was bound. Immunofluorescence photomicrograph by A. Malcolm Campbell.

**Immuno Enzyme Techniques in Cytochemistry**

**Summary**

The introduction of light and especially electron optic systems for morphological studies of cellular and subcellular structures has enabled significant advances to be made in the know-ledge of cell biology and normal and diseased organs.However, spatial and temporal aspects of cellular processes and the functional or evolutionary significance of the increasing comple-xity of higher organisms cannot be elucidated simply by comparing fine structures. In this context, classical histochemistry and new specific cytological procedures under development allow relationships between biological structure and function to be more readily discerned. In order to understand the molecular composition of organs at the cellular level, the combina-tion of immunological and histological concepts is a promising line of research which already proved extremely useful for histopathology and cell biology. Antibodies possess a high degree of specificity towards antigenic determinants. Because of the narrow range of specificity of an antibody molecule to bind with its antigenic determinant, immunochemical methods are part of the most sensitive techniques in molecular biology and biomedicine. With respect to the definition of antigenic molecules (substances which initiate the formation of and react with antibodies are called antigens), immunoserological analyses of organs of normal state and in disease are of great importance. To this aim, qualitative and quantitative approaches have been described since the very early years of this century e.g. by Ehrlich, Landsteiner, Witebs-ky, Heidelberger, Marrack, Kabat, Oudin, Grabar and schools derived from these pioneers in immunochemistry.

In our day, further developments of highly sensitive techniques like those based on radio- or enzyme-immuno-assays are still in progress. The principle of an immunoserological analysis of organs relies on the use of immune sera produced by heteroimmunization of animals, on the use of antibodies produced by hybridomas or other biotechnical procedures or on the occurrence of autoantibodies in connection with certain diseases. A number of phenomena which resemble antibody reaction are shared by lectins. These occur in a variety of plants, invertebrates and vertebrates and are used for the study of carbohydrate moieties.

The immunofluorescent approach introduced by Coons and co-workers (1941, 1950) opened specific investigations on cellular structure and function at the light microscopic level. In the meantime, immunofluorescent methods have progressed from pure scientific research towards routine in histopathology. It is evident that analogous techniques will also be useful and important for ultrastructural studies.

In principle, the resolution of the electron microscope enables the demonstration of an anti-body molecule which has reacted with its antigen. However, after usual resin embedment single protein molecules in the tissue cannot be identified because such molecule groups are not more electron dense than the surrounding matrix. In consequence, unlabeled antibodies are only suitable for the demonstration of isolated particles when measurable and reproducible changes in density or definite structural changes are obtained.

The purpose of most immunohistological procedures is the identification and characterization of cellular structure or function *in situ* rather than immuno-staining of physicochemically iso-lated constituents. Hence, the respective immunological ligand must be “labeled” in a way so that the antigen-antibody complexes become readily visible. Suitable substances for labeling purposes are those which lead to distinct color/fluorescent reactions (e.g. light or fluorescent microscopes, laser scanning microscopes) or which give significant deflection of electrons in the electron microscope. A milestone in immuno-electron microscopy was then the conjuga-tion of the metalloprotein ferritin with antibodies by Singer (1959) which opened a new era of ultrastructure research.

It is now well established that immunological concepts of cellular ligand assays at both light and electron microscopic levels are important for the study of histogenesis, histodifferentia-tion and

histopathology of organs. In this publication, the major steps in preparation of immu-nohistological reagents on the one hand and tissue sampling on the other hand are described.
The detection of intracellular molecules is especially emphasized which is much more intri-cate than that of extracellular spaces and cell surface membranes. The use of solid tissues instead of single cell suspensions or monolayer cultures is preferentially treated for the reason that tissues or their fragments represent the majority of specimens in histopathology and that, according to current experience, pitfalls are mainly observed with such solid organ prepara-tions. In any case, principles in the preparation of immunohistological reagents are the same and theoretical as well as practical considerations of tissue sampling are quite similar for both tissue fragments and single cells.
When cells are to be studied, preservation of their structure and minimal alteration from the living state should be considered. The adaption of a fixation method is in most cases necessa-ry. Yet, one of the most limiting factors impeding full utilization of immunological reagents is fixation and embedment of biological specimens (e.g. embedment in epoxy resins). Numerous publications during the last decades have shown that by experimental testing and methodolo-gical improvement of immunochemical and cytological parameters, conditions can be obtai-ned under which suitable intracellular labeling of cellular molecules is obtained.
A general and ideal procedure for the detection of cellular ligands is not available. Light and electron microscopic immuno-stainings must be usually established for each biological model. Thus, immunohistology may consider two particular parts:

- the preparation of immunohistological reagents as well as
- the cytological assays.

In this way, at least two different areas meet together, namely immunochemistry (even if numerous reagents can be now purchased) and cellular morphology.

**Fig. 1: Steps of tissue processing in preembedment immunohistology from incubation to Epon embedment.** Frozen cut sections are immuno-stained, washed, dehydrated and infiltrated with embedding mixture in small glass vials (right). Then, single sections are placed into polyethylene lids from Beem capsules or equivalent containing a droplet of fresh Epon mixture (middle). From polymerized Epon molds which are prepared in advance in lids of Beem capsules, Epon is removed and put with the flat surface on top of the section. After subsequent polymerization a desired part of the section is selected by light microscopy. Then, tissue and Epon excess are cut away with a razor blade. The preparation is mounted with adhesive (or Epon) on a casule filled with Epon (and poly-merized in advance). Finally, the selected tissue area of the flat embedded section is trimmed for ultramicrotomy (left).

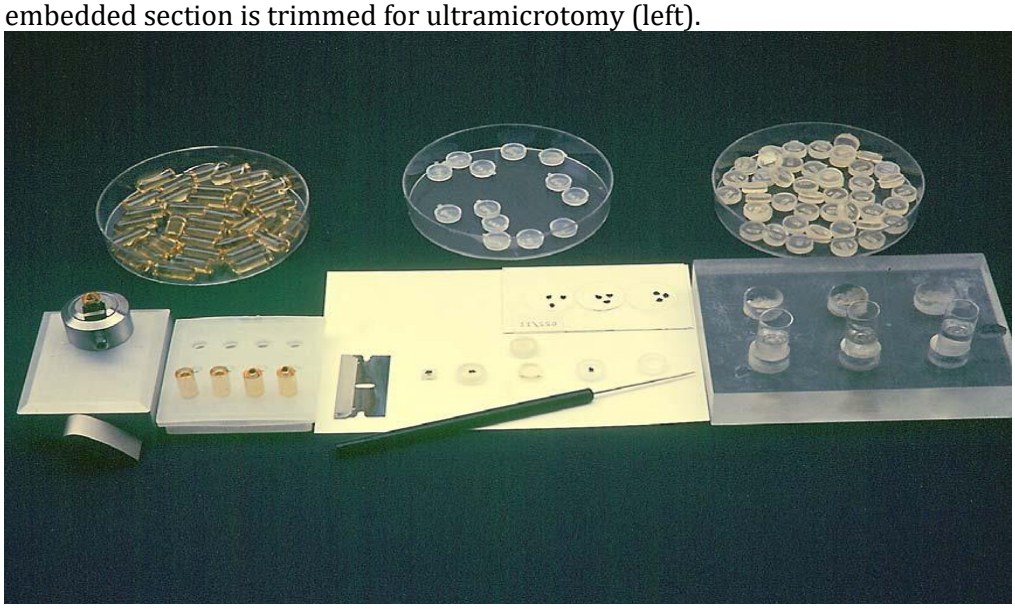


**Fig. 2a-d: Complete 40 µm thick frozen section after immuno-staining, dehydration and Epon embedment (see Fig. 1); a-c) several light microscopic views of an area of interest (marked with a razor blade) which is selected for further studies; d) semithin section of same preparation. Positive immuno-stainings in c) and d) are indicated by arrows.**

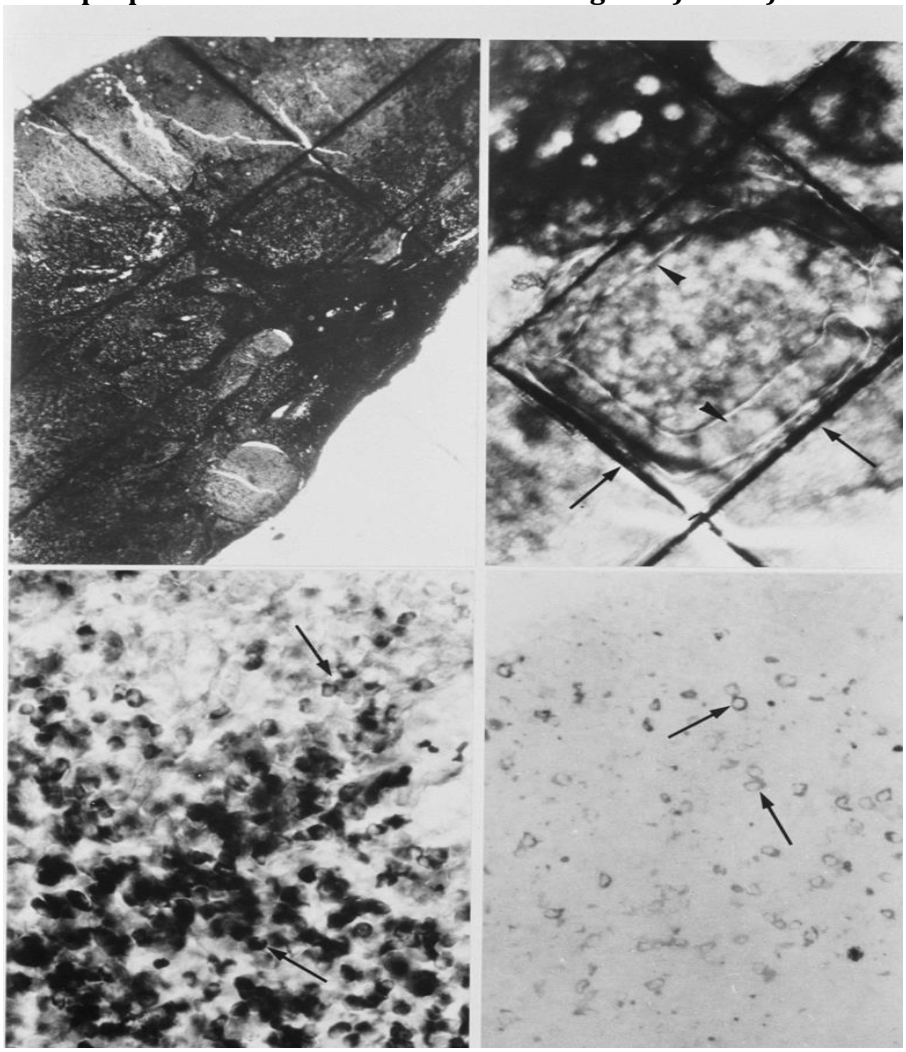
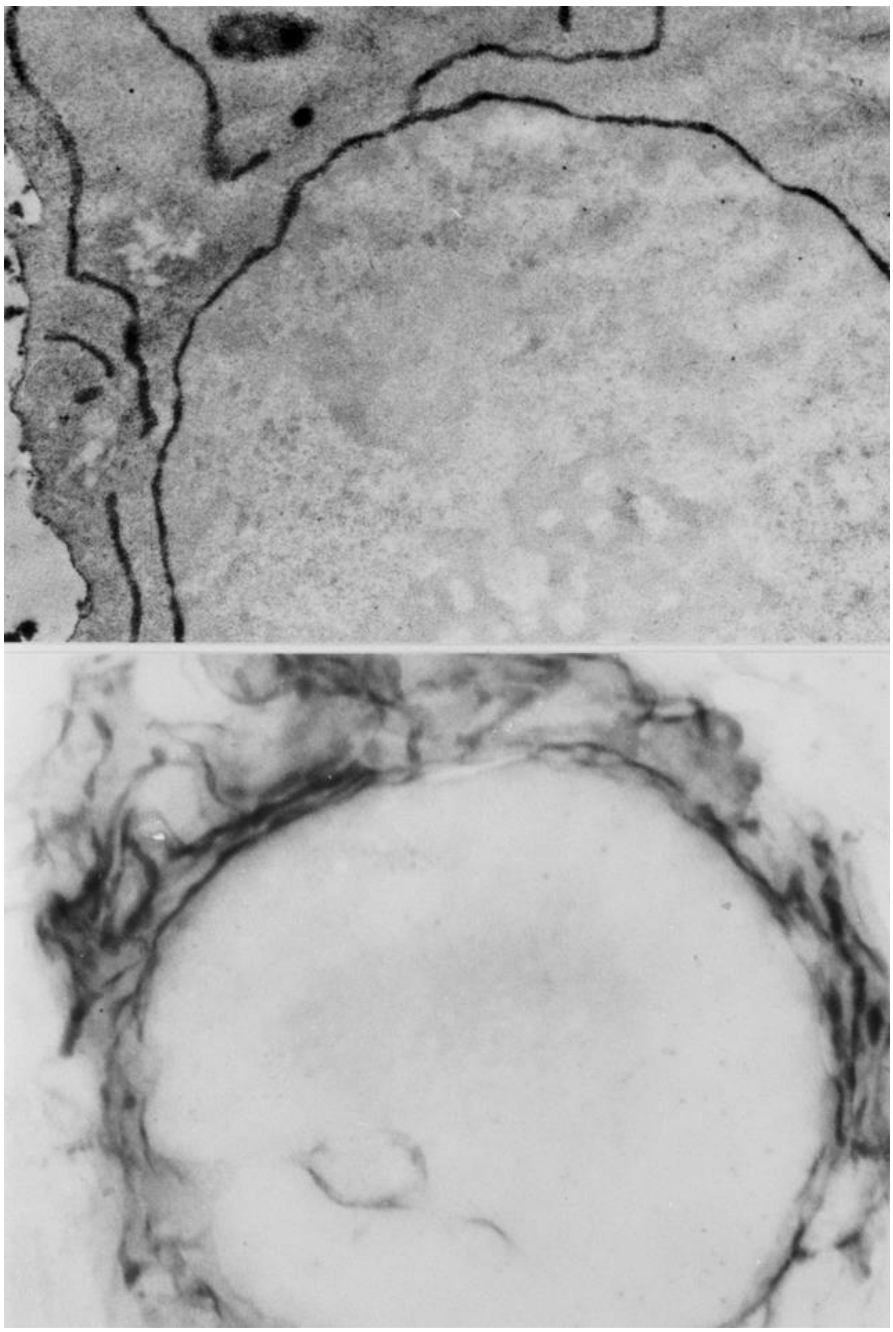


**Fig. 3a-c: Transverse sec-tions of 40µm thick frozen sections, prepared as des-cribed in Fig. 1 and Fig. 2. Note positive immuno-staining and good pene-tration of immunocytoche-mical reagents across the 40 µm thick frozen sections. Fig. 3c represents an immu-nocytological control incu-bation.**

**Fig. 4a-b: Detection of in-tracellular IgG by use of antibody-HRP conjugates in preembedment immuno-histology; a) ultrathin sec-tion, note immuno-staining of some RER lamellae; b) one µm thick Epon section of the same tissue in the electron microscope, note the interconnected system of stained RER lamellae.**



**Immuno Ferrtin Techniques**

**Immunoferritin Labelling**

This technique is an adaptation of the labelling principle of fluorescent antibody staining. In this method of the antibody is conjugated to an electrondense molecule, such as ferritin, and is made

visible, under the electron microscope. Ferritin is an organic complex of ferric hydroxide and ferric phosphate associated with apoferritin, a protein of the liver and spleen that serves as a storage place of iron. Cells are incubated with ferritin-conjugated antibody. Cell sections are then examined under the election microscope. Ferritin molecules can be observed on the cell surface where antibodies are bound.

A general method for the ultrastructural localization of intracellular proteins and antigens by immunoferritin techniques has been developed. The method involves direct staining of ultrathin sections of mildly glutaraldehyde- fixed and frozen tissues cut by means of a cryo- ultramicrotome. Bovine pancreatic sections were cut, mounted on grids, and stained with ferritin-rabbit antibovine RNase conjugates. After negative staining with 0.2%
phosphotungstic acid, electron micrographs revealed specific labeling of all of the zymogen granules and the cisternae of the rough endoplasmic reticulum. No significant
labeling was seen in the nucleus, mitochondria, or cell sap regions. The observation that no significant labeling was found in any region of rat pancreatic sections was consistent
with the fact that rat RNase is immunologically noncrossreactive with bovine RNase. In addition, the labeling seen in bovine pancreas was completely absent if the sections
were first incubated with free antibody. The method used here avoids prolonged fixation, dehydration, and other harsh chemical or physical treatments, and should extend the usefulness of immunoferritin techniques to the intracellular localization of many protein antigens beyond previously available methods.

**Immuno Electron Microscopy**

This study evaluated a variety of fixatives and methods of tissue preparation for application cf the direct peroxidase-labeled antibody technique to rat kidney specimens. Tissue ultrastructure was most satisfactorily preserved and the antigens studied (rabbit IgG, human IgG, and rat Tamm-Horsfall protein (THP)) were adequately preserved after brief fixation with 1%
glutaraldehyde ( 15 mm), a mixture of paraformaldehyde ( l7c) and glutaraldehyde (0.05%), or a paraformaldehyde, lysine, periodate fixative. Glycerol substitution was considered
an important step which minimized ice crystal artifacts. Freezing and thawing were essential steps that facilitated adequate penetration of labeled antibody to ı specific antigenic sites. The distribution of injected rabbit IgG (anti-rat glomerular basement membrane (GBM) antibody) was predominately on the lamina rara interna and externa of the GBM. Injected aggregated human IgG ı was found primarily within the spaces between glomeruılar mesangial cells. Rabbit anti-rat THP was localized primarily on the infolding membranes of cells of the ascending thick limb of Henle. We suggest that the methods described may have wide application.

**UNIT V MOLECULAR IMMUNOLOGY**

**Vaccine**

A **vaccine** is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe or its toxins. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters.

Vaccines can be prophylactic (e.g. to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen), or therapeutic (e.g. vaccines against cancer are also being investigated; see cancer vaccine).The term *vaccine* derives from Edward Jenner's 1796 use of the term *cow pox* (Latin *variolæ vaccinæ*, adapted from the Latin *vaccīn-us*, from *vacca* cow), which, when administered to humans, provided them protection against smallpox.

### *History*

Sometime during the 1770s Edward Jenner heard a milkmaid boast that she would never have the often-fatal or disfiguring disease smallpox, because she had already had cowpox, which has a very mild effect in humans. In 1796, Jenner took pus from the hand of a milkmaid with cowpox, inoculated an 8-year-old boy with it, and six weeks later variolated the boy's arm with smallpox, afterwards observing that the boy did not catch smallpox. Further experimentation demonstrated the efficacy of the procedure on an infant. Since vaccination with cowpox was much safer than smallpox inoculation, the latter, though still widely practiced in England, was banned in 1840. Louis Pasteur generalized Jenner's idea by developing what he called a rabies vaccine, and in the nineteenth century vaccines were considered a matter of national prestige, and compulsory vaccination laws were passed.

The twentieth century saw the introduction of several successful vaccines, including those against diphtheria, measles, mumps, and rubella. Major achievements included the development of the polio vaccine in the 1950s and the eradication of smallpox during the 1960s and 1970s. Maurice Hilleman was the most prolific of the developers of the vaccines in the twentieth century. As vaccines became more common, many people began taking them for granted. However, vaccines remain elusive for many important diseases, including malaria and HIV.

### *Types*

Avian flu vaccine development by reverse genetics techniques.

Vaccines are dead or inactivated organisms or purified products derived from them.

There are several types of vaccines currently in use. These represent different strategies used to try to reduce risk of illness, while retaining the ability to induce a beneficial immune response.

**1. Killed**

Some vaccines contain killed, but previously virulent, micro-organisms that have been destroyed with chemicals or heat. Examples are the influenza vaccine, cholera vaccine, bubonic plague vaccine, polio vaccine, hepatitis A vaccine, and rabies vaccine.

**2. Attenuated**

Some vaccines contain live, attenuated microorganisms. Many of these are live viruses that have been cultivated under conditions that disable their virulent properties, or which use closely-related but less dangerous organisms to produce a broad immune response; however, some are bacterial in nature. They typically provoke more durable immunological responses and are the preferred type for healthy adults. Examples include the viral diseases yellow fever, measles, rubella, and mumps and the bacterial disease typhoid. The live Mycobacterium tuberculosis vaccine developed by Calmette and Guérin is not made of a contagious strain, but contains a virulently modified strain called "BCG" used to elicit immunogenicity to the vaccine.

**3. Toxoid**

Toxoid vaccines are made from inactivated toxic compounds that cause illness rather than the micro-organism. Examples of toxoid-based vaccines include tetanus and diphtheria. Toxoid vaccines are known for their efficacy. Not all toxoids are for micro-organisms; for example, *Crotalus atrox* toxoid is used to vaccinate dogs against rattlesnake bites.

**Subunit**

Protein subunit – rather than introducing an inactivated or attenuated micro-organism to an immune system (which would constitute a "whole-agent" vaccine), a fragment of it can create an immune response. Examples include the subunit vaccine against Hepatitis B virus that is composed of only the surface proteins of the virus (previously extracted from the blood serum of chronically infected patients, but now produced by recombination of the viral genes into yeast), the virus-like particle (VLP) vaccine against human papillomavirus (HPV) that is composed of the viral major capsid protein, and the hemagglutinin and neuraminidase subunits of the influenza virus.

**Conjugate**

Conjugate – certain bacteria have polysaccharide outer coats that are poorly immunogenic. By linking these outer coats to proteins (e.g. toxins), the immune system can be led to recognize the polysaccharide as if it were a protein antigen. This approach is used in the *Haemophilus influenzae* type B vaccine.

**Experimental**

A number of innovative vaccines are also in development and in use:

- Dendritic cell vaccines combine dendritic cells with antigens in order to present the antigens to the body's white blood cells, thus stimulating an immune reaction. These vaccines have shown some positive preliminary results for treating brain tumors.
- Recombinant Vector – by combining the physiology of one micro-organism and the DNA of the other, immunity can be created against diseases that have complex infection processes
- DNA vaccination – in recent years a new type of vaccine called *DNA vaccination*, created from an infectious agent's DNA, has been developed. It works by insertion (and expression, triggering immune system recognition) of viral or bacterial DNA into human or animal cells. Some cells of the immune system that recognize the proteins expressed will mount an attack against these proteins and cells expressing them. Because these cells live for a very long time, if the pathogen that normally expresses these proteins is encountered at a later time, they will be attacked instantly by the immune system. One advantage of DNA vaccines is that they are very easy to produce and store. As of 2006, DNA vaccination is still experimental.
- T-cell receptor peptide vaccines are under development for several diseases using models of Valley Fever, stomatitis, and atopic dermatitis. These peptides have been shown to modulate cytokine production and improve cell mediated immunity.
- Targeting of identified bacterial proteins that are involved in complement inhibition would neutralize the key bacterial virulence mechanism.

While most vaccines are created using inactivated or attenuated compounds from micro-organisms, synthetic vaccines are composed mainly or wholly of synthetic peptides, carbohydrates or antigens.

**Valence**

Vaccines may be *monovalent* (also called *univalent*) or *multivalent* (also called *polyvalent*). A monovalent vaccine is designed to immunize against a single antigen or single microorganism. A multivalent or polyvalent vaccine is designed to immunize against two or more strains of the same microorganism, or against two or more microorganisms. In certain cases a monovalent vaccine may be preferable for rapidly developing a strong immune response.

### *Developing immunity*

The immune system recognizes vaccine agents as foreign, destroys them, and "remembers" them. When the virulent version of an agent comes along the body recognizes the protein coat on the virus, and thus is prepared to respond, by (1) neutralizing the target agent before it can enter cells, and (2) by recognizing and destroying infected cells before that agent can multiply to vast numbers.

When two or more vaccines are mixed together in the same formulation, the two vaccines can interfere. This most frequently occurs with live attenuated vaccines, where one of the vaccine components is more robust than the others and suppresses the growth and immune response to the other components. This phenomenon was first noted in the trivalent Sabin polio vaccine, where

the amount of serotype 2 virus in the vaccine had to be reduced to stop it from interfering with the "take" of the serotype 1 and 2 viruses in the vaccine. This phenomenon has also been found to be a problem with the dengue vaccines currently being researched, where the DEN-3 serotype was found to predominate and suppress the response to DEN-1, -2 and -4 serotypes.

Vaccines have contributed to the eradication of smallpox, one of the most contagious and deadly diseases known to man. Other diseases such as rubella, polio, measles, mumps, chickenpox, and typhoid are nowhere near as common as they were a hundred years ago. As long as the vast majority of people are vaccinated, it is much more difficult for an outbreak of disease to occur, let alone spread. This effect is called herd immunity. Polio, which is transmitted only between humans, is targeted by an extensive eradication campaign that has seen endemic polio restricted to only parts of four countries (Afghanistan, India, Nigeria and Pakistan).The difficulty of reaching all children as well as cultural misunderstandings, however, have caused the anticipated eradication date to be missed several times.

### *Effectiveness*

Vaccines do not guarantee complete protection from a disease.Sometimes, this is because the host's immune system simply does not respond adequately or at all. This may be due to a lowered immunity in general (diabetes, steroid use, HIV infection, age) or because the host's immune system does not have a B cell capable of generating antibodies to that antigen.

Even if the host develops antibodies, the human immune system is not perfect and in any case the immune system might still not be able to defeat the infection.

Adjuvants are typically used to boost immune response. Most often aluminium adjuvants are used, but adjuvants like squalene are also used in some vaccines and more vaccines with squalene and phosphate adjuvants are being tested. Larger doses are used in some cases for older people (50–75 years and up), whose immune response to a given vaccine is not as strong.

The efficacy or performance of the vaccine is dependent on a number of factors:

- the disease itself (for some diseases vaccination performs better than for other diseases)
- the strain of vaccine (some vaccinations are for different strains of the disease)
- whether one kept to the timetable for the vaccinations
- some individuals are "non-responders" to certain vaccines, meaning that they do not generate antibodies even after being vaccinated correctly
- other factors such as ethnicity, age, or genetic predisposition

When a vaccinated individual does develop the disease vaccinated against, the disease is likely to be milder than without vaccination.[

The following are important considerations in the effectiveness of a vaccination program:[

1. careful modelling to anticipate the impact that an immunization campaign will have on the epidemiology of the disease in the medium to long term
2. ongoing surveillance for the relevant disease following introduction of a new vaccine and
3. maintaining high immunization rates, even when a disease has become rare.

In 1958 there were 763,094 cases of measles and 552 deaths in the United States.With the help of new vaccines, the number of cases dropped to fewer than 150 per year (median of 56).In early 2008, there were 64 suspected cases of measles. 54 out of 64 infections were associated with

importation from another country, although only 13% were actually acquired outside of the United States; 63 of these 64 individuals either had never been vaccinated against measles, or were uncertain whether they had been vaccinated.

### *Trends*

Vaccine development has several trends

- Until recently, most vaccines were aimed at infants and children, but adolescents and adults are increasingly being targeted.
- Combinations of vaccines are becoming more common; vaccines containing five or more components are used in many parts of the world.
- New methods of administering vaccines are being developed, such as skin patches, aerosols via inhalation devices, and eating genetically engineered plants.
- Vaccines are being designed to stimulate innate immune responses, as well as adaptive.
- Attempts are being made to develop vaccines to help cure chronic infections, as opposed to preventing disease.
- Vaccines are being developed to defend against bioterrorist attacks such as anthrax, plague, and smallpox.
- Appreciation for sex and pregnancy differences in vaccine responses "might change the strategies used by public health officials".

Principles that govern the immune response can now be used in tailor-made vaccines against many noninfectious human diseases, such as cancers and autoimmune disorders.For example, the experimental vaccine CYT006-AngQb has been investigated as a possible treatment for high blood pressure.Factors that have impact on the trends of vaccine development include progress in translatory medicine, demographics, regulatory science, political, cultural, and social responses.

**Current approaches to vaccine preparation**

Numerous conventional vaccines for animal use are currently available, and many of these vaccines have been instrumental in the control of infectious diseases of major economic importance. A vaccine has even been instrumental in global eradication of smallpox, an important human disease. However, many of the current vaccines are deficient in efficiency, potency, or safety. It has been recognized that the conventional methodologies are a limitation to further vaccine development. Introduction of monoclonal antibodies, recombinant DNA, and protein engineering techniques has facilitated a rather rapid increase in the knowledge of pathogenetic mechanisms, as well as of protective antigens at the molecular level. This knowledge provides the basis for development of a new generation of vaccines. As a rule, these vaccines contain purified immunogens, or even isolated epitopes, identified and prepared by molecular biological techniques. The efforts to find better delivery systems and better adjuvants accompany the research on vaccines.

**Applications of Recombinant DNA Technology**

- **Polymerase Chain Reaction (PCR)** has a wide range of applications in many disciplines
  - Molecular Biology/Research
  - Diagnostics
  - Genetic Counseling
  - Criminology/Forensics
  - Paternity testing
  - Archeology
  - Food testing

- Evolutionary studies

**Advantages over traditional methodologies**

- Fast and efficient amplification of specific DNA sequences
- No requirement for cloning or subcloning
- Tiny amounts of material are usually sufficient
- Disease diagnoses will be greatly expedited by PCR to identify microorganisms in infected people who would prove falsely negative by other diagnostic procedures

**1. Detection of HIV in T-lymphocytes**

- Serological techniques reguire humoral immune responses to become activated for successful detection of anti-HIV antibodies (seroconversion)
- Acquired immune responses can take 10-14 days before Ab titers reach maximum levels.
- Individual may test negative and transmit HIV unknowingly (False negative)

**2. Human Papilloma Virus (HPV)**

- Causes genital warts and cervical cancer
- Tissue sample from cervix used in pCR reaction
- Treatment can begin earlier
- Acyclovir
- Gancyclovir
-

**Abzyme**

An **abzyme** (from antibody and enzyme), also called *catmab* (from *catalytic monoclonal antibody*), is a monoclonal antibody with catalytic activity. Molecules which are modified to gain new catalytic activity are called synzymes. Abzymes are usually artificial constructs, but are also found in normal humans (anti-vasoactive intestinal peptide autoantibodies) and in patients with autoimmune diseases such as systemic lupus erythematosus, where they can bind to and hydrolyze DNA. Abzymes are potential tools in biotechnology, e.g., to perform specific actions on DNA.

Enzymes function by lowering the activation energy of the transition state, thereby catalyzing the formation of an otherwise less-favorable molecular intermediate between reactants and products. If an antibody is developed to a stable molecule that's similar to an unstable intermediate of another (potentially unrelated) reaction, the developed antibody will enzymatically bind to and stabilize the intermediate state, thus catalyzing the reaction. A new and unique type of enzyme is produced.

***HIV treatment***

In a June 2008 issue of the journal Autoimmunity Reviews, researchers S Planque, Sudhir Paul, Ph.D, and Yasuhiro Nishiyama, Ph.D of the University Of Texas Medical School at Houston announced that they have engineered an abzyme that degrades the superantigenic region of the gp120 CD4 binding site. This is the one part of the HIV virus outer coating that does not change, because it is the attachment point to T lymphocytes, the key cell in cell-mediated immunity. Once infected by HIV, patients produce antibodies to the more changeable parts of the viral coat. The antibodies are ineffective because of the virus' ability to change their coats rapidly. Because this protein gp120 is necessary for the HIV virus to attach, it does not change across different strains and is a point of vulnerability across the entire range of the HIV variant population.

The abzyme does more than bind to the site, it actually destroys the site, rendering the HIV virus inert, and then can attach to other viruses. A single abzyme can destroy thousands of HIV viruses. Human clinical trials will be the next step in producing treatment and perhaps even preventative vaccines and microbicide.

**Application Of PCR Technology To Produce Antibodies And Other Immunological Reagents**

Antibody production tests have traditionally been used to test biological materials for viral contamination.Now molecular biology techniques have emerged as an alternative. The authors compare MAP testing with PCR-based detection methods, focusing on differences in animal

use, laboratory requirements, sample size, and limits of detection.

Recently human antibody genes can easily be amplified by PCR method from lymphocytes. Thus, genetically engineered libraries of human antibodies were prepared by using phage, *E. coli* and animal cells as host cells. It is reported, however, that recombinant *S. cerevisiae* could secret human IgG antibody into fermentation broth but failed to secret human IgM antibody.

We firstly tried to produce a human IgM-type antibody by secretion from recombinant *S. cerevisiae*. The gene of a human anti-exotoxin A antibody (IgM-type), which could be secreted from a human-mouse hybridoma, was used as a model antibody gene. We also propose a new method to obtain a larger size of combinatorial libraries of antibody by use of two kinds of mating types in yeast cells, a-type and alpha-type. By transforming libraries of genes of heavy and light chains to a- and alpha-types respectively, for example, it should be possible to obtain a larger size of libraries of antibodies by mating them. If we use heavy and light chain libraries containing 10n kinds of genes,respectively, a library containing 102n of antibody should theoretically be obtained. The genes of heavy and light chains were amplified by PCR method from the hybridoma and cloned on vectors. We amde a large size of a library and confirmed that *S. cerevisiae* could secret the human-type antibody into broth.

Effective production methods and fermentation conditions for recombinant proteins by secretion form yeast cells were also studied. Secretion of recombinant proteins from *P. pastoris* can be induced with methanol by use of AOX1 promotor. The effects of DO and the methanol concentration of the productivity were studied, and the secrete proteins were characterized by use of anti-peptide antibodies.

**Immuno Therapy With Genetically Engineered Antibodies**

**Genetically Engineered Antibodies**

Expression of antibody heavy- and light-chain genes by transfection permits the production of monoctonal antibodies with improved biological and antigen-binding properties. The immunoglobulin genes are placed in vectors containing a gene for encoding a protein that provides a biochemically selectable function in eukaryotic cells; these vectors are transfected into myeloma and hybridoma cells. Selection of drug-resistant cells permits the efficient isolation of the rare cells that express the transfected DNA. By placing heavy and light chains on plasmids with different selectable markers, one can deliver heavy- and light-chain genes simultaneously to the same cell. The transfected immunoglobulin genes are efficiently expressed and the proteins produced are a faithful mirror of the genes that were introduced. Using the standard techniques of genetic engineering and gene transtection, we can now produce antibodies of widely varying structures, including chimeric antibodies with segments derived from different species. These antibodies provide useful reagents to study structure-function relationships within the antibody molecule. Ultimately it will be possible to produce a new generation of antibody molecules with improved antigenbinding properties and effector functions.

**Examples for Immuno Therapy With Genetically Engineered Antibodies**

**AIM** Recent progress in phage antibody display technology has revolutionized our ability to select and engineer human monoclonal antibodies for therapy. Antibody fragments with desirable specificities are selected from very large libraries consisting of billions of engineered phage particles each expressing an antibody fragment with a unique specificity. Our aim is to exploit subtractive phage selection methods to obtain antibody fragments that selectively bind to tumor cells and tumor-associated endothelial cells. In addition, we have exploited this approach to obtain antibody fragments that bind to disease-specific conformations of a non cell-bound molecule. These antibodies have been engineered into fully human monoclonal antibodies of various isotypes and affinities and tested in tumor models for their therapeutic efficacy. **METHODS:** We have used phage antibody display libraries in combination with flow cytometry to select for phages that bind

to intact leukemic or solid tumor cells or to tumor-associated endothelial cells from freshly obtained tumor samples. Thus, antibodies can be obtained against cells that have not been modified by in vitro cell culture. In addition, the procedure allows the isolation of antibodies against very rare cells present in a heterogeneous mixture. Furthermore, we have used subtraction approaches to select for antibodies that bind to the active but not the native conformation of vitronectin, an extracellular matrix protein involved in various (patho)physiological processes, including cancer. Antibodies were tested for their specificity by extensive flow cytometric and immunohistochemical analysis of normal and tumor tissues and target antigens were identified by conventional methods. Antibody fragments with relevant binding patterns were converted into intact IgG1 or IgA antibodies and analyzed for their functional activities in vitro and in vivo assays. **RESULTS:** Using phage display libraries, we have generated a panel of fully human monoclonal antibodies that have therapeutic potential in the treatment of solid and leukemic tumors. The antibodies bind to tumor-associated antigens expressed by the tumor cells proper, to antigens expressed by tumor-associated endothelial cells or to a tumor-associated form of the extracellular matrix protein vitronectin. At least in some cases, we found that the antibodies did not bind to de novo expressed molecules but rather to tumor-associated conformations or glycosylation forms of molecules also expressed on the membrane of non-tumor cells. Fully-human IgA and IgG monoclonal antibodies had tumor cell killing properties based on the recruitment of immunological effector cells and molecules. In addition, some antibodies displayed additional tumor cell killing properties independent of immunological mechanisms. **CONCLUSIONS:** Phage display libraries are a source of human monoclonal antibodies that bind to tumor-associated molecules and that have potential therapeutic application. By using subtractive phage selection methods, antibodies that detect subtle alterations of molecules can be obtained such as alterations in conformation or glycosylation pattern. These approaches yield targets for tumor therapy that are not uncovered by genomics approaches

**1. Application of genetically-engineered anti-CEA antibodies for potential immunotherapy of colorectal cancer.**

Hitherto anti-CEA monoclonal antibodies (MAbs), normally of mouse origin, have been used primarily for clinical diagnosis of colorectal cancer, either as a tumor marker in serum to monitor tumor recurrence, or latterly as a means to localize in vivo CEA-bearing tumors, and metastases in patients. In vivo diagnosis using mouse anti-CEA MAbs has so far had limited clinical utility because the antibodies elicit a strong anti-mouse immunoglobulin immune response on repeated administration in man. This problem has been addressed by the development of various strategies for "humanization" of mouse anti-CEA MAbs by genetic manipulation of immunoglobulin genes. Such humanized, engineered antibodies markedly attenuate the antigenic response directed against the MAb, such that safe, repeated administration to patients has become feasible. Such humanized anti-CEA antibodies can thus be radioactively-labelled and applied for in vivo monitoring and detection of recurrent malignant disease, or used for therapeutic strategies which similarly take advantage of the ability of the antibodies to target cytotoxic agents selectively to tumor cells. The application of these novel procedures for manipulating MAb structure presents entirely new opportunities for diagnosis and treatment of human colorectal cancer.

## Human Gene Therapy

## Genetically Engineered Antibodies in Gene Transfer and Gene Therapy

**ABSTRACT**

Our ability to produce and engineer human monoclonal antibodies provides a basis for the development of novel therapeutical strategies against a variety of diseases. These strategies not only include improved passive immunotherapy but also more sophisticated antibody-based gene therapies involving gene transfer approaches. Four of the major applications of antibody gene engineering in the field of gene therapy are reviewed here. These are (1) the redefinition of viral vector tropism of infection for better transduction of cells of therapeutical interest, (2) the grafting of new cell recognition activities to effector cells of the immune system to kill cancer and pathogen-infected cells, (3) the inhibition of cellular and viral functions through intracellular expression of

antibody-derived molecules, and (4) the systemic delivery of therapeutic monoclonal antibodies by non-B cells in living organisms.

**Overview summary**

Monoclonal antibodies are potentially useful for the treatment of many diseases. In this context, our ability to construct human monoclonal antibodies has constituted a major advance toward the passive immunization of human beings. Moreover, gene engineering not only permits the cloning of immunoglobulin genes, but also allows modifications of various types. These include improvement of kinetic/thermodynamic properties of antibodies, grafting of additional biological activities, and development of new classes of molecules. This extends considerably the scope of clinical applications of antibodies. Research by numerous groups worldwide indicates that antibody engineering may also prove advantageous in the field of gene therapy. Antibodies and antibody-related molecules may be used either for directing gene transfer vectors oward cells of therapeutic interest or as molecules with intrinsinc therapeutic activity.

**Genetically engineered antibodies for direct antineoplastic treatment and systematic delivery of various therapeutic agents to cancer cells**

Classical antineoplastic therapeutic modalities such as surgery, radiation, and chemotherapy not only fail to cure the great majority of neoplasms, but their employment often leads to severe and debilitating side effects associated with severe neoplasm-related morbidity. Immunotherapy as a fourth modality of anti-cancer therapy has already been proven to be quite effective. The astonishing immunophenotypic (IP) heterogeneity of neoplastic cells, the different cytotoxic activity associated with the moiety linked to given monoclonal antibodies (mAb), and mostly the impressive genetic modulation capabilities of cancer cells still remain as yet unsolved difficulties in the present immunotherapy of human neoplasms. The advances in mAb production have revitalised the initial concept of use of cancer cell specific "magic bullets." Antibodies represent new approaches to anti-cancer therapy: they are neoplastic cell-specific and lethal to neoplastically transformed cells via immune effector mechanisms with no toxicity to normal tissues. They are being observed and developed, adhering to the old prayer: "Destroy the diseased tissues, preserve the normal." Strategies for the employment of antibodies include: 1) immune reaction directed destruction of neoplastic cells; 2) interference with the growth and differentiation of malignant cells; 3) antigen epitope directed transport of anti-cancer agents to neoplastic cells; 4) anti-idiotype tumour vaccines; and 5) development of engineered (humanized) mouse mAbs for anticancer therapy. In addition, a variety of agents (e.g. toxins, radionuclides, chemotherapeutic drugs) have been conjugated to mouse and human mAbs for selective delivery to neoplastic cells.

**UNIT VI CURRENT TOPICS IN IMMUNOLOGY**

**Summary of all sections discussed above**