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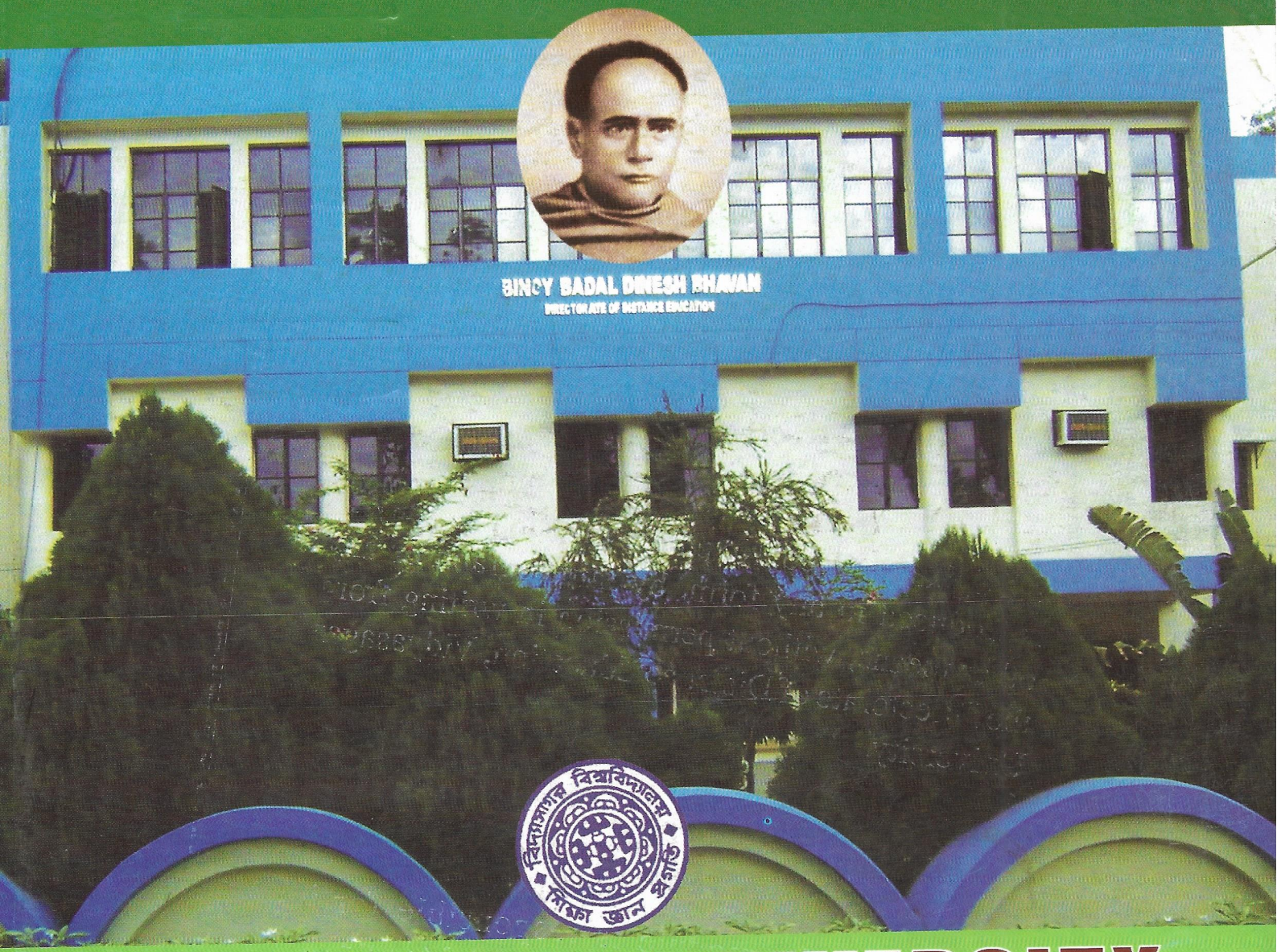

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION

MIDNAPORE - 721 102

M. Sc. in Botany

PART - I • Paper : I

Module No. - 1, 2, 3, 4, 5, 6, 8, 9, 10 & 11

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Contents

Botany

Moudule No. - 1

Part - I Paper - I (1st Half)

Microbiology

Contents

Chapter - 1

Microbiology - A Relevant Science

Contents :

* History of Discovery of Microbial World.
* Scope of Microbiology.
* Areas of Microbiology.
* Microbiology and the origin of life.
* Place of Microorganisms in the living world.
* Haeckle's kingdom - Protista.
* Whittaker's five kingdom concept.
* Kingdom of Procaryotae after Bergey's Manual of systematic Bacteriology.
* Groups of micro - organisms.
* Broad classification of microorganisms.

Microbilogy is the study of living organisms of microscopic size. Roughly speaking, organisms with a diameter of 1 mm or less are microorganisms and fall into the broad domain of microbiology. Microorganisms have a wide taxonomic distribution, they include some metazoas animals, protozoa, many algae and fungi, bacteria and viruses.

Microbilogy is one of the youngest among the biological sciences. Its beginning is in the middle of to Late nineteenth Century. It is contemporaneous with other great discovery in biology such as Natural selection and Mendelian genetics. Now it stand in partnership with older biological sciences - viz Botany and Zoology.

The existence of this microbial world was unknown until the invention of microscopes, optical instrument that serve to magnify objects so small that they can not be clearly seen by the unaided human eye. Invention of microscopes at the beginning of the seventeenth Century opened the biological realm of the very small to systematic scientific exploration.

The discoverer of microbial world was a Dutch merchant Anton Van Leeuwenhock. His lucid reports on the ubiquity of microbes enabled Louis Pasteur 200 years later to discover the involvement of these creatures in fermentation reactions and allowed Robert Koch, Theobold Smith and many others to discover the association of microbes with disease. Koch is remembered for his isolation of the bacteria that cause anthrax and tuberculosis and for the rigid criteria he demanded before a specific bacterium be held as the cause of disease. His important contribution to the creation of the science of microbiology won him the 1905 Nobel Prize.

A chronological Arrangement of Events Important in the History of Microbiology is given in Table 1.

Table 1 : A Chronological Arrangement of Events Important in the History of Microbiology

Era	Investigation	Contribution
1500-1600	Girolame Fracastoro (1483-1553)	Theory that invisible living seeds caused disease
1600-1700	Francesco Redi (1626-1697)	Performed experiments to disprove spontaneous generation.
	Antony van Leeuwenhoek (1632-1723)	First to observe and accurately record and report microorganisms.
1700-1800	John Needham (1713-1781)	Performed experiments, results supported concept of spontaneous generation.
	Lazaro Spallanzani (1729-1799)	Did experiments, results disproved spontaneous generation
	Edward Jenner (1749-1823)	Discovered vaccination for smallpox using cowpox vaccine.
1800-1900	Theodor Schwann (1810-1882)	Performed experiments, results disproved spontaneous generation.
	Franz Schultze (1815-1873)	Performed experiments, results disproved spontaneous generation.
	Justus Von Liebig (1803-1873)	Supported concept of physicochemical theoryof fermentation.
	Jacob Henle (1809-1885)	Established principles for germ theory of disease.
	Oliver Wendell Holmes (1809-1894)	Stressed contagiousness of puerperal fever; that agent was carried from one mother to another by doctors.
	Ignaz Philipp Semmelweis (1918-1865)	Introduced use of antiseptics.
	Louis Pasteur (1822-1895)	Established germ theory of fermentation and germ theory of disease, developed immunization techniques.
	Florence Nightingale (1820-1910)	Organized hospitals which minimized cross-infection.

Era	Investigation	Contribution
	Joseph Lister (1827-1912)	Developed aseptic techniques; isolated bacteria in pure culture.
	Thomas J. Burill (1839-1916)	Discovered bacterial disease of plants.
	John Tyndall (1820-1893)	Developed fractional sterilization to kill spores (Tyndallization)
	Fanny Hesse (1850-1934)	Suggested use of agar as a solidifying material for microbiological media.
	Robert Koch (1843-1910)	Developed pure culture technique and Koch's postulates discovered causative agents of anthrax and tuberculosis.
	Paul Metchnikoff (1845-1916)	Discovered phagocytosis.
	hans Christian Gram (1853-1933)	Develo, ed important procedure for differ ential staining of bacteria, the Gram stain.
	Sergai N. Winogradsky (1856-1953)	Discovered nitrogen-fixing bacteria in soil.
	William Henry Welch (1850-1934)	One of first great American microbiologist, discovered relation of clostridia to gas gangrene.
	Theobald Smith (1859-1934)	Early american microbilogist, discovered tansmission of Texas fever by cattle tick.
1900-1910	Walter Reed (1851-1902)	Reported transmission of yellow fever by mosquito.
	Jules Bordet (1870-1961) and Octave Gengou (1875-1957)	Discovered complement-fixation reaction.
	August Von Wasserman (1866-1925)	Introduced complement-fixation reaction test for syphilis.
	Martinus Willem Beirjerinck (1851-1931)	Utilised principle of enrichment cultures confirmed finding of first virus.
	Frederick W. Twort (1877-1950)	Independently discovered bacteriophages.

Era	Investigation	Contribution
	Felix H. d'Herelle (1873-1949)	Viruses that destroy bacteria.
	Howard T. Ricketts (1871-1920)	Reported Rocky Mountain spotted fever transmitted by wood tick and Mexican typhus transmitted by body louse.

THE SCOPE OF MICROBIOLOGY

Microbilogists have been exploiting the microorganisms in nearly every field of human activity-in industry, in agriculture, in the preparation of food, in connection with problems of shelter or clothing, in the conservation of human and animal health and to combating diseases.

The biological principles can be demonstrated through the study of microbes because they have many characteristics which make them ideal specimens for the investigation of numerous fundamental life processes. This is possible because at the cellular level, many life processes are performed in the same manner whether they be in microbe, mouse or human Microbes can be grown conveniently in test tubes or flasks, thus requiring less space and maintenance is easier compare to higher plants and animals. They grow rapidly and reproduce at an unusually high rate, some species of bacteria undergo almost 100 generations in a 24 h period. The metabolic processes of microorganisms follow patterns that occur amongh higher plants and animals. For example, yeasts utilize glucose in the same manner as cells of mammalian tissue do. The same system of enzymes is present in these diverse organisms. The energy liberated during the breakdown of glucose is trapped and made available for the work to be performed by the cells are same in bacteria, yeasts, protozoa or muscle cells. In fact, the mechanism by which organisms utilize energy is fundamentally the same throughout the biological world. But the source of energy does, of course vary amongh organisms. Plants utilize radiant energy, where as animals require chemical substances as their fuel. In this respect some microorganisms are like plants other like animals, and some have the unique ability of using either radiant energy or chemical energy and thus are like both plants and animals.

Furthermore some microorganisms, the bacteria in particular, are able to utilize a great variety of chemical substances as their energy source - ranging from simple inorganic substances to complex organic substances.

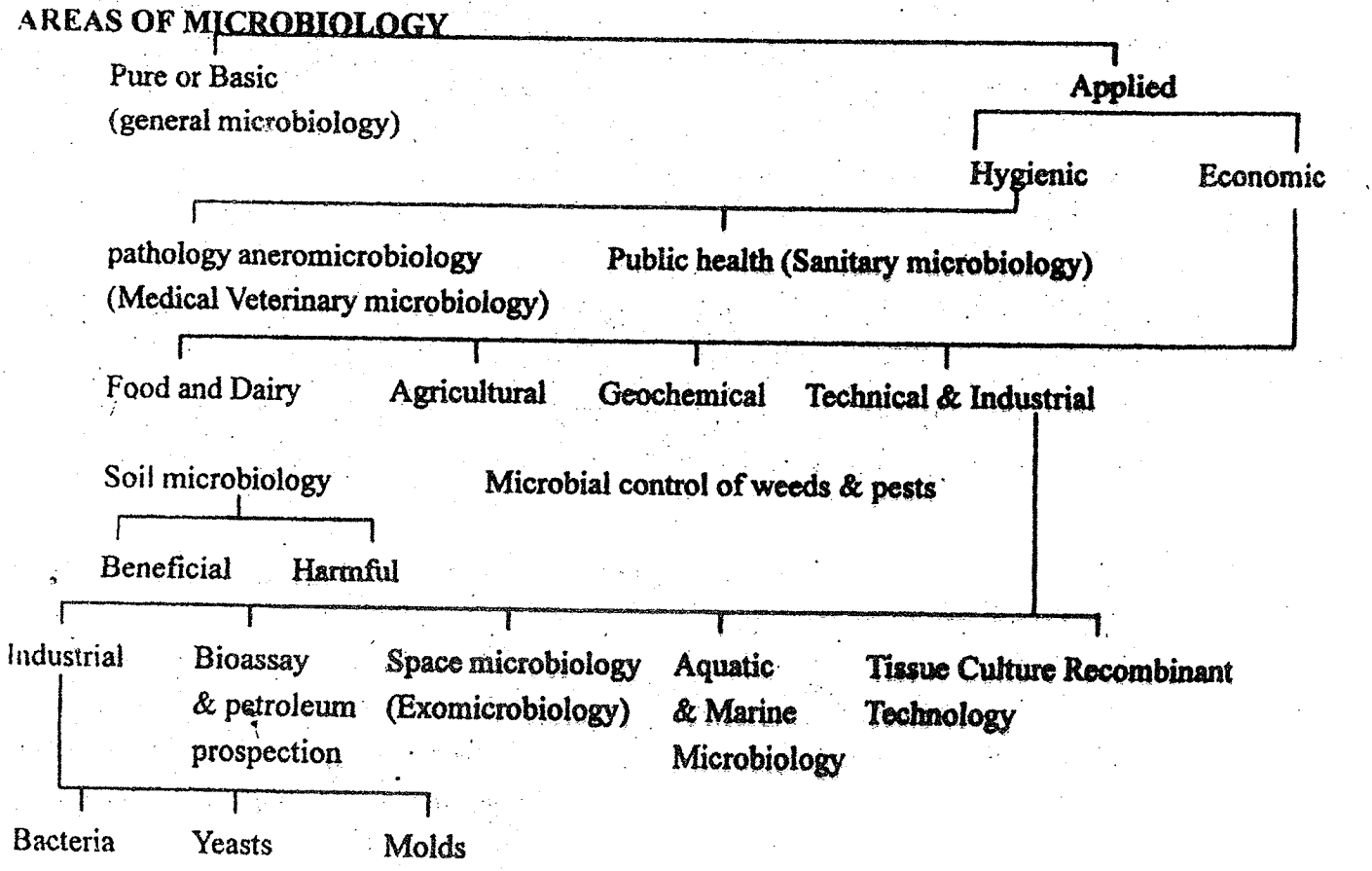
In microbiology we can study organisms in great detail and observe this life processes while they are actively metabolizing growing reproducing aging and dying. By modifying their environment we can alter metabolic activities, regulate growth and even change some details of their genetic pattern - all without destroying the organisms. For example, bacteriophages demonstrate the complete sequence of host-parasite reactions and provide a model by which virus-host-cell reactions can be postulated for infections in higher plants and animals. Thus they elucidate many bilogical phenomena, specially those concerned with genetics.

Micro organisms show considerable versatility in their synthetic capacities. For example- some bacteria are able to utilize atmospheric nitrogen for the synthesis of proteins and other complex organic nitrogenous compounds. So researches are being carried on to exchange the N_2 fixing "nif" gene to higher organisms particularly in rice plant through genetic engineering. If this is successful then the fertilizer problem will be solved.

The most dramatic current development in applied microbiology is the ability to alter an organisms genetic make up, commonly referred to as genetic engineering. The detailed knowledge that has been obtained about the structure and function of DNA, together with the discovery of enzymes that "cut unzip or rebuild" the molecule, has made it possible to alter the DNA structure

For further details consult ch. 3

- Microbiology, by Relczar, Char & Krieg, 5th Edition of microorganisms. New pieces of DNA can be inserted into a DNA molecule in a process called recombination. Thus a micro organism can be engineered, through modification of its DNA, to produce new substances, such as human proteins. Bacteria have been genetically modified to produce human insulin and interferon for example. Genetically engineered micro organisms hold great potential for the production of drugs and vaccines, for improvement of agricultural crops and for other products and processes.



CRITERIA OF PURE MICROBIOLOGY :

1) Morphology.
2) Physiology
3) Taxonomy
4) Genetics
5) Ecological behaviors]

1,2, 3 & 4 utilised for classification and identification of microorganisms

6) Nature of certain unusual type of organisms for example PPLO (Pleuro Pneumonia like organisms or Microplasma Nature of bacteriophages)

For further details consult Ch.3
- Microbiology by Pelczar Chan & Krieg, 5th Edition

Major Fields of Applied microbiology and their areas :

Field	Areas
Medical microbilogy	Causative agents of disease, diagnostic procedures for identifications causative agents, preventive measures
Aeromicron biology Sanitary microbiology	Contaimination & spoilage & disemination of diseases Provision of Potable water for human consumption Disposal of domestic & industrial sewage. (potable i,e, clear clean odorless, virtually tasteless and free from pathogenic microorganisms and deleterious chemicals for drinking and other domestic purposes)
Food & Dairy microbiology	Food preservation and preparation, food borne disease and their prevention food spoilage. [food preparation micro biological method - a) making of wine, beer, bread etc. b) making of fermented milk products - cheese, butter, curd etc. Various practices utilised for food preservation - I. Aseptic Handling II. High temperatures a) Bioling b) Steam under pressure c) pasteurization d) Sterilization e) Aseptic Processing

Field	Areas
	III. Low temperatures a) Refrigeration b) Freezing IV. Dehydration V. Osmotic pressure a) in concentrated sugar b) with brine VI. Chemicals a) Organic acids b) Substances developing during processing (smoking) c) Substances continued by microbial fermentation (acids) VII. Radiation a) Ultraviolet b) Ionizing radiations
	For further details consult Chapter 28 of Microbiology by Pelczar, Chaz & Kreig, 5th ediction.
	Food spoilage :- The major food stuff serve as a good medium for the growth of microorganisms and this growth may cause the food to undergo decomposition & spoilage.
Agricultural microbiology	Microbial flora of soil. Interaction among soil micro organisms & Soil microbiology Biogeochemical Role of soil microorganisms. Biochemical.
	Transformations of Nitrogen & Nitrogen compounds :-
	Proteolysis, Amino acid Degradation, Ammonification Recombinant DNA and Nitrogen fixation Biochemical Transformation of Carbon & Sulphur and other compounds.
Microbial Control of & pests	Biofertilizer. Biopesticides for example (*Bacillus thurengensis Weeds* used for the control of insects]
	For further details consult general Microbiology (5th edition) by Stanier et al. (Chapter 33) & for Soil Microbiology by Pelczar, Char & Kreig.
Geochemical microbiology	Coal, mineral and gas formation, Prospection for deposits of coal, oil and gas, recovery of minerals from low-grade ores.
Exo microbiology	Exploration of life in outer space.

Field	Areas
Industrial Microbiology	
a) Bacteria	i) For the production on industrial chemicals like acetone, butanol. ii) Production of amino acids iii) Production of antibiotics iv) Steroid transformation v) Production of Enzymes vi) Production of vitamins vii) Dextran production viii) Flavoring agents for tea & coffee ix) Manufacture of vinegar x) Retting of fibre xi) Single cell protein (SEP)
	For further reading - Some Indstrial Products produced by bacteria by Pelczar, Char & Kreig, page - 650 & by Mold (Table 29-3) pg. 658 & by Yeasts (29-2) pg. 655. from Pelezar Char Kreig.
Yeasts	i) As a food (SEP) ii) Bread making iii) Preparation of beverages (wine, beer etc.) iv) Misc. chemicals v) Vitamins (Riboflavin) vi) Enzymes (invertase)
Molds	i) Production of organic acids (Citric, gluconic, itaconic, gallic) ii) Enzymes iii) Antibiotics iv) Transformation of steroids.
Bioassay and Petroleum prospection (Analytical microbiology)	i) Petroleum formation ii) Petroleum Exploration iii) Petroleum Recovery iv) Microbiological assays used for the measurement of vitamin, amino acids and antibiotics.
Aquatic microbiology	Water purification microbiological examination Biological degradation of waste, Ecology.
Tissue Culture	Production of monoclonal antibodies.
Recombinant technology	Genetic Engineering of micro organism for industrial purposes.

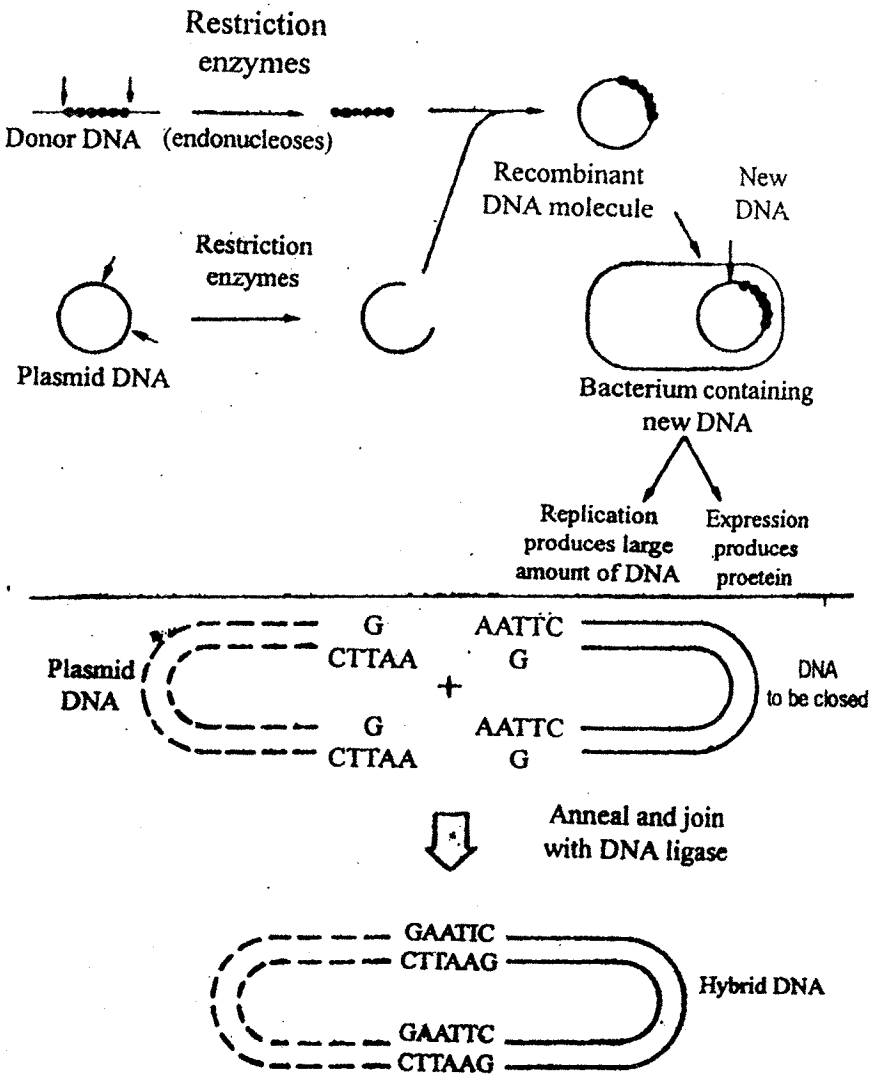


Fig. 1(A) The major steps in producting a "generically engineered" bacterium.

(B) Fragments of donor DNA and Plasmid DNA excised endonucleases and formation of hybrid DNA by joining these fragments using DNA ligase. (Erwin F. Lessel. illustrator.)

Some Industrial products produced by bacteria (Table 1-I-A) by Mold (Table 1-I-B) & by Yeast (Table 1-I-C) is given below :

Table 1-I-A. **Some Industrial Products (Other than Antibiotics) Produced by Bacteria**

Product	Microorganism	Uses
Acetone-butanol	Clostridium acetobutylicum and others	Solvent; chemical manufacturing
2, 3-Butanediol	Bacillus Polymyxa Enterobactor aerogenes	Solvent; humectant; chemical intermediate
Dihydroxyacetone	Gluconobacter suboxydans	Fine chemical

Product	Microorganism	Uses
2-Ketogluconic acid	Pseudomonas spp.	Intermediate for D-arabiascorbic acid
5-Ketogluconic acid	G.suboxydans	Intermediate for tartaric acid
Lactic acid	Lactobacillus delbrueckii L. bulgaricus	Food products; textile and laundry; chemical manufacturing, deliming hides
Bacterial amylase	Bacillus subtils	Modified starches, sizing paper; desizing textiles
Bacterial protease	B. subtilis	Bating-hides; desizing fibers; spot remover, tenderizing meat
Dextran	Leuconstoc mesenteroides	Stabilizer in food products; blood- plasma substitue.
Sorbose	G. suboxydans	Manufacturing of ascorbic acid
Cobatamin (vitamin B_{12})	Streptomyces olivaceus Propionibacterium freudenreichii	Treatment of pernicious anemia; food and feed supplementation
Glutamin acid	Brevibacterium spp.	Food additive
Lysine	Micrococcus glutamicus	Animal-feed additive
Streptokinase-streptodornase	Streptococcus equisimilis	Medical use (dissolving blood clots)
Bioinsecticides	Bacillus thuringiensis Baccilus popilliae	Control of insects
Insulin, interferon, somatostatin (human growth hormone)	Recombinanant DNA Varieties of *E.coli*	Human therapy
Microbial protein (SEP)	Methane-oxidizing bacteria	Food supplement

Table 1-I-B. Some Industrial Products (Other than Antibiotics) Produced by Molds

Product	Microorganism	Uses
Citric acid	Aspergillus niger or Aspergillus Wentii	Food products, medicinal citates; blood for transfusion
Fumaric acid	Rhizopus nigricans	Manufacture of alkali resins, wetting agent.
Gluconic acid	A. niger	Pharmaceutical products, textiles, leather, photography.
Itaconic acid	Aspergillus terreus	Manufacture of alkali resins, wetting agents.
Pectinases, proteases	A. wentii or Aspergillus aureus	Clarifying agents in fruit juice industries.
11-g-Hydroxy-progesterone	Rhizopus arrhizus, R. nigricans, others.	Intermediate for the ghydroxycorticosterone
Gibberellic acid	Fusarium moniliforme	Setting of fruit, seed production
Lactic acid	Rhizopus oryzone	Foods and pharmaceuticals.

Table 1-I-C. Some Commercial Products of Yeast

Product	Microorganism	Uses
Bakers yeast, beer, wine, ale, bread	Saccharomyces cerevisiae	Baking industry; brewing industry
Soy sauce	Saccharomyces rouxir	Food condiment
Sour French bread	Candida milleri	Baking
Commercial alcohol (ethanol)	S. cerevisiae Kluyveromyces fragilis	Fuel; solvent
Riboflavin	Eremothecium ashbyi	Vitamin supplement
Microbial Protein	Candida utils	Animal food supplement (single-cell protein) from paper - pulp waste
	Saccharomycopsis lipolytica	Microbial protein from petroleum products

MICROBIOLOGY AND THE ORIGIN OF LIFE

The time scale of chemical evolution biological evolution and the emergence of microbial life is given in Figure 1A.

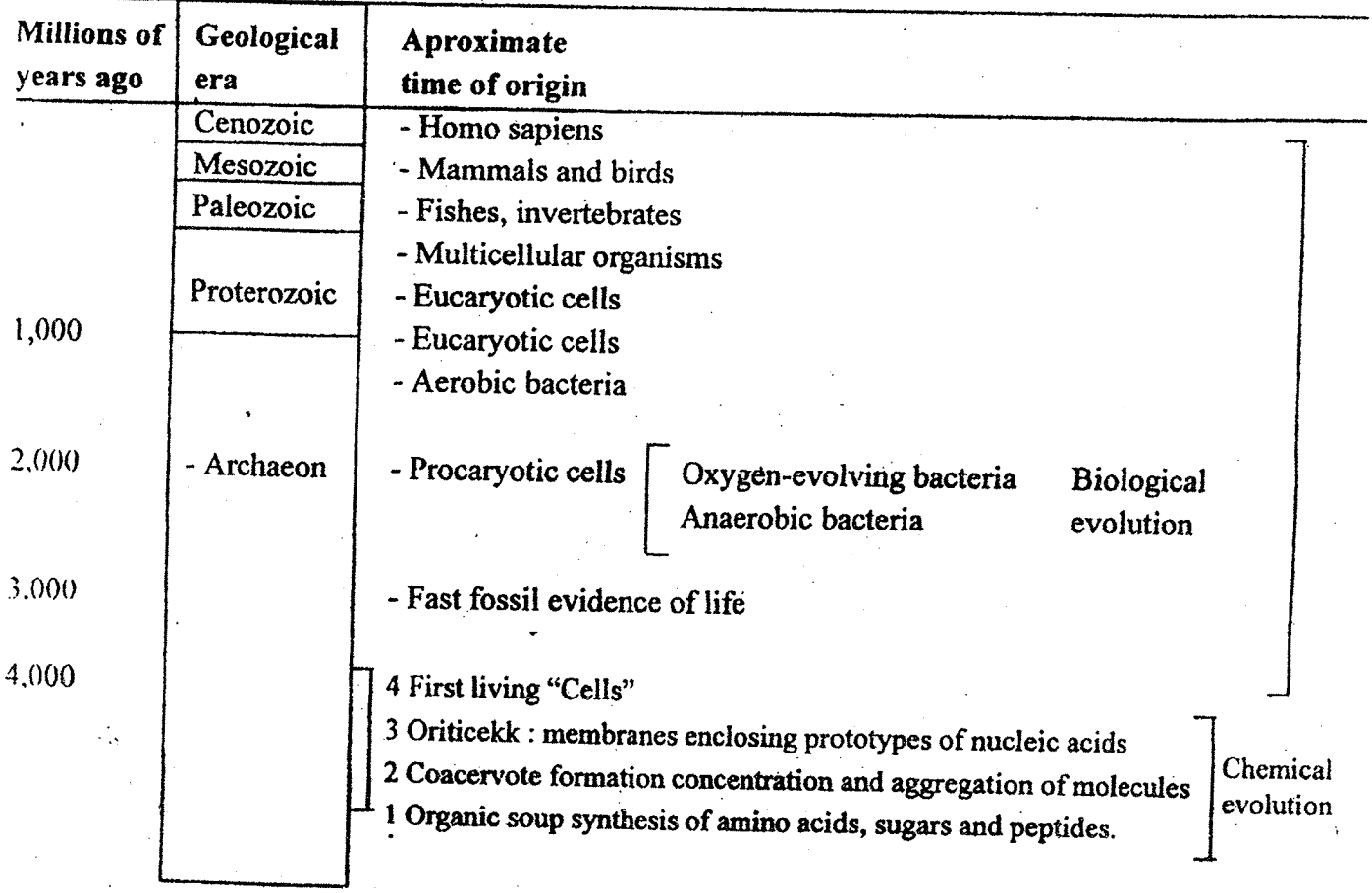


Figure 1-A. Time scale of the chemical evolution, the biological evolution, and the occurrence of microbial life.

PLACE OF MICROORGANISM IN THE LIVING WORLD

In biology as in any other field, classification means the orderly arrangement of units under study into groups of larger units. Present day classification in biology was established by the work of Carulus Linnaeos (1707-1778) a Swedish botanist. His boo on the classification of plants & animals are considered to be the beginning of modern botanical & zoological nomenclature, system of naming plants and animals. Nomenclature in microbiology which came much later, was based on the principles established for the plant and animal kingdoms.

Until the 18th Century, the classification of living organisms placed all organisms into one of two kingdoms, plant and animal. In microbiology we study some organisms that are predominantly plant like others that are animal like and some that share characteristics common to both plants and animals. Since there are organisms that do not fall naturally into either the plant or the animal kingdom, so it was proposed that new kingdoms be established to include those organisms which typically are neither plants nor animals.

HACKLE'S KINGDOM- PROTISTA

One of the earliest proposals was made in 1866 by a German Zoologist E.H. Hackle. He suggested that a third kingdom, Protista be formed to include those unicellular micro organisms that are typically neither plants nor animals. These organisms the protists, include bacteria algae fungi and protozoa (viruses are not considered as they are not cellular organisms).

Bacteria are referred to as lower protists, the others - algae, fungi and protozoa - are called higher protists.

Procaryotic and Eucaryotic protists :-

Hackle's kingdom Protista left some questions unanswered. For example what criteria could be used to distinguish a bacterium from a yeast or certain microscopic algae? Satisfactory criteria were unavailable until the discovery of Electron microscope. In the late 1940's the internal cell structure was revealed through electron microscope. It was discovered that in typical bacteria cell, the nuclear substance was not enclosed by nuclear membrane but in typical algae and fungi cell, the cuclers was enclose in a membrane. This discovery - the absence of membrane bound internal structure in one group of Protists (bacteria) and the presence of membrane-bound structures in all others (fundi, algae, protozoa) - was a discovery of fundamental significance so, these two cell types have been designated as procaryotic and eucaryotic and organisms of each cell type are called Procaryotes and Eucaryotes respectively. Distinguishing features of procaryotic and eucaryotic cells are give in Table - 2.

Table 2. Features Distinguishing Procaryotic from Eucaryotic Cells

Feature	Procaryotic Cells	Eucaryotic Cells
Groups where found as	Bacteria structure	Algae, fungi, protozoa, plants, unit of and animals
Size range of organisms	1-2 by 1-4 mm or less	Greater than 5 mm width or diameter
Structure of nucleus	Not bounded by nuclear membrane; one circular chromosome	Bounded by nuclear membrane; more than one chromosome
	Chromosome does not contain histones no mitotic division	Chromosomes have histones; mitotic nuclear division
	Nucleolus absent; functionally related genes may be clustered	Nucleolus present; functionally related genes not clustered
Sexuality	Zygote nature is merozygotic (partial diploid)	Zygote is diploid
	Cytoplasmic nature and structures	
Ctoplasmic streaming	Absent	Present
Pinocytosis	Absent	Present
Gas vacuoles	Can be present	Absent
Mesosome	Present	Absent
Ribosomes	70S.* distributed in the	80S arrayed on membranes as in endoplasmic reticulum; 70S in mitochondria and chloroplasts
Mitochondria	Absent	Present
Chloroplasts	Absent	May be present
Golgi structures	Absent	Present
Endoplasmic reticulum	Absent	Present
Membrane-bound (true) vacuoles	Absent	Present
Outer cell structures		
Cytoplasmic membranes	Generally do not contain sterols; contain part of respiratory and, in some, photosynthetic machinery	Sterols present; do not carryout respiration and Photo-synthesis
Cell wall	Peptidoglycan (murein or mucopeptide) as component	Absence of peptidoglycan
Locomtor organelles	Simple fibril	Multifibrilled with "9+2" microtubules

Bacteria are procaryotic micro organisms. The eucaryotic micro organisms include the protozoa, fungi and algae (plant & animal cell are also encaryotic) viruses are left out of this scheme of classification.

WATTAKER'S FIVE KNGDOM CONCEPT

A more recent and comprehensive system of classification, the five kingdom system was proposed by R.H. Whittaker (1969). A schematic representation of Whittaker's five-kingdom system is give in Fig.2

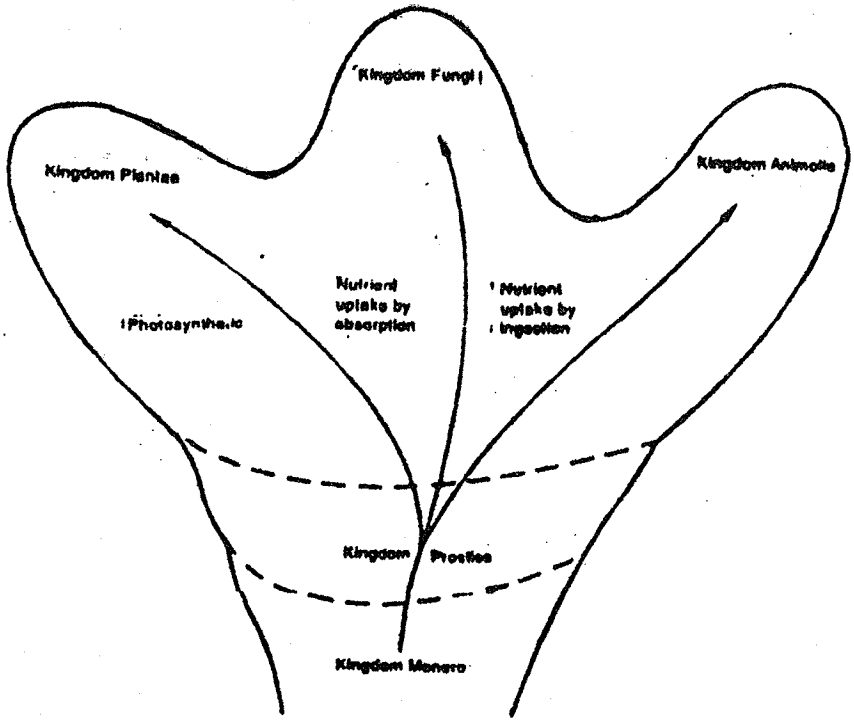


Figure 2 : A simplified schematic representation of Whittaker's five-kingdom system.

(Erwin F. Lessel, illustrator)

This system of classification is based on three levels of cellular organization which evolved to accommodate three principal modes of nutrition, Photosynthetic, absorption and ingestion.

a) Procaryotes are included in the kingdom Monera, they lack the ingestive mode of nutrition (e,g, Bacteria & Cyanobacteria)

b) Unicellular Eucaryotic micro organisms are placed in the kingdom Protista all three nutritional types are represented here (e, g, Microalgae- Photosynthetic, Protozoa - ingestive some other protist - absurptive.

c) Multicellular and multinucleal eucaryotic organisms are placed in teh kingdom plantae (e, g, multicelular green plants and higher algae).

d) Animalia (multicellular animals)

e) Fungi (multicellular higher fungi)

Micro-organisms are found in three of the five kingdoms : Monera (bacteria & cyanobacteria) Protista (microalgae & protozoa) and Fungi (Yeasts & Molds).

KINGDOM PROCARYOTAE AFTER BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY:

Bergey's Manual of systematic Bacteriology places all bacteria in the kingdom procaryotae which in turn divided into 4 division as follows :

Div. I. Gracilicutes - Complex cell wall structure characteristic of Gram negative bacteria.

Div. II. Firmicutes - Cell was structure characteristic of Gram negative bacteria.

Div. - III. Tenericutes - Lacks cell wall

Div. - IV. Mendosicutes -

Show evidence of an earlier phytogenetic origin than those bacteria included in Division 1 and 2.

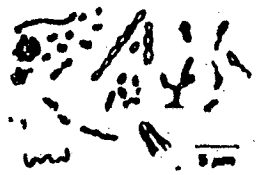
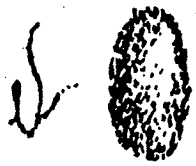
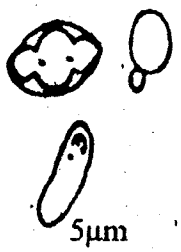
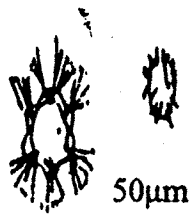
A comparable manual of classification does not exist in for fungi, algae or protozoa. However schemes of classification for each group that have wide acceptance & usage. An international system for classification and nomenclature of viruses is in the process of development.

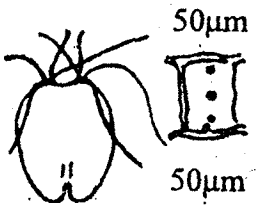
GROUPS OF MICRO ORGANISMS

The major groups of Protists are Algae, Fungi, Bacteria, Protozoa & viruses. Although viruses are not protist or cellular organisms they are included for two reasons :

1) the techniques used to study viruses are microbiological in nature.

2) viruses are causative agents of diseases, hece diagnostic procedures for their identification are employed in the clinical microbiological laboratory as well as the plant pathology laboratory. Some characteristics of major groups of micro organisms are given in Table - 3.

Group	Morphology	Size	Important Characteristics	Practical Significance
Bacteria		Typical 0.5-1.5μm	Procaryotic; Unicellular simple internal structure grow on artificial laboratory media: Reproduction asexual, Characteristically by simple cell division.	Some causes disease; some perform important role in natural cycling of elements which contributes to soil fertility; used in industry for manufacture of valuable compounds, some spoil foods and some make foods.
Viruses	 100nm	Range : 0.015-0.2μm	Do not grow on artificial laboratory media-require living cells within which they are reproduced; all are obligate parasites electron microscope required to see viruses.	Cause disease in humans, other animals, plants; also infect microorganisms.
Fungi :Yeasts	 5μm	Range:	Eucaryotic; unicellular; laboratory cultivation much like that of bacteria; reproduction by asexual cell division, budding, or sexual processes.	Production of alcoholic beverages also used as food supplement; some cause disease.
Fungi:Moulds	 20μm	Range : 2.00-10.0μm	Eucaryotic; multicellular, with many distinctive structural features; cultivated in laboratory much like bacteria reproduction by asexual and sexual and sexual processes.	Responsible for decomposition (deterioration) of many materials; useful for industrial production of many chemicals, including penicillin, cause diseases of humans, other animal and plants.
Protozoa	 50μm	Range 2.0-200μm	Eucaryotic; unicellular, some cultivated in laboratory much like bacteria; some are intracellular parasites; reproduction by asexual and sexual processes.	Food of aquatic animals; some cause disease.

Group	Morphology	Size	Important Characteristics	Practical Significance
Algae		Range1.0µm	Eucaryotic; unicellular and multicellular; most occur in aquatic environments; contains chlorophyll and are photosynthetic; reproduction by asexual and sexual processes.	Important to the production of food in aquatic environments; used as food supplement and in pharmaceutical preparations source of agar for microbiological media; some produce toxic substances.

BROAD CLASSIFICATION OF MICRO ORGANISMS (BACTERIA) :-

The most widely used reference for bacterial classification is Bergey's Manual systematic Bacteriology, now published in four volumes.

Volume -1 : includes ordinary Gram negative chemoheterotrophic eubacteria. The major sections are listed below in **Table -4.**

Table 4. **Gram Negative Bacteria Included in Bergey's Manual, Volume 1**

Section	Other Major Characteristics
THE SPIROCHETES	Flexible, helical, have periplasmic flagella; saprophytes or parasites
AEROBIC/ MICROAEROPHILIC, MOTILE, HELICAL/ VIBRIOID, GRAM - NEGATIVE BACTERIA	Rigid, motile by polar flagella; oxidative type of metabolism; saprophytes or parasites
NONMOTILE (OR RARELY MOTILE) GRAM-NEGATIVE CURVED BACTERIA	Rigid; curved, ring-shaped, or helical cells lacking flagella; saprophytes or parasites
AEROBIC GRAM NEGATIVE RODS AND COCCI	Rigid; straight or slightly curved (but not helical) rods, and cocci; oxidative type of metabolism; saprophytes and parasites
FACULTATIVELY ANAEROBIC GRAM-NEGATIVE RODS	Rigid; straight or curved rods have both and oxidative and a fermentative type of metabolism; saprophytes and parasities
ANAEROBIC GRAM-NEGATIVE STRAIGHT, CURVED AND HELICAL RODS	Rigid; obtain energy by fermentation or by an anaerobic that does not use sulfur compounds as electron acceptors; parasiters.

Section	Other Major Characteristics
DISSIMILATORY SULFATE-OR SULFUR-REDUCING BACTERIA	Rigid; anaerobic use sulfur compounds as electron acceptors; saprophytes and parasites.
ANAEROBIC GRAM-NEGATIVE COCCI THE RICKETTSIAS AND CHLAMYDIAS	Rigid; nonmotile; fermentative; parasites Rigid; tinny cells; intracellular parasites of humans, other animals, and arthopods, can be isolated and cultivated in host cells and sometimes on culture media.
THE MYCOPLASMIC	Soft and plastic; nonmotile; lack cell walls; parasites and saprophytes
ENDOSYMBIONTS	Bacteria-like forms that are obligate parasites of protozoa, anthropods, or other hosts, often benficial to their.

For studying importance of the different members belongs to different sections. Further reading microbiology Plezar, Chan & King Ch. 13

Volume -2. includes ordinary Gram positive Bacteria. The major sections of volume 2 are listed below in Table - 5.

Table 5. **Gram positive Bacteria Included in Bergey's Manual, Volume- 2**

Section	Other Major Characteristics
GRAM - POSITIVE COCCI	May have a strictly respiratory type of metabolism, a respiratory plus a fermentative metabolism, or a strictly fermentative metabolism; in the latter category they may be able to grow in air (aerotolerant), or they may be anaerobic.
ENDOSPORE-FORMING GRAM-POSITIVE BACTERIA	Mainly rod-shaped, but some are cocci; range from aerobic to facultatively anaerobic to anaerobic; most of the anaerobic libe by fermentation, but some respire anaerobically with sulfate.
NONSPOREFORMING GRAM-POSITIVE RODS OF REGULAR SHAPE	The cells have a uniform appearance without swellings, branching or other types of variation; some occur in characteristic trichomes; aerobes, facultative anaerobes, or aerotolerant anaerobes are included.

Section	Other Major Characteristics
NONSPOREFORMING GRAM-POSITIVE RODS OF REGULAR SHAPE	The cells may exhibit swelling, Y or V shapes, rod/ coccus cycles, or other deviations from a uniform morphology; some are filamentous during at least some stage or their growth; aerobic facultatively anaeerobic, and anaerobic genera are included.
MYCOBACTERIA	Aerobic, slightly curved or straight rods which sometimes show branching, stain acid-fast.
NOCARDIOFORMS	Aerobic organisms that tend to form a substrate mycelium and sometimes and aerial mycellium, the hyphae fragment into rod-shaped or cocoid elecments; conditiospores may develop from the aerial hyphae Volume - 3. includes Bacteria with unusual proterties.

Volume 3 includes Bacteria with unusual properties.
The major sections of volume 3 are listed in Table - 6.

Table 6 : **Bacteria Included in Bergey's Manual, Volume 3.**

Section	Other Major Characteristics
ANOSYGENIC PHOTOTROPHIC BACTERIA	Gram-negative bacteria that contain bacteriochlorophyll and can use light as an energy cource, the organisms are anaerobic and do not evolve O_2 during photosynthesis.
OXYGENIC PHOTOTROPHIC BACTERIA	Bacteria contain chlorophyll, can use light as an energy source, and evolve O_2 in a manner similar to that of green plants; the group includes the cyanobacteria ("blue - green algae").
GLIDING, FRUITING BACTERIA	Gram-negative nonphototrophic bacteria that lack flagella, yet can glide across solid surfaces; they have a complex life cycle in which the cells swarm together in massage and form fruiting bodies.
GLIDING NONFRUITING BACTERIA	Gram-negative nonphototrophic rods, filaments, or multicellular-trichomes that guide across solid surfaces; fruiting bodies are not produced.
THE SHEATHED BACTERIA	Gram-negative nonphototrophic bacteria that form an external sheath that covers the chains or trichomes
BUDDING AND/ OR APPENDAGED BACTERIA	Gram-negative nonphototrophic bacteria that reproduce asymmetrically by budding and or form prosthecae or stalks.

Section	Other Major Characteristics
CHEMOLITHOTROPHIC BACTERIA	Gram-negative nonphottrophic bacteria that obtain energy for carbon dioxide fixation from the oxidation of ammonia, nitrate, reduced sulfur compounds, or ferrous iron.
ARCHAEOBACTERIA	Gram-prositive or Gram-negative bacteria that are phylogenetically distinct from eubacteria; some produce methane gas; some require unusually high levels of NaCI for growthl others are distinguished by their ability to grow at a low pH and a high temperature.

For further study consult Ch. 15 of Microbiology by Pelzar, Chan & Krieg 5th edn.

Volume - 4. includes Gram positive Filamentous Bacteria.
The major sections of volume 4 are given below in table - 7.

Table - 7 : **Gram - Positive Filamentous Bacteria included in Bergey's Mannual, Volume 4.**

Section	Some Major Characteristics
FILAMENTOUS BACTERIA THAT DIVIDE IN MORE THAN ONE PLANE	The hyphae divide not only transversely but also longitudinally to produce clusters or packets of cells or spores; cell-wass type III*; soil organisms, animal pathogens, and symbiotic nitrogen-fixters are represented.
FILAMENTOUS BACTERIA THAT FORM TRUE SPORANGIA	Harmless soil and water organisms whose hypdivide in a single plane; the spores are fromed within special sacs; cell-wall types II or III*.
STREPTOMYCES AND SIMILAR GENERA	The hyphae divide in a single plane; long chains of condiospores are formed at the tips of sporogenic hyphae; the organisms are mainly harmless soil organisms that are noted for production of antibiotics; a few are human or plant pathogens; cell wall type I*.
ADDITIONAL FILAMENTOUS BACTERIA HAVING UNCER-TAIN TAXONOMIC PLACEMENT	A heterogenours collection of organisms whose relationships to the major groups of Gram-positive filamentous bacteria is not yet agreed upon; some have remarkable morphological or physiological properties; a few organisms are pathogenic for humans; the cell-wall types vary.

For further sutdy consult Ch. 16 of Plezar's Microbiology 5th edn.

Chapter 2

Nutrition of micro organisms :-

* The principles of Microbial nutrition
* Nutritional requirements.
* Nutritional Types of Bacteria.
* The construction of culture media
* Types of media.
* Enrichment culture Technique.
* Preparation of media.
* Physical conditions required for growth- Temperature, Gases requirements.
* Cultivation of Aerobic & Anaerobic bacteria.
* Choice of media and conditions of Incubation.

PRINCIPLES OF MICROBIAL NUTRITION :-

All forms of life, from microorganisms to human beings, share certain nutritional requirements for growth and normal functioning. In Natural habitats micro organisms obtained these nutrients from the environments. Where as for cultivation of microorganisms in the laboratory, culture media is required. Therefore, a culture medium must contain all necessary nutrients. Literally thousands of different media have been proposed for their cultivation. The various components used for designing a culture medium should be based on scientific principles. The principles of nutrition is based on the chemical composition of cells. These composition is constant throughout the living world and it also give indication about the major nutritional requirements for their growth.

NUTRITIONAL REQUIREMENTS :-

* Water (the major essential nutrient as because it accounts for 80-90% of the total weight of cell).
* Source of energy (for growth).
* Source of electron (for their metabolism).
* Carbon source (for synthesizing cell components)
* Nitrogen source (for cell component).
* Oxygen, sulfur & phosphorus (for cell component).
* Metal ions like K^+, Ca^{2+}, Mg^{2+}, Fe^{2+} (for normal growth).

Other metal ions like Zn^{2+}, Cu^{2+}, Mn^{2+}, Mo^{6+}, Ni^{2+}, B^{3+}, Co^{2+} at very low concentration (Trace elements) (for growth) Most bacteria do not require Na but marine bacteria & cyanobacteria & Photosynthetic bacteria do require 12 to 15% Nacl.

* Growth factors

Vitamin & Vitamin like compounds (function as coenzyme or building block of coenzymes)

Amino acids (constituents of Protein).

Purines and pyrimidines (constituents of nucleic acids).

NUTRITIONAL TYPES OF BACTERIA ARE CATAGORISED IN THE FOLLOWING HEADS:-

I. On the basis of energy requirement

a) phototrphs (use radiant energy)

b) chemotrophs (use chemical compound for energy)

II. On the basis of electron requirement :-

a) lithotrophs

(reduced inorganic compound as electron donors)

Photolithotroph

Chemolithotrophs

b) organotrophs

(organic compounds as electron donors)

Photo organotrophs chemo organotrophs

III. On the basis of Carbon requirement :-

a) autotrophs

(CO_2 - major or sole source of carbon)

b) Heterotrophs

organic compounds - as carbon source.

Nutritional characterization of Bacteria is given in Table - 8.

Table 8 : **Nutritional Characterization of Bacteria**

	Energy		**Electron Donor**		**Carbon for Assimilation**	
Bacteria	***Phototropic***	***Chemotropic***	***Lithotropic***	***Organotropic***	***Autotropic***	***Heterotropic***
Chromatium okenii	+		+		+	
Rhodospirillum rubrum						
(anaerobic conditions)	+				+	+
(aerobic conditions)		+		+		
Nitrosomonas europaea	+	+		+		
Desulfovibrio desulfuricans		+	+			+
Pseudomonas pseudoflava						
(H_2 supplied)		+	+		+	
(no H_2 supplied)		+		+		+
Escherichia coli		+		+		+

THE CONSTRUCTION OF CULTURE MEDIA :-

In constructing a culture medium for any microorganism the primary goal is to provide a balanced mixture of the required nutrients, a concentrations that will permit good growth.

For the formulation of media 1st thing is mineral base which provides all those nutrients that can be supplied to any organism in inorganic form. This base can then be supplemented as required with a carbon source, an energy source, and any required growth factors. These supplements will, of course, vary with nutritional properties of the particular organism that one wishes to grow.

A medium composed entirely of known chemical compounds is termed a synthetic medium or chemically defined medum. A medium composed of ingredients complex medium.

Table 9 . **Four Media of Increasing Complexity**

Common Ingredients	**Additional Ingredients**			
	MEDIUM1	**MEDIUM2**	**MEDIUM3**	**MEDIUM4**
Water, 1 liter K_2HPO_4, 1g $MgSO_4$. $7H_2O$, 10mg $FeSO_4.7H_2O$, 10mg $CaCL_2$, 10 mg Trace elements (Mn, Mo, Cu, Co, Zn) as inorganic salts 0.02, 0.5 mg of each.	NH_4 Cl 1g	Glucose 5g NH_4cl, 1g	Glucose 5g NH_4cl, 1g 0.1 mg	Glucose 5g Yeast extract

For the three media i.e., Medium 1,2,3 so far described the chemical nature of every ingredient is known, these are good example of synthetic media. Medium 4 is a complex medium in which NH_4 CL & nicotinic acid of medium 3 have been replaced by a nutrient of unknown composition yeast extract at a concentration 5g/lit.

Examples of relatively simple liquid and solid media that support the growth of many common heterotrophs are nutrient broth and nutrient agar (Table - 10).

Table 10 **Composition of Nutrient Broth & Nutrient agar medium :-**

Nutrient Broth

Beef extract - 5g

Peptone - 5g

Water - 1000 ml

Nutrient Agar

Beef extract - 3g.
Peptone - 5g
Agar - 15 g
Water - 1000 ml

The addition of Yeast extract to each of these formulas improves the nutrient quality, since yeast extract contains several of the B-vitamins & other growth promoting substances. Addition of Yeast extract, a nutrient of unknown chemical composition, to the medium formulates complex medium, other complex supplements such as bovine remen fluid, animal blood, blood serum of extracts of plant and animal tissues may be required for the cultivations of certain tastidious heterotrophs.

Characteristics of several complex materials used an ingredients of media. Table - 11.

Table 11 : **Characteristics of several Complex Materials used as Ingredients of Media.**

Raw Material	Characteristic	Nutritional Value
Beef extract	An queous extract of lean beef tissue concentrated to paste.	Contains the water soluble substances of animal tissue, which include carbohydrates, organic nitrogen compounds, water soluble vitamins and salts.
Peptone	The product resulting from the digestion of protenaceous materials, e.g., meat, casein, and gelatinl digestion of the protein material is accomplished with acids or enzymes; many different peptones (depending upon the protein used and the method of digestion) are available for use in bacteriological media; peptones differ in their ability to support growth of bacteria.	Principle soruce organic nitrogen; may also contain some vitamins and sometimes carbohydrates, depending upon the kind of proteinaceous material digested.
Agar	A complex carbohydrate obtained from certain marine algae; processed to remove extraneous substances.	Used as a solidification agent for media; agar dissolved in aqueous solutions, gels when the temperature is reduced below 45°C; agar not considered a source of nutrient to the bacteria.
Yeast extract	An aqueous extract of Yeast cells, commercially available as a powder.	A very rich source of the B vitamins; also contains organic nitrogen and carbon compounds.

The control of PH :-

Although a given medium may be suitable for the initiation of growth, the subsequent development of a bacterial population may be severely limited by chemical changes that are brought about by the growth and metabolism of the organism themselves. For example, in glucose containing media organic acids that may be produced as a result of fermentation may become inhibitory to growth. In contrast the decomposition of proteins & amino acids may also make a medium alkaline as a result of ammonia production.

To prevent excessive changes in hydrogen ion concentration either fuffers or insoluble carbonates are often added to the medium.

The phosphate buffers, which consists of mixture of monohydrogen and dihydrogen phosphates (e.g. K_2HPO_4 and KH_2PO_4) are the most useful ones.

The phosphates are used widely in the preparation of media because they are the only inorganic agents that buffer in the physiologically important range around neutrality and that are relatively non toxic to microorganism. In addition they provide a source of phosphorus, which is essential element for growth.

The avoidance of Mineral precipitates :-

Chelating Agents

A trouble some problem often encountered in the preparation of synthetic media is the formation of a precipitate upon sterilization, particularly if the medium has a relatively high phosphates and certain cations, particularly calcium and iron. Although it usually does not affect the nutrient value of the medium, it may make the observation or quantitation of microbial growth difficult. The problem can be avoided by sterilizing separately the calcium and iron slats in concentrated solution and adding them to the sterilized and cooled medium. alternatively addition of .01% chelating agent like EDTA (ellylene diaminete traacete acid), which will form a soluble complex with these metals and thus prevent them form forming an insoluble complex with phosphates.

The Control of oxygen concentration:-

Oxygen is an essential nutrient for the obligately aerobic bacteria. In unshaken liquid cultures growth usually occurs at the surface. Below the surface, howver, conditions became anaerobic and growth is impossible. To obtain large population in liquid cultures, it is therefore necessary to aerate the medium. Another method of aeration is the continuous pasrage of a steam of sterile air through a culture.

The provision of Light :

For the cultivation of phototrophic micro organisms (algae, photosynthetic bacteria) light is a

requirement. Direct exposure to sunlight should be avoided, because the intensity may be too high, and the temperature may rise to a point where growth is prevented.

Types of media :-

Selection media : These media provide nutrients that enhance the growth and predominance of a particular type of bacterium and do not enhance other types of organisms that may be present. For instance, a medium in which cellulose is the only carbon source with specifically select for or enrich the growth of cellulose utilizing organisms when it is inoculated with a soil sample containing may kinds of bacteria.

Differential media :

Certain reagents of supplements, when incorporated into culture media may allow diferentiation of various kinds of bacteria. For example, it a mixture of bacteria is inoculated into a blood containing agar medium (blood agar) some of the acteria may hemolyze (destroy) the RBC others do not. Thus one can distinguish between demolytic and non hemolytic bacteria on the same medium.

For Assay Media, Media for Enumeration of Bacteria Media for characterization of bacteria, Maintenance media solid and semi solid media. Consult Pelzar's Microbiology

ENRICHMENT CULTURE TECHNIQUE :-

It is possible to design growth media that will favour the growth of particular microorganism based on their nutritional requirements. This is the basis of the Enrichment Culture Technique.

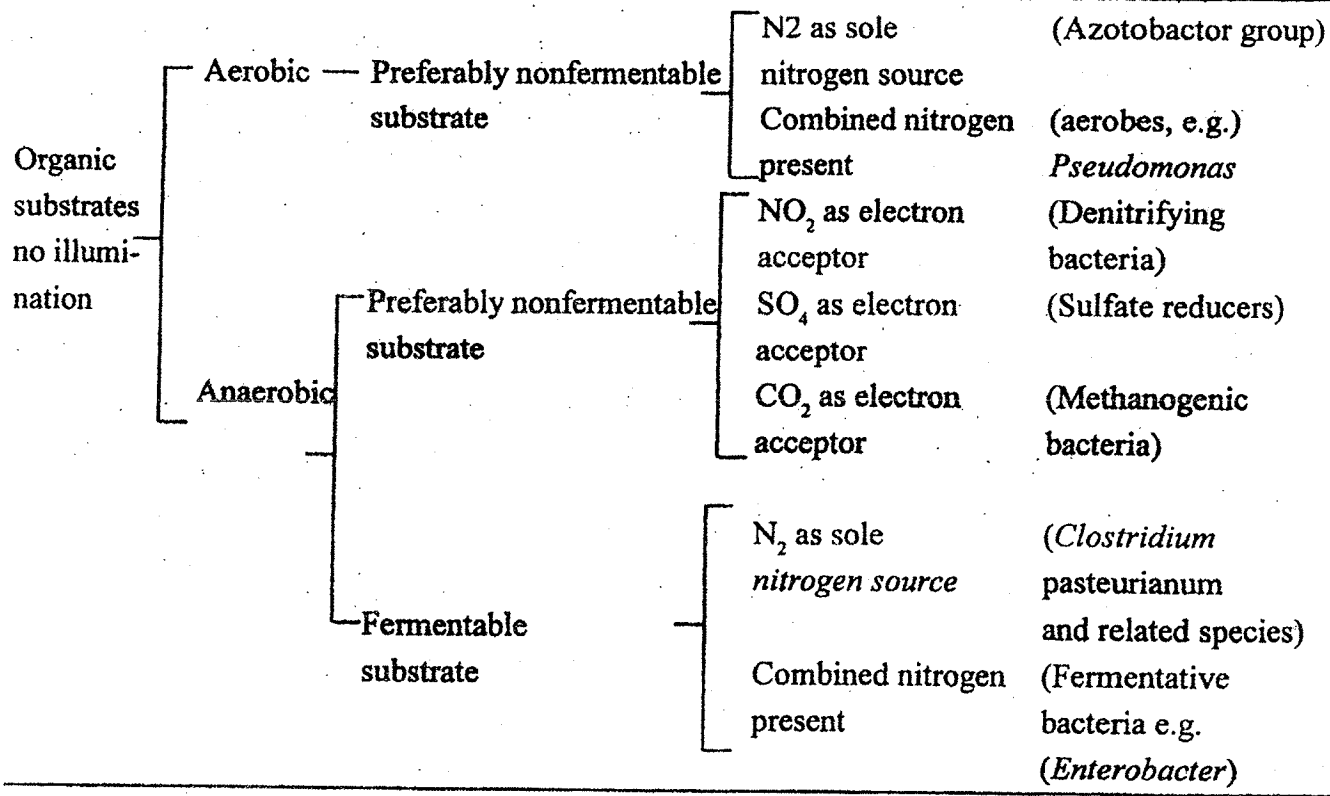
The selectivity of an enrichment culture is not determined solely by the chemical composition of the medium used. The outcome of enrichment in a medium can be significantly modified by variation of such other factors as temperature, PH, ionic strength, illumination, aeratic or source of inoculum.

In the isolation of endospore-forming bacteria, competition from nonsporalating bacteria, can be largely eliminated by a pretreatment of the inoculum. Pasteurization of the inoculum, involving brief exposure to a high temperature (2-5 minutes of 80^0C) will destroy most vegetative cells, leaving the much more heat-resistant spores relatively unaffected.

Synthetic Enrichment media for chemoheterotrophs.

Table 12

Primary Environmental Factors That Determine the OUtcome of Enrichment Procedures for Chemoheterophic bacteria with the Use of Synthetic Media



The Enrichment of chemoautotrophic & photosynthetic organisms.

Table 13

Primary Environmental Factors That Determine the Outcome of Enrichment Procedures for Some Chemoautotrophic Bateria

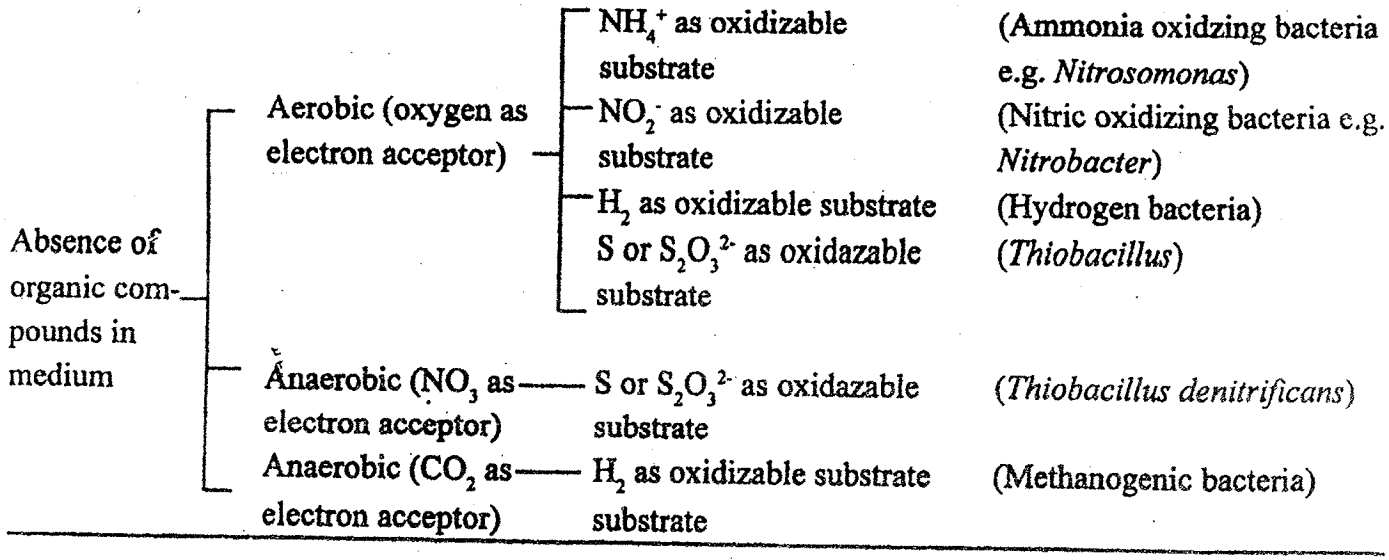
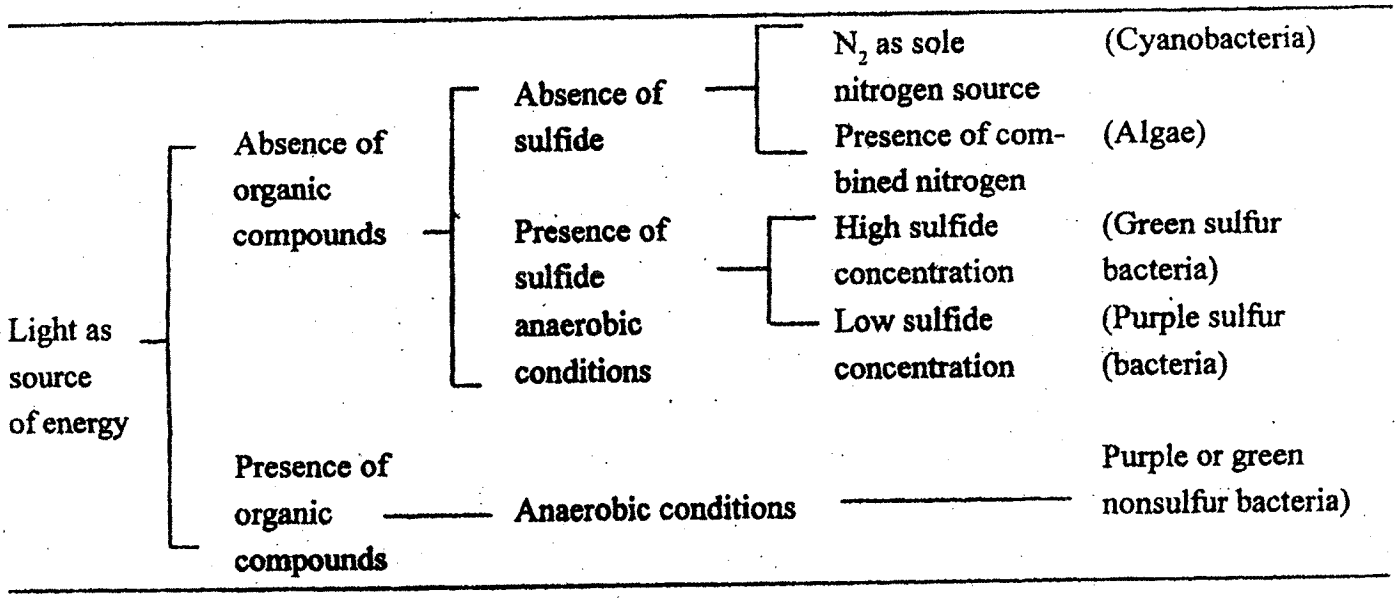


Table 14
Primary Environmental Factors That Determine Outcome of Enrichment Procedures for Photosynthetic Microorganisms



PREPARATION OF MEDIA :-

The preparation of bacteriological media usually involves the following steps :-

1. Each ingredient is dissolved in the appropriate volume of distilled water.
2. The PH of the fluid medium is adjusted if necessary.
3. It a solid medium is designed, agar is added and the medium is boiled to disslove the agar, (silica gel is some times used as an inorganic solidfying agent for autotrophic bacteria).
4. The medium is dispensed in to tubes or flasks.
5. The medium is sterilized generally by auto claving. Some media that are heat-labile are sterilized by filtration.

PHYSICAL CONDITION REQUIRED FOR GROWTH :-

In addition to the proper nutrients, for the cultivation of bacteria, it is also necessary to know the physical environment in which the organisms will grow best. They exhibit diverse responses to physical conditions such as temperature gaseous conditions and PH.

Temperature :-

Since all processes of growth are dependent on chemical reactions and since the rates of these reactions are influence by temperature, the pattern of bacterial growth can be profoundly influenced by

this condition. The temperature that allows for most rapid growth during a short period of time (12-24th) is known as optimum growth temperature. The maximum temperature at which growth occurs is usually quite close to the optimum temperature, where as minimum temperature for growth is usually much lower than the optimum.

On the basis of their temperature relationships bacteria are divided into three main groups :-

1. Psychrophiles : They are able to grown at 0°C or lower, though they are grow best at higher temperatures. It can grow at 0°C but have an optimum temperature of 15°C or lower and a maximum temperature of about 20°C psychrotroph or Facultative psychrophile are those organisms able to grow at 0°C but they range of about 20°C to 30°C. During isolation of strict psychrophles, (for example, Antarctic soil sample) it is usually necessary to maintain the source sample at cold temperatures from the time they are collected and also to chill all media before attempting isolation. The reason behinds it is that the strict psychrophiles usually die if they are even temporarily exposed to room temperature. Even at optimum growth temperature it often takes two of three weeks for colonies of phychrophiles to develop.

The physiological factors responsible for the low temperature maxima for stict psychrophiles are not entirely clear, but some factors that have been implicated are heat instability of ribosomes and various enzymes increased leakage of cell components and impaired transport of nutrients above the maximum temperature.

Mesophiles grow best within a temperature range approximately 25° to 40°C for example, all bacteria that are pathogenic for humans and warm blooded animals are mesophiles (grow best at 37°C).

Thermophiles grow best at temperatures above 45°C. the grow the range of many thermophiles extends into mesophilic region, these species are designated facultative thermophiles other thermophiles can not grow in the mesophilic range, these are termed true thermophiles obligate thermophiles or stenottermophiles.

Factors that are responsible for the growth at high temperature are an increased thermal stability of ribonsomes, membranes, and various enzymes hoss of the fluidity that exists with in the lipid bilayer of the cytoplasmic membrane may be factor governing the maximum temperatures.

Gaseous requirements :-

The principal gases that affect bacterial growth are oxygen and CO_2 Bacteria display such a wide variety of convenient to divide them into four groups on the following bases.

1. Aerobic bacteria required oxygen for growth and can grow when incubated in an air atmosphere (i, e, 21% O_2).

2. Anaerobic bacteria do not use oxygen to obtain energy, moreover oxygen is toxic for them and

they cannot grow when incubated in an air atmosphere. Some can tolerate low levels of oxygen (non stricngentor tolerant anaerobes) but others (stringent or strict anaerobes) can not tolerate even low levels and may upon brief exposure to air.

3. Facultative anaerobic bacteria do not require oxygen for growth, although they may use it for energy production if it is available. They are not inhibited by oxygen and usually grow as well under an air atmosphere as they do in the absence of oxygen.

4. Microaerophilic bacteria require low levels of oxygen for growth but cannot to create the level of oxygen present in an air atmosphere. **Fig. 3.**

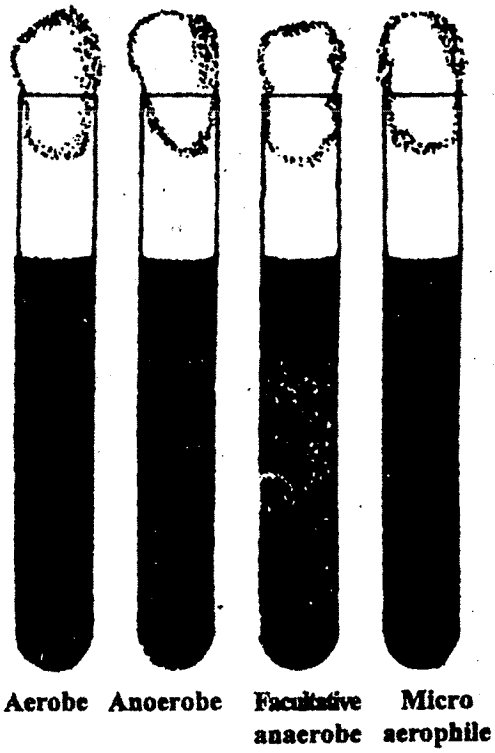


Figure 3. *Scheatic illustration of the growth of bacteria in deep agar tubes, showing differences in response to atmospheric oxygen.*

Oxygen Toxicity :-

O_2 is both beneficial and poisonours to living organisms. It is beneficial because its strong oxidizing ability makes it an excellent terminal electron acceptor for the energy yielding process known as respiration. However O_2 is also a toxic substance. Aerobic and facultative organisms have developed protective mechanism that greatly mitigate this toxicity but micro-aerophilis and anaerobes are deficient in these mechanisms and are restricted to habitats where little or no oxygen is present.

Factors responsible for oxygen toxicity :-

1. **Oxygen inactivation of enzymes :** Molecular oxygen can directly oxidize certain essenttial reduced groups, such as teh 01 (-5H) groups or enzymes resulting in enzyme inactivation. For example, the enzyme complex known as nitrogenase, responsible for nitrogen fixation, is irreveribly destroyed by even small amounts of oxygen.

2. **Damage due to toxic derivatives of oxygen :** Various cellular enzymes catalyze chemical reactions involving molecular oxygen, some of these reactions can result in addition of a single electron to an oxygen molecule, thereby farming a super oxide radical (O_2).

$O_2 + e^- - O_2^-$ (1)

Super oxide radicals can inactivate cell components, and produced more toxic substances such as hydrogen peroxide (H202) and hydroxyl radicals (OH).

$2O_2 + 2H^+ - O_2 + H_2O_2 -$ (2)

$O_2^- + H_2O_2$ chelated iron - $O_2 + OH + OH$ - (3)

Hydroxyl radicals can damage almost every kind of molecule, found in living cells. H_2O_2 is not a free radical, but it is a powerful oxidizing agent that is highly toxic to many kinds of cells. Another toxic derivative of oxygen is an energized is produced in biological system by certain photochemical reactions.

Aerobic and facultative organisms have developed various protective mechanksm agaist the toxic forms of oxygen. One is the enzyme known as super oxide dismutase, which eliminates super oxide radicals by greatly increasing the rate of reaction (2) mentioned above. The H_2O_2 produced by catabase and peroxidase enzymes.

$2 H_2O_2$ Catalase - $2H_2O + O_2$ - (4)

H_2O_2 + reduced substrate - Puroxidase - $2H_2O$ + Oxidized substrates - (5)

Elimination of either O_2 - or H_2O_2 can prevent the formation of the highly dangerous OH. Since both reactants are required for reaction (3).

In general anaerobic bacteria have either no super oxide dismutase or only relatively low levels compared to aerobes. Many anaerobes are also deficient in catalase and/or at least input, peroxidase. For the above reasons, the anaerobes are sensitive to oxygen although other factors are probably involved as well.

CULTIVATION OF AEROBIC BACTERIA :-

To grow aerobic or facultative bacteria in tubes or small flasks, incubation of the medium under normal atmospheric condition is generally satisfactory. However, in large scale production of aerobic organisms, it is accomplished by dispensing the mediums in shallow layers, in kille flask/ Roux bottle/ Fernbach Flask. Fig. 4

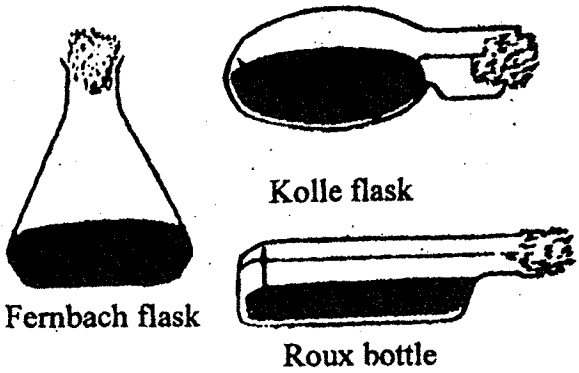


Figure 4 : *Methods for providing incresed areation during incubation. (A) Culture vessels of several designs that provide a large surface area for a shallow layer of medium.*

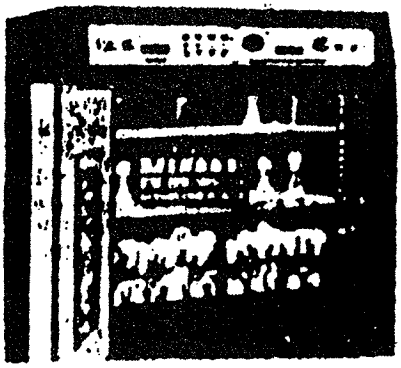


Fig 5

Aeration can also be increased by constantly shaking the inoculated liquid cultures (in incubator shaker). Fig. 5.

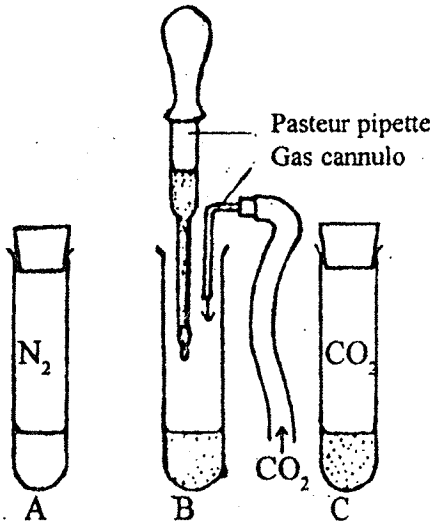
CULTIVATION OF AEROBIC BACTERIA :-

Stringent anaerobes can be grown only by taking special precautions to exculde all atmospheric oxygen from the medium. These includes :-

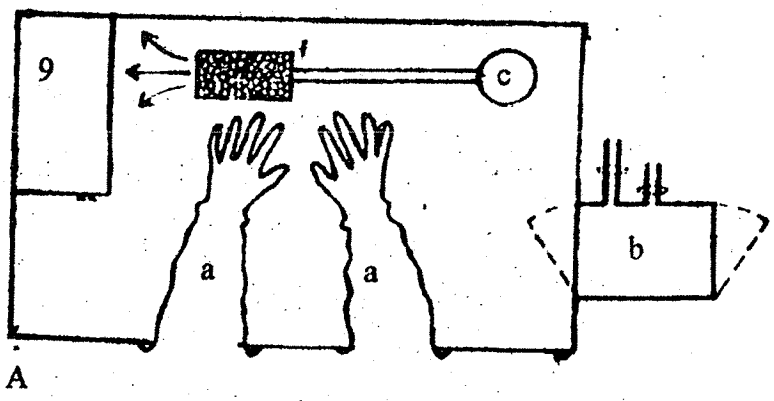
1. prereduced media :- Here culture medium is boilded for several minutes to drive off most of the dissolved oxygen.

A reducing agent e, g, cysteine, is added to further lower the oxygen content O_2 free N is bubbled through the medium to keep it anaerobic the medium is then dispensed into tubes which are being fused with O_2 free N_2, stoppered tightly, and sterilized by autoclaving. During inoculation, the tubes are continuously flushed with oxygen free CO_2 by means of cannula restoppered and incubated. Fig. 7.

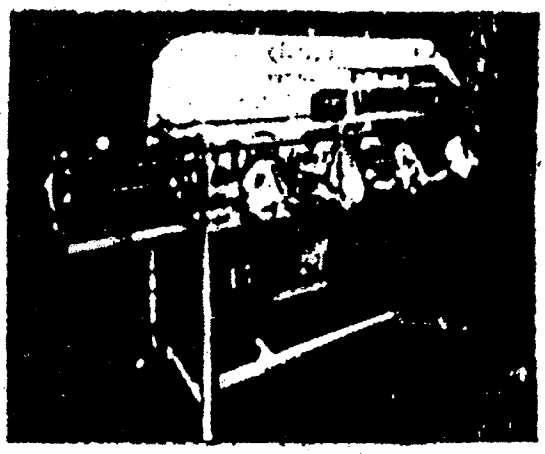
Figure 7 . ***Use of precluded media for cultivation of stringent anaerobes.*** *(A) Tube of prereduced medium containing an atmosphere of oxygen free N_2. (B) To inoculate, the stopper is removed and a gas cannula inserted to flush the tube continuously with oxygen free CO_2 and maintain anaerobic conditions. The medium is onoculated with a few drops of culture by means of a Pasteur pipette. (C) After inoculation the tube is restoppered and incubated.*



2. **Anaerobic chamber :-** It is nothing but a plastic anaerobic glove box (see fig. 8 (A, B) below) that illustration contains an atmosphere of H, CO and N culture media are placed within the chamber by means of an air lack which can be evacuated and refilled with N. From teh ari lock the media are placed within the main chamber. any O in the media is slowly removed by reaction with the H forming water this reaction is added by a palladium catalyst. After being rendered oxygen free the media are inoculated with in the chamber (by means of the glove ports) and ncubated also within the chamber).



A



B

Fig. 5

Figure 8 (A) . *Schematic diagram of the various parts of an anaerobic chamber (top view). (a) Glove ports and rubber gloves that allow the operator to perform manipulations within the chamber. (b) Air lock with inner and outer doors. Media are placed within the air lock with the inner door remaining sealed; air is removed by a vacuum pump connection (c) and replaced wtih N_2 through (d). The inner door is opened and the media are placed within the main chamber, which contains an atmosphere of H_2 + CO_2 + N_2. A circular (e) circulates the gas atmosphere through pellets of palladium catalyst (f), causing any residual oxygen in the media to be used up by reaction with H_2. After media have become completely anaerobic they can be inoculated and placed in an incubator (g) located within the chamber (B) Photograph of an anaerobic chamber. (Courtesy of the Germ free Laboratories, Inc.)*

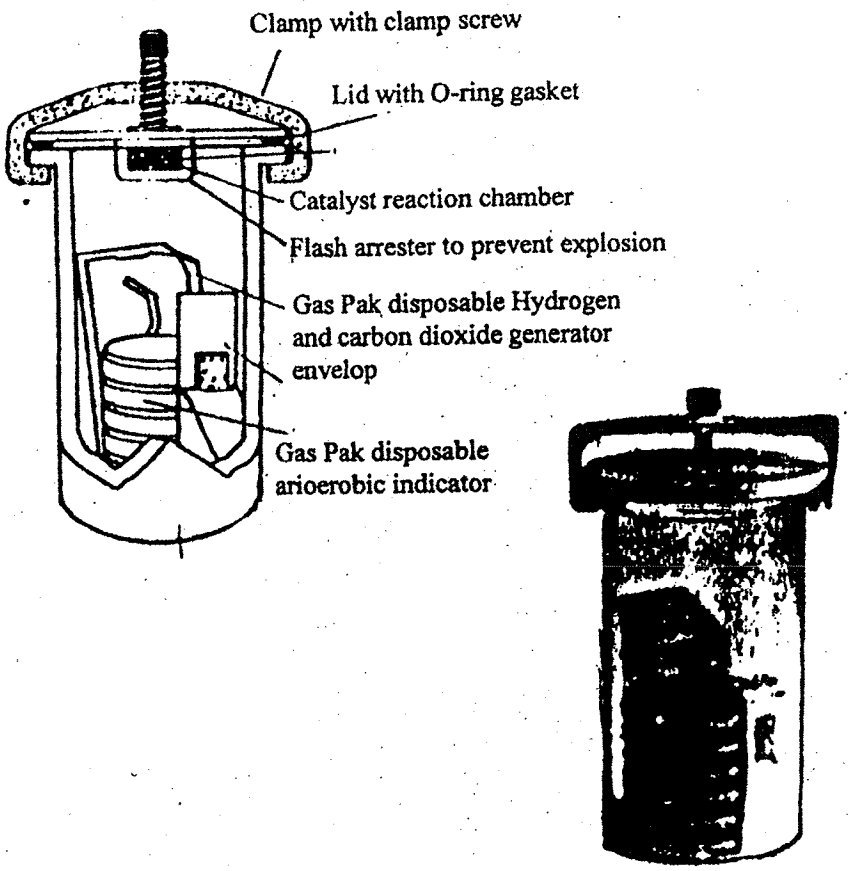


Figure 9 *Ariaerobic jar; GasPac system. (A) Media are inoculated and then placed in the jar. Water is added to the GasPak generator envelop, causing the evolution of H_2 and CO_2. The H_2 reacts with O_2 on the surface of the palladium catalyst, forming water and establishing anaerobic conditions. The CO_2 aids growth of fastidious anaerobes which sometimes fail to grow, or grow only poorly, in its absence. an anaerobic indicator strip (a pad saturated with methylene blue solution) changes from blue to colorless in the absence of oxygen. (B) The GasPak Anaerobic System with inoculated Petri dishes, the GasPak generator envelope, and the anaerobic indicator strip. (Courtesy of BBL Microbiology Systems).*

Nonstringent anaerobic can be cultured an anaerobic jar (Fig. 9). Inculated media are placed in the jar along with an $H_2 + CO_2$ generating system. After the jar is sealed, the oxygen present in the atmosphere with in the jar, as well as that dissolved in the culture medium, is gradually used up through reaction with the hydrogen in the presence of a catalyst.

Chapter - 3

Principel Characteristics used in the classification and identification of microbes

Characterization, classification and identification are major objectives in all branches of the biological sciences.

It is necessary to learn the characteristics of microorganisms for their smooth identification and classification. It is usually not feasible to study the characteristics of a single microorganism because of its small size, therefore, it is necessary to study the characteristics of a culture - a population of microorganism.

A culture that consists of a single kind of microorganism (one living species) regardless of the number of individuals, in an environment free of other living organisms it called a pure culture (axenic culture). In the strict, technical sense a pure culture is one grown from a single cell.

The major observable features of microorganism which together are used to identify the species are enumerated below :

I. **Direct microscope Examination**

- A) Staining reaction
  - a) Gram staining
  - b) Acid fast staining
- B) Cell size
- C) Cell shape
- D) Presence of spore
- E) Capsule formation
- F) Motility

II. **Chemical compositions** : The various chemical constituents of the cell.

III. **Cultural characteristics :**

- a) Food requirement
- b) Media most suitable for growth
- c) Appearance of growth in different media.
- d) Oxygen requirements.
- e) Optimum growth temperature.
- f) Characterization of pure culture (i) Size (ii) Shape (iii) Texture (iv) Elevation of the colonies (v) Pigmentation.

IV. **Metabolic characteristics :** The way in which cells obtain and use their energy, carry out chemical reactions and regulate these reactions.

V. **Antigenic characteristics :** Special large chemical components (antigens) of the cell, distinctive for certain kinds of micro organisms.

VI. **Genetic characteristics :** Characteristics of the hereditary material of the cell (DNA) (or more briefly mul% G+C) and occurrence and function of other kinds of DNA, that may be present, such as plasmid.

VII. **Pathogenicity :** The ability to cause disease in various plants of animals of even other micro organisms.

VIII. **Ecological characteristics :** Habitat and the distribution of the organisms in nature and the interaction between among species and natural environments.

Staining reaction:

(a) Gram staining : A differential staining procedure of great value in the identification of eubacteria is the gram stain 1st developed by Christian gram (1884).

On the basis of the staining behaviour bacteria have been grouped into 2 groups.

1) Gram positive bacteria.
2) Gram negative bacteria.

i) A heat fixed smear of bacteria is stained successively with a solution of crystal violet (or a related basic dye) and with a solution of iodine. Thus the crystal violet and iodine formed crystal violet - Iodine complex (CV-I).

ii) The preparation is then treated with an organic solvent such as alcohol or acetone.
Gram negative bacteria are completely decolourised (CV-I complex released) by the organic solvent.
Gram positive bacteria resist decolourization (CV-I complex not released) retaining the deep purple colour.

iii) Gram negative bacteria are stained with satranin when they exhibit the red colour of the counterstain.

As a result of this difference in colour gram positive and gram negative cells can be readily distinguished from one another under the microscope.

(b) Acid fast staining : The walls of some bacteria contain large amounts of waxy materials. These waxes confer on the cell a special staining property, termed acidfastness, which is a very valuable diagnostic character.

Acid fastness is determined by a procedure known as Ziel-Neelson stain. The fixed cells are treated for 10 minutes with a hot, dilute phenolic solution of the red basic dye, carbol fuschin and heated to 90°C. over a steam bath for four minutes. This softens the wax and the dye supposedly penetrates. After washing of the excess dye, the smear is treated for five minutes with cold 95 percent alcohol containing 5 to 10 percent HCL or H_2SO_4.

The organism retain the red dye in spite of the acid alcohol, which removes the colour from everything else. Organism retaining the red stain are said to be acid fast. If methylene blue or brilliant green is now applied as a counter stain, the acid fast bacteria stand out as bright red objects in a blue or green field. This stain is also a differential stain because from non acid-fast ones.

It is used especially for staining tuberculosis bacilli (Mycobacterium tuberculosis) and related organisms (genus Mycobacterium) having an abundance of particular acid fast waxy materials (mycolic acids) on the cell. Such organism are gram-positive, but the Gram stain does not give an useful information about them as the Ziehl Neelson or acid fast stain.

Size : Microorganism are very small and their size is usually expressed in micro meters (um) [approximately 0.5 to 1.0 um in diameter].

Shape & arrangement : The shape of bacteria is governed by its rigid cell wall, Typical bacterial cells are spherical (cocci/coccus), straight rods (bacilli/bacillus) or rods that are helically curved (spirilla/ spirillum). Some home cells that are pleomorphic (a variety of shapes) cocci may be of different types on the basis of plane of cellular division and whether the daughter cells stay together following division Bacilli are not arranged in pattern as complex as those of cocci and must occur singly or in pari (diplobacilli) some form chains (strepto bacilli) others such as Beggiatoa and Saprospira species form trichomes, and streptomyces species form long branched multicellular filaments called hypae.

Cocci may be of different types :

i) Diplococci : Cells divide in one plane and remain attached predominantly in pairs.
ii) Streptococci : Cells divide in one plane and remain attached to form cheins.
iii) Tetra cocci : Cells divide in two planes and characteristically form groups of four cells.
iv) Staphylocci : Cells divide in three planes in irregular pattern, producing "punches of locci".
v) Sarcinae : Cell divide in three planes in a regular pattern, producing cuboidal arrangement of cells.

Presence of spore : Some organism can produce spore and other can not. This property helps in the identification of an organism. For example it a grant straight rod can produce spore and is aerobic, the bacterium may be baccillus, if anaerobic it may be clostridium. The gram positive straight rod if do not produce spores, Lactobacillus and some other bacteria are considered gram negative straight rods generally do not produce spores.

Capsule formation : In case of bacteria presence or absence of Capsule help in identifying the organism.

Motility : The possession of flagella, and hence the capacity for active moment is of importance in identification.

Microbial classification, Nomenclature and Identification :

Classification : In microbiology taxa are initially constructed from strains. A strain is made up of all the descendents of a pure culture.

For example, strain ATCC 19554 is a strain of spirilla isolated originally from pond water Blackspurg Virginia in 1965 by wells and Krieg and cultures of the strain are maintained at the American Type Culture Collection (ATCC) Rock Ville, Maryland. Cultures of the same species that were isolated from other sources would be considered different strains.

The Basic taxonomic group (Taxon) is the species, i,e, a collection of strains having similarcharacteristics. Bacterial species consist of a special strain called type strain together with all other strains that are considered sufficiently similar to the type strain as to warrant inclusion in the species. Similarly bacterial genus is composed of a collection of similar species.

Taxonomic groups of higher rank than genus :
Family : a group of similar genera
Order : a group of similar families
Class : a group of similar orders
Division : a group of similar classes
Kingdom : a group fo similar divisions.

Nomenclature : One rule in bacteriological nomenclature is that name must be written as a Latin or Latinized binomial (two words) and must follow certain rules of Latin grammar. The first word in the binomial is the genus name and is always capitalized. The second word is the specific epithet and is never capitalized. Both the names (genus and species) and written in italics or underlined).

Principles of International code of Nomenclature of Bacteria.

1. Each distinch kind of organism is designated as a species.
2. The species is designated by a Latin binomial to provide a characteristic international label (binomial system of nomenclatue).
3. Regulation is established for the application of names.
4. A law of priority ensured the use of oldest availabel legitimate name.
5. Designation of categories is required of classification of organisms.
6. Requirements are given for effective publication of new specific names as well as guidance in coining new names.

Identification : An organism must be classified before it can be identified - that is given a name.

Many identification schemes are in the form of keys, which give identifying characteristics arranged in logical fusion. Identification tables are very useful and generally contain more characteristics than do keys, the the information arranged in an easy-to-read, summarized form. Bergey's Mannual of systematic Bacteriology is very helpful for the identification of Bacteria.

Suggested questions :

1. What is type strain ?

   Why is the type strain the most important strain in a bacterial species.

2. What is differential staining? Mention few differential stain with special reference to Acid fast stain indicating its application.

3. Why are micro organisms useful as subjects for research in the field of biology.

4. Name several applied areas of micro biology. Describe the importance of bacteria, mold and yeast in industry?

5. What condition of cultivation would allow you to grow selectively.

   a) **Thiobacillus thioosxydexns** from a mixed culture of bacteria.

   b) an extreme halophile form a sample of sea salt.

   c) a nitrogen fixing bacterium form a soil sample.

   d) a spore forming bacterium form a soil sample.

   or

   Indicate the various toxic derivatives of oxygen and explain how aerobic organisms might protect themselves against these deravatives.

BOTANY

Modue No. - 2
FIRST PAPER FIRST HALF

Microbiology

Chapter - 1
Bacterial Growth

Contents

* Definition
* Overview of Cell Growth
* Growth and Generatiion time
* Growth Cycle (Growth Curve) of bacteria
* Synchronours Growth
* Continuour Culture
* Quantitative measurement of Growth
* Effect of environmental factors on Growth
    a) Temperature
    b) Acidity and Alkalinity
    c) Water availability
    d) Oxygen

OVERVIEW OF CELL GROWTH

In any biological system, growth can be defined as the orderly increase of all chemical components. Microbial growth involves an increase in the number of cells rather than in the size of individual cells. Growth of most microorganisms occurs by binary fission. Cell division and chromosome replication are normally coordinately regulated.

The bacterial cell is essentially a synthetic machine that is able to duplicate itself, the synthetic processes of bacterial cell growth involve as many as 2000 chemical reactions of a wide variety of types, some of these reactions involve energy transformation. Other reactions involve biosynthesis of small molecules – the building blocks of macromolecules – as well as the various cofactors and coenzymes needed for enzymatic reactions. However, the main reactions of cell synthesis are *Polymerization reactions*, the processes by which polymers (macromolecules) are made from monomers, DNA synthesis, RNA synthesis, and protein synthesis. Once polymers are made, the stage is set for the final events of cell growth assembly of macromolecules and formation of cellular structures such as the cell wall, cytoplasmic membrane, flagella, ribosomes, inclusion bodies enzyme complexes, and so on.

In most prokaryotes, growth of an individual cell continues until the cell devides into two new cells, a process called binary fission (*binary* to express the fact that *two* cells have arisen from *one* cell). In a growing culture of a rod-shaped bacterium such as *Escherichia coli*, for example, cells are observed to elongate to approximately twice the length of an average cell and then form a partition that eventually

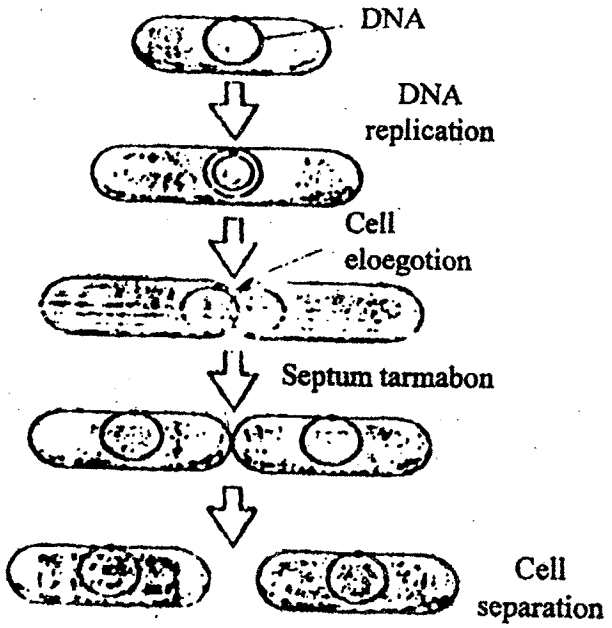


Figure 1.1. : *The process of binary fission in a rod shapped prokaryote. For simpheity, the nucleoid is depicted as a single circle*

separates the cell into two daughter cells (Figure 1). This partition is referred to as a *seption* and is a result of the inward growth of the cytoplasmic membrane and cell wall from opposing directions until the two daughter cells are pinched off (Figure 1.1). During the growth cycle all cellular constituents increase in number such that each daughter cell receives a complete chromosome and sufficient copies of all other macromolecules, monomers, and inorganic ions to exist as an independent cell. Partitioning of the replicated DNA molecule between the two daughter cells depends on the DNA remaining attached to membranes during division, with septum formation leading to separation of chromosome copies, one going to each daughter cell (Figure 1.1).

The time required for a complete growth cycle in bacteria is highly variable and is dependent on a number of factors, both nutritional and genetic. Under the best nutritional conditions the bacterium *Escherichia coli* can complete the cycle in about 20 min., a few bacteria can grow even faster than this, but many grow much slower. The control of cell division is a complex process and appears to be immediately tied to chromosomal replication events.

Population Growth

As growth is defined as an increase in the number of microbial cells in a population, which can also be measured as an increase in microbial mass. Growth rate is change in cell number or cell mass *per unit time*. During this cell division cycle, all the structural components of the cell double. The interval for the formation of two cells from one is called a generation and the time required for this to occur is called generation time. The generation time is thus the time required for the cell population to double. Because of this, the generation time is also sometimes called the *doubling time*. Note that during a single generation, both the cell number and cell mass double. Generation times vary widely among organisms. Many bacteria have generation times of 1-3 hr., but a few very rapidly growing organisms are known that divide in as little as 10 min, and others have generation times of several hours or even days.

Growth and Generation Time

The most common means of bacterial reproduction is binary fission, one cell divides, producing two cells. Thus, if we start with a single bacterium, the increase in population is geometric progression:

$$1 \longrightarrow 2 \longrightarrow 2^2 \longrightarrow 2^3 \longrightarrow 2^4 \longrightarrow 2^5 \longrightarrow 2^n$$

when n = the number of generations. Each succeeding generation, assuming no cell death, doubles the population. The total population N at the end of a given time period would be expressed

$$N = 1 \times 2^n \quad (1)$$

However, under practical conditions, the number of bacteria N_0 inoculated at time zero is not 1 but more likely several thousand, so the formula becomes

$$N = N_0 \times 2^n \quad (2)$$

Solving Eq. (2) for n, we have

$$\log_{10} N = \log_{10} N = n \log_{10} 2$$

$$n = (\log_{10} N - \log_{10} N_0)/\log_{10} 2 \quad (3)$$

If, we now substitute the value of $\log_{10} 2$, which is 0.301, in the above equation.

$$N = (\log_{10} N - \log_{10} N_0) / 0.301$$

$$n = 3.3 (\log_{10} N - \log_{10} N_0) \quad (4)$$

Thus, by use of Eq.(4), we can calculate the number of generations that have been taken place, providing we know the initial population and the population after growth has occurred.

The generation time g (the time required for the population to double) can be determined from the number of generations n that occur in a particular time interval *t*. Using Eq.(4) for *n*, the generation time can be calculated by the following formula.

$$G = t\, n = 1\, 3.3 (\log_{10} N - \log_{10} N_0) \quad (5)$$

Not all bacteria have the same generation time; for some as *E. coli*, it may be 15 to 20 minutes, for others it may be many hours. Similarly, the generation time is not the same for a particular species under all conditions. it is strongly dependent upon the nutrients in the medium and on prevailing physical growth, the growth rate (i.e., the number of generations per hour), termed r, is the reciprocal of the generation time g. It is also the slope of the straight line obtained when the log number of cells is plotted against time.

$$R = 3.3 (\log_{10} N - \log_{10} N_0)/t \quad (6)$$

GROWTH CURVE

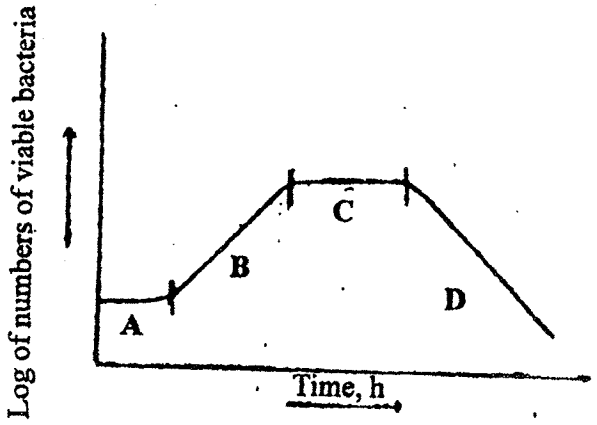


Fig. 2: *Typical bacterial growth curve. A-Lag phase; B-Log or exponential phase; C-Stationary phase; D-Death or decline phase.*

In reality when we inoculate a fresh medium with a given number of cells, determine the bacterial population intermittently during an incubation period of 24 h [more or less], and plot the logarithms of the number of cells versus time, we obtain a curve of the type illustrated in fig. 2. From this it can be seen that there is an initial period of what appears to be no growth [the lag phase], followed by rapid growth [the exponential or logarithmic phase] then a leveling [stationary phase], and finally a decline in the viable population [death of decline phase], between each of these phases there is a transitional period [curve portion]. This represents that time required before all cells enter the new phase.

The Lag Phase

The addition of inoculum to a new medium is not followed immediately by a doubling of the population instead, the population remains temporarily unchanged, as illustrated in fig.2 but this dose mean that the cells are quiescent or dormant, on the contrary, during this stage the individual cells increase in size beyond their normal dimensions. Physiologically they are very active and are synthesizing new protoplasm. The bacteria in this new environment may be deficient in enzymes or coenzymes with much first be synthesized in amounts required for optimal operation of the chemical machinery of the cell. Time for adjustment in the physical environment around each cell may be required. The organisms are metabolizing, but there is a lag in cell division.

At the end of the lag phase, each organism divides. However, since not all organisms complete the lag, period simultaneously, there is a gradual increase in the population until the end of this period, when all cell are capable of dividing at regular intervals.

The logarithmic or Exponential phase

During this period the cells divide steadily at a constant rate, and the log of the number of cells plotted against time results in a straight line [fig. 2]. Moreover, the population is most nearly uniform in terms of chemical composition of cells, metabolic activity, and other physiological characteristics.

Time (hr)	Total number of cells
0	1
0.5	2
1	4
1.5	8
2	16
2.5	32
3	64
3.5	128
4	256
4.5	512
5	1,024
5.5	2,048
6	4,096
a) 10	1,048,576

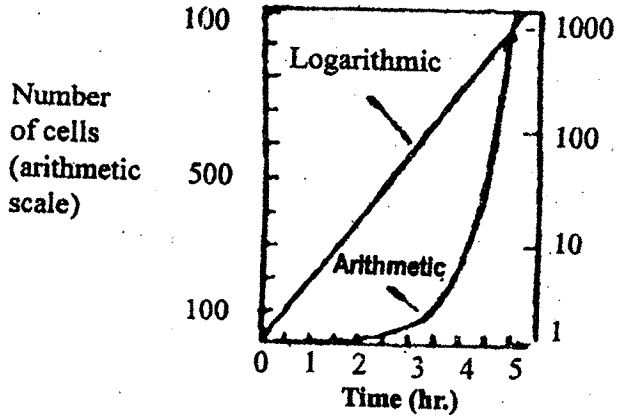


Fig.1.2 *The rate of growth of a culture (a) Data for a population that doubles every 30 min. (b) Data plotted on an arithmetic (left ordinate) and a logarithmic (right ordinate) scale.*

A growth experiment beginning with a single cell having a doubling time of 30 min is presented in Figure 2a. This pattern of population increase, where the number or cells *double* during each unit time period, is referred to as **exponential growth**, when the cell number from such an experiment is graphed on arithmetic coordinates as a functions of elapsed time, one obtains a curve with a constantly increasing slope (Figure 2b). However, deriving growth rate information from such curves is difficult. The number of cells on a logarithmic (log_{10}) scale is presented in Figure 2b in a graph in which cell number is plotted logarithmically and time is plotted arithmetically (a semilogarithmic graph), resulting in a straight line. This straight line function is an immediate indicator that the cells are growing exponentially. Semilogarithmic graphs are also convenient and simple to use for estimating generation times for a set of results. The doubling time may be read directly from the graph (Figure 1.3).

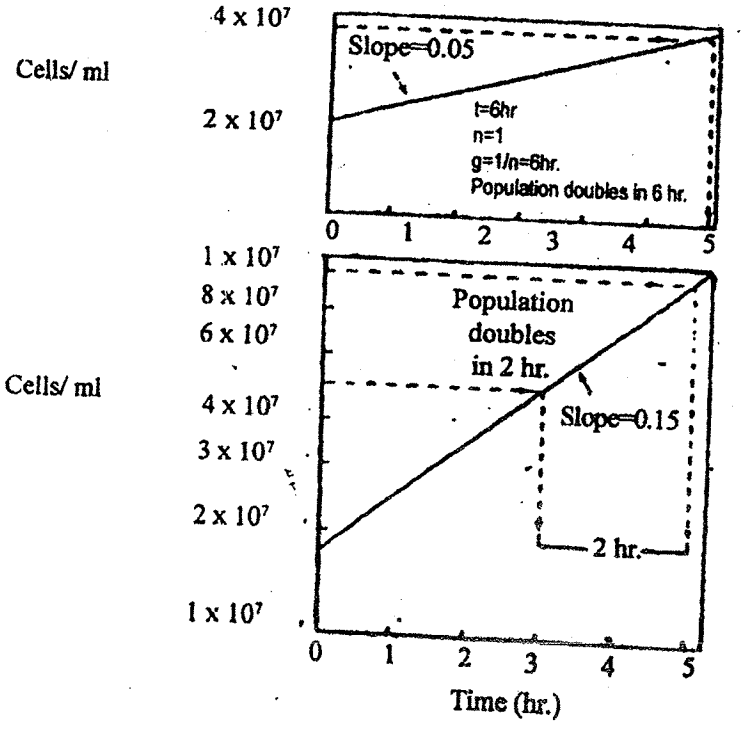


Fig.1.3 *Method of estimating the generation times(g) of exponentially growing populations with generation times of 6 and 2 hr. respectively, from data plotted on semilogarithmic graphs. The slope of each line is 0.301/g. All numbers are expressed in scientific notation; that is 10,000,000 is 1 x 10⁷, 60,000,000 is 6 x 10⁷, and so on.*

This growth rate can remain constant during the logarithmic phase of growth even though the concentration of substrate (i.e., some essential nutrient in the culture medium, usually the carbon and energy source) is continually decreasing through utilization by the organisms. To understand this, one must recognize that the relationship between R and substrate concentration is not a simple linear relationship, as shown in Fig. 2-A, when the substrate concentration is high, a change in the concentration has very little effect on the growth rate. It is only when the substrate concentration becomes quite low that the growth begins to decrease significantly. Since bacteria are commonly "overfed" in laboratory culture, (i.e. are supplied with far greater substrate concentration that they need), they can multiply at a constant exponential rate for many generations before the substrate level becomes low enough to affect this rate.

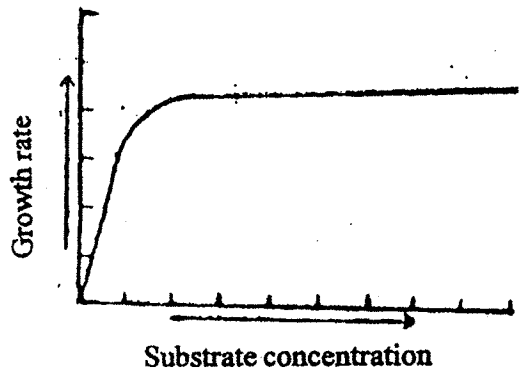


Fig.2A *The effect of nutrient (substrate) concentration upon the growth rate of a bacterial culture. The level of substrate commonly provided in a bacterial culture is sufficiently high (right portion of the curve) so that, even through the bacteria use up some substrate during the log phase of growth, the growth rate does not decrease appreciably. It is only when substrate levels become very low (left portion of curve) that the growth rate brings to be severely affected.*

The stationary phase

The logarithmic phase of growth begins to taper off after several hours, again in a gradual fashion represented by the transition from a straight line through a curve to another straight line, the stationary phase, as shown in Fig. 2. This trend toward cessation of growth can be attributed to a variety of circumstances, particularly the exhaustion of some nutrients, and, less often, the production of toxic products during growth. The population remains constant for a time. Perhaps as a result of complete cessation of division or perhaps because the reproduction rate is balanced by an equal death rate.

The phase of Decline or Death

Following the stationary phase the bacteria may die faster than new cells are produced, if indeed some cells are still reproducing. Undoubtedly a variety of conditions contribute to bacterial death, but the most important are the depletion of essential nutrients and the accumulation of inhibitory products, such as acids. During the death phase, the number of viable cells decreases exponentially, essentially the inverse of growth during the log phase. Bacteria die at different rates, just as they grow at different rates some species

of Gram-negetive cocci die very rapidly, so that there may be very few viable cells left in a culture after 72 hr. or less. Other species die so slowly that viable cells may persist for months or even years.

Transitional Period between Growth Phases

Note that culture proceeds gradually from one phase of growth to the next (Fig. 2). This means that not all the cells are in an identical physiological condition toward the end of the given phase of growth. Time is required for some to catch up with others.

Synchronomous Growth

Information about the growth behaviour of individual bacteria can be obtained by the study of synchronomous cultures i.e., cultures composed of cells that are all at the same stage of the cell cycle. Measurement made on such cultures are equivalent to the measurements made on individual cells. Synchronomous cultures of bacteria can be obtained by a number of techniques. Synchromy can be induced by manipulation of environmental condition viz.

i) manipulating the physical environment. The cells may be inoculated into a medium at a suboptional temperature, if they are kept in this condition for some time they will metabolize slowly but will not divide, when the temperature is subsequently raised, the cell will undergo synchronised division.

ii) Manipulating the chemical composition of the medium.

iii) By physical separation (by differential filteration or centrifugation).

An excellent selective method for obtaining synchronomous culture is the Helmstetter-Cummings technique. The technique involves filtering an unsynchronized culture of bacteria through a (membrane) filter, then inverting the filter and allowing fresh medium to flow through it (Figure 4). After loosely associated bacteria have been washed from the filter, the only bacterial cells in the effluent stream of medium are those that arise through division. Hence, all cells in the effluent are newly formed and are therefore at the same stage of the cell cycle.

The growth of a culture of *E. coli* so synchronized is shown in Figure 5. The number of cells in the culture remains approximately constant for about one hour while the newly formed cells grow in size. Then, rather abruptly, the number of cells doubles. In the second division cycle, the plateau is less distinct and the population rise extends over a longer period indicating that synchrony is already being lost. In the third division cycle, almost no indication of synchrony remains.

Synchronous cultures rapidly lose synchrony because various cells of a population do not all divide at the same size (age, or time following the previous division).

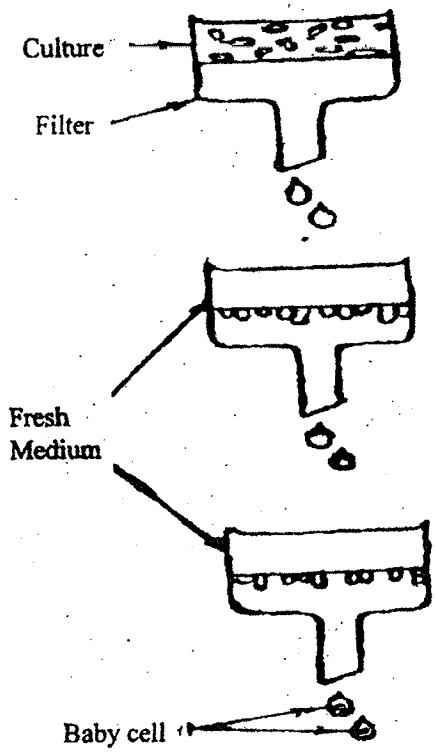


Fig.4 *Helmstetter-Cummings technique of obtaining synchronous cultures.*

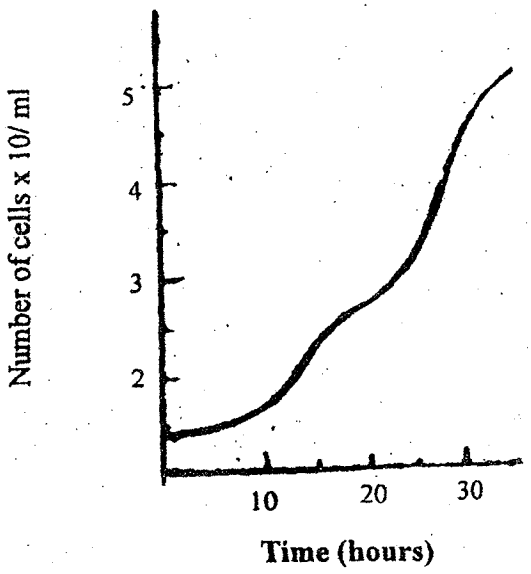


Fig.5. *Synchronous growth of E. coli in glucose munimal medium.*

Continuous Culture

In both experimental research and in industrial processes, it is often desirable to maintain a bacterial population growing at a particular rate in the exponential or log phase. This condition is known as steady-state growth. The culture volume and the cell concentration are both kept constant by allowing fresh sterile medium to enter the culture vessel at the same rate that "spent" medium containing cells, is removed from the growing culture (see Figure 6). Under these conditions, the rate at which new cells are produced in the culture vessel is exactly balanced by the rate at which cells are being lost through the overflow from the culture vessel.

One type of system that is widely employed for continuous cultivation is the **chemostat**. This system depends on the fact that the concentration of an essential nutrient (substrate) within the culture vessel will control the growth rate of the cells. The concentration of substrate within the culture vessel is in turn controlled by the dilution rate, i.e., the rate at which fresh medium is being added to the culture (flow rate) divided by the volume of the culture vessel.

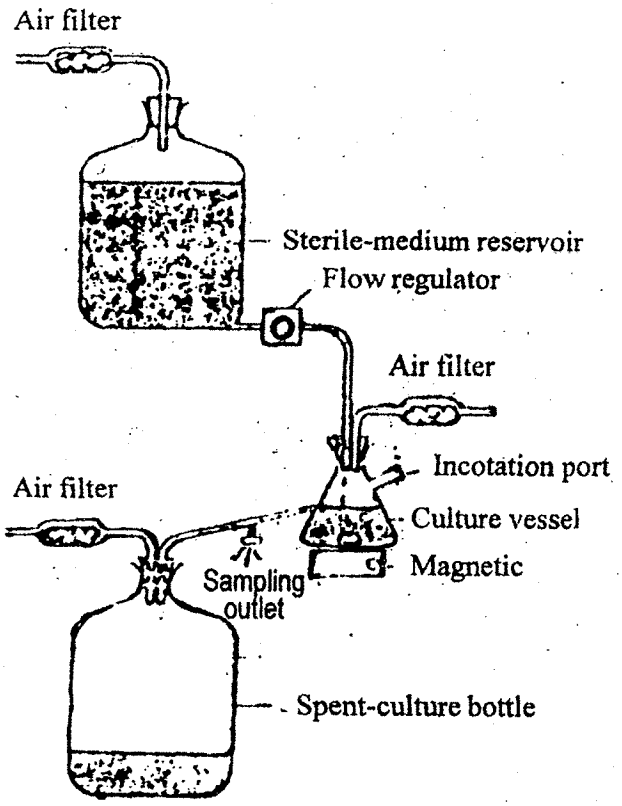


Fig.6. *Apparatus for continuous cultivation of bacteria. The system can be regulated for continuous addition of fresh sterile medium to and removal of spent medium (and cells) from the vessel.*

Therefore, by adjusting the dilution rate we can control the growth rate, for example, suppose that the dilution rate is very low. The cells reach a high density because they are leaving the culture vessel at a very slow rate, moreover, they have time to use the substrate almost completely. Therefore, the substrate concentration is maintained at a low level within a vessel. This low substrate concentration permits the cells to grow at only a slow rate. On the other hand, if the dilution rate is high, the cell density is low because the cells are leaving the vessel at high rate, moreover, they have little time to utilize the substrate that is entering the vessel, and therefore, the substrate concentration is maintained at a high level within the vessel (but still less than that in the sterile-medium reservoir). This high concentration allows the cells to grow at a high rate. In each case the growth rate automatically adjusts to match the dilution rate. However, if the dilution rate is increased to the point where it exceeds the maximum growth rate of the cells, then washout occurs; that is, the cells cannot grow as fast as the rate at which the culture is being diluted by fresh medium, and they are soon eliminated from the culture vessel.

A second type of continuous culture apparatus is the turbidostat. Here a photoelectric device continuously monitors the cell density within the culture vessel and controls the dilution rate to maintain the cell density at a constant value. If the density becomes too high the dilution rate is increased, if the density becomes too low, the dilution rate is decreased.

QUANTITATIVE MEASUREMENT OF BACTERIAL GROWTH

The term growth as commonly applied in microbiology refers to the magnitude of the total population. Growth in this sense can be determined by numerous techniques based on one or more of the following types of measurements :

1. ***Cell count***. Directly by microscopy or by using an electronic particle counter, or indirectly by a colony count.
2. ***Cell mass***. Directly by weighing or by a measurement of cell nitrogen, or indirectly by turbidity.
3. ***Cell activity***. Indirectly by relating the degree of biochemical activity to the size of the population.

Certain specific procedures will illustrate the application of each type of measurement.

Direct Microscopic count

Bacteria can be counted easily and accurately with the Petroff-Hausser counting chamber. This is a special slide accurately ruled into squares that are 1/400 mm^2 in area; a glass cover slip rests 1/50 mm above the slide, so that the volume over a square is 1/20,000 mm^3 (1/20,00,000 cm^3). A suspension of unstained bacteria can be counted in the chamber, using a phase-contrast microscope. If, for example, an average of five bacteria is present in each ruled square, there are 5 x 20,000,000 or 10^8, bacteria per millimeter. Direct microscopic counts can be made rapidly and simply with a minimum of equivalent; moreover, the morphology of the bacteria can be observed as they are counted. Very dense suspensions can be counted if they are diluted appropriately; however, suspensions having low numbers of bacteria, e.g., at the beginning of a growth curve, cannot be counted accurately.

Electronic Enumeration of Cell Numbers

In this method, the bacterial suspension is placed inside an electronic particle counter, within which the bacteria are passed through a tiny orfice 10 30 μm in diameter. This orfice connects the two compartments of the counter which contain an electronically conductive solution. As each bacterium passes through the orfice, the electrical resistance between the two compartments increases momentarily. This generates an electrical signal which is automatically counted. Although this method is rapid, it requires sophisticated electronic equipment; moreover, the orfice tends to become clogged.

The main disadvantage of direct counting of cell numbers is that there is no way to determine whether the cells being counted are viable. To determine the viable count of a culture, we must use a technique that allows viable cells to multiply, such as the plate-count method or the membrane-filter method.

The Plate-Count Method

This method, illustrated in Fig. 7, allows determination of the number of cells that will multiply under certain defined conditions. a measured amount of the bacterial suspension is introduced into a Peri dish,

after which the agar medium (maintained in liquid form at 45°C) is added and the two thoroughly mixed by rotating the plate. When the medium solidifies, the organisms are trapped in the gel. Each organism grows, reproducing itself until a visible mass of organisms – a colony – develops; i.e., one organism gives rise to one colony. Hence, a colony count performed on the plate reveals the viable microbial population of the inoculum. The original sample is usually diluted so that the number of colonies developing on the plate will fall in the range of 30 to 300. Within this range the count can be accurate, and the possibility of interference of the growth of one organism with that of another is minimized. Colonies are usually counted by illuminating them from below (dark-field illumination) so that they are easily visible, and a large magnifying lens is often used (see Fig. 7-11A). Various electronic techniques have been developed for the counting of colonies (Fig. 7.11B).

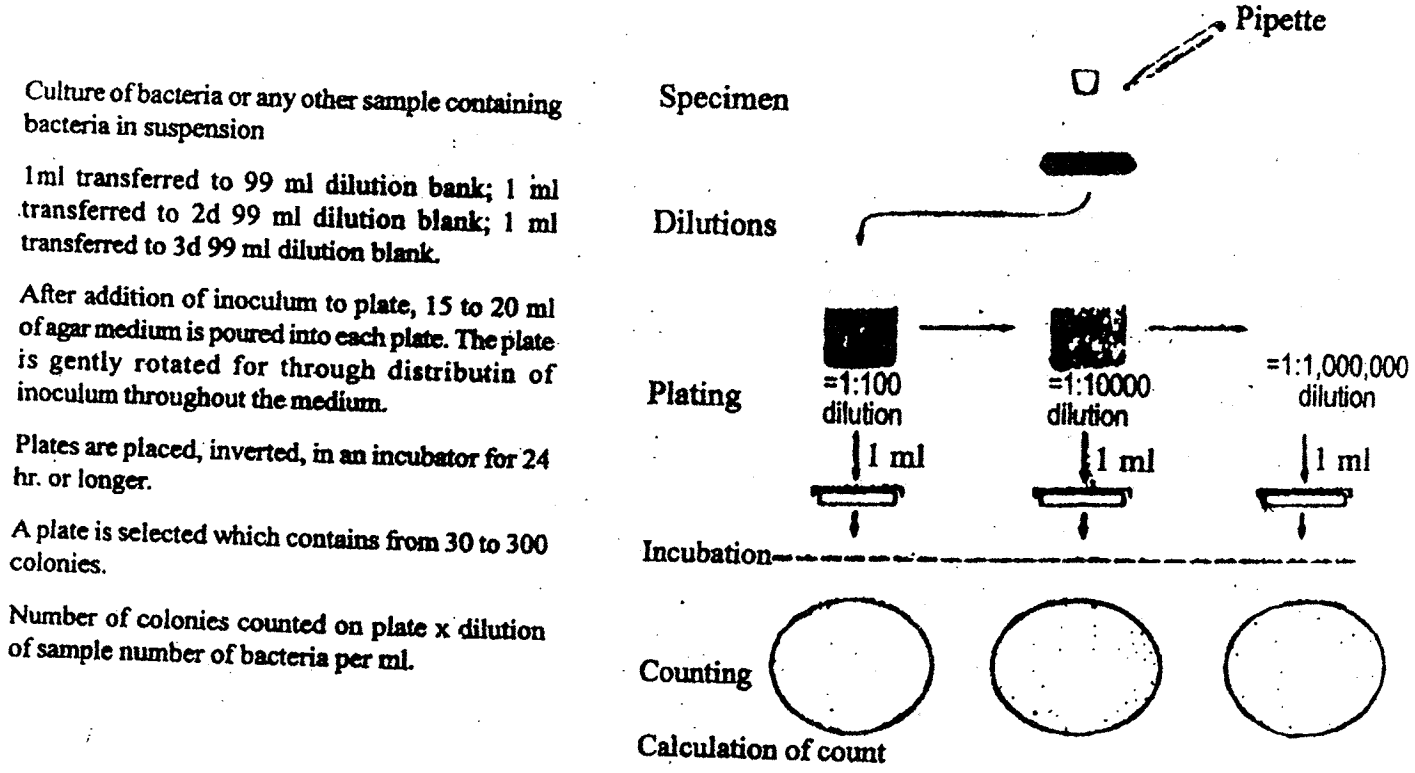


Fig.7. *The plate-count technique in which the sample is diluted quantitatively and measured amounts of the dilution are cultured in Peri dishes.*

One limitation of the plate-count technique is that the only bacteria that will be counted are those which can grow on the medium used and under the conditions of incubation provided. This can be an important consideration if a mixture of bacteria is to be counted. Another limitation is that each viable organism that is capable of growing under the culture conditions provided may not necessarily give rise to one colony. The development of one colony from one cell can occur when the bacterial suspension is homogeneous and no aggregates of cells are present; however, if the cells have a tendency to aggregate,

e.g., cocci in clusters (staphylococci), chains (stretococci), or pairs (diplococci), the resulting counts will be lower than the number of individual cells. For this reason the "counts" are often reported as colony-forming units per milliliter rather than number of bacteria per milliliter.

The plate-count technique is used routinely and with satisfactory results for the estimation of bacterial populations in milk, water, foods, and many other materials. It is easy to perform and can be adopted to the measurement of populations of any magnitude. It has the advantage of sensitivity, since very small numbers of organisms can be counted. Theoretically, if a specimen contains as few as one bacterium per milliliter, one colony should develop upon the plating of 1 ml.

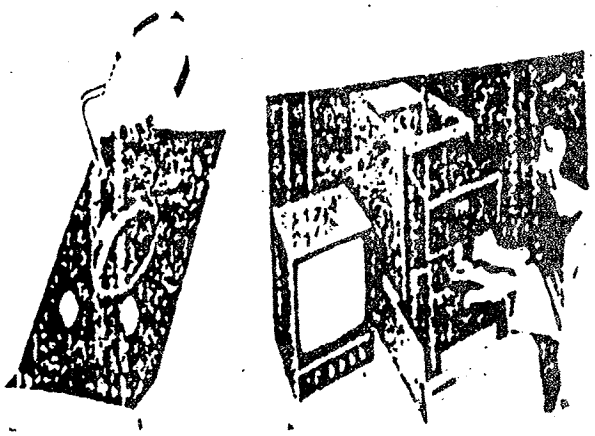


Fig.7. *Bacterial colony counters. (A) Quebec colony counter. A Petri dish fish into the recess in the platform. The Petri dish is illuminated from beneath while the lens provides x 1.5 magnification. (B) An electronic colony counter. The Petri dish is placed on the illuminated stage, the count bar is depressed, and the precise number of colonies is instantly displayed on a digital readout. (Courtesy of New Brunswick Scientific Company, Inc.).*

Membrane-Filter Count

A very useful variation on the plate-count technique is based on the use of molecular or membrane filters. These filters have a known uniform porosity of predetermined size sufficiently small to trap microorganisms. This technique is particularly valuable in determining the number of bacteria in a large sample that has a very small number of viable cells; e.g., the bacteria in a large volume of air or water can be collected simply by filtering them through an assembly as illustrated in Figure 7-C. The membrane with its trapped bacteria, is then placed in a special plate containing a pad saturated with the appropriate medium. Special media and dyes can be used to make it easier to detect certain types of organisms than with the conventional plate count. During incubation, the organisms grow into colonies which appear on the membrane surface (see Fig. 7-D).

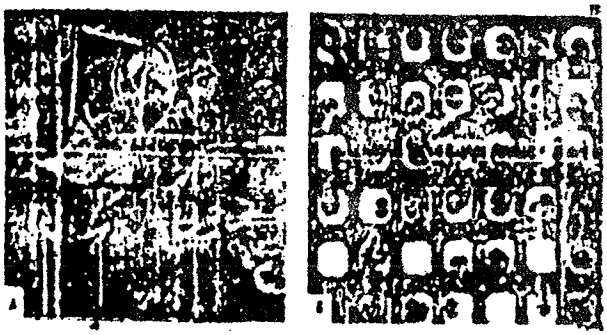
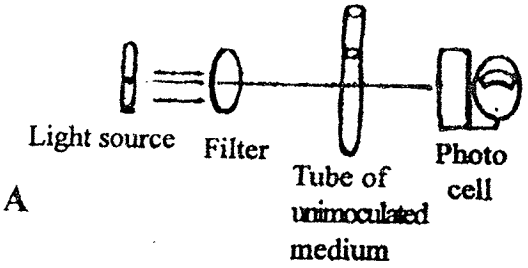


Fig.7(C) *Filteration apparatus for use with a membrane filter. After placing the filter on the support, the hinged upper part of the apparatus clamps it in place. A known volume of the bacteria-containing sample is then passed through the filter. (D) The filter is then incubated on a suitable culture medium. In the particular type of filter shown, a grid divides the filter into 1,600 small square compartments, and colony growth is restricted to these compartments. This greatly facilitates the counting of the colonies.*

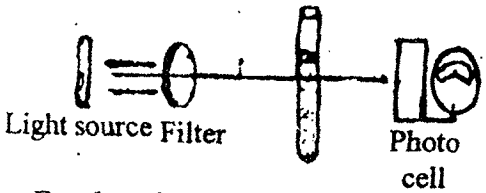
Turbidimetric Methods

Bacteria in a suspension absorb scatter the light passing through them, so that a culture of more than 107 to 108 cells per millimeter appears turbid to the naked eye. A spectrometer or colorimeter can be used for **turbidimetric measurements** of cell mass (see Fig. 8). Turbidimetry is a simple, rapid method for following growth; however, the culture to be measured must be dense enough to register some turbidity on the instrument. Moreover, it may not be possible to measure cultures grown in deeply colored media or cultures that contain suspended material other than bacteria. It must also be recognised that dead as well as living cells contribute to turbidity.



A

Instrument adjusted to read zero optical density



B Broth culture of bacteria; replaces tube shown in A

Reading on instrument will be greater than zero; the more turbid the culture, the higher the reading.

Fig.8. *Schematic illustration of the use of a photoelectric colorimeter for measuring bacterial populations. The instrument measures optical density (also termed absorbance). A function of light intensity which is almost linearly proportional to cell mass. (A) Adjustment of Instrument. A glass tube (cuvette) filled with uninoculated culture medium is used to set the instrument to give a basal optical density reading of 0. (B) The "blank" cuvette is replaced by a similar cuvette containing the broth culture (i.e., medium + cellls), and the increase in optical density is recorded.*

Table 1. **Summary of Methods for Measuring Bacterial Growth.**

Method	Some Application	Manner in which Growth is expressed
Microscopic count	Enumeration of bacteria in vaccines and cultures	Number of cells per ml.
Electronic enumeration	Same as for microscopic count	Same as for microscopic count
Plate count	Enumeration of bacteria in Milk, water, foods soil Cultures etc.	Colony-forming units per ml.

Method	Some Application	Manner in which Growth is expressed
Membrane filter	Same as plate count	Same as plate count
Turbidimetric Measurement	Microbiological assay, estimation of cell crop in broth, cultures, or aqueous suspensions.	Optical density (absorbance)
Nitrogen determination	Measurement of cell crop from heavy culture suspensions to be used for research in metabolism.	Mg. nitrogen per ml.
Dry weight determination	Same as for nitrogen determination	Mg. dry weight of cells per ml.
Measurement of Biochemical activity e.g. acid production per culture	Microbiological assays	Milliequivalents of acid per ml or per culture

Determination of Nitrogen Control

The major constituent of cell material is protein, and since nitrogen is a characteristic part of proteins, one can measure a bacterial population or cell crop in terms of bacterial nitrogen. Bacteria average approximately 14 percent nitrogen on a dry-weight basis, although this figure is subject to some variation introduced by changes in cultural conditions or differences between species. To measure growth by this technique, you must first harvest the cells and wash them free of medium and then perform a quantitative chemical analysis for nitrogen. Bacterial nitrogen determinations are somewhat laborious and can be performed only on specimens free of all other sources of nitrogen. Furthermore, the method is applicable only for concentrated populations. For these and other reasons, this procedure is used primarily in research.

Determination of the Dry Weight of Cells

This is the most direct approach for quantitative measurement of a mass of cells. However, it can be used only with very dense suspensions, and the cells must be washed free of all extraneous matter. Moreover, dry weight may not always be indicative of the amount of living material in cells. For example, the intracellular reserve material poly-β-hydroxybutyrate can accumulate in *Azotobacter beijerinckii* at the end of the log phase of growth and during the stationary phase and finally can comprise up to 74 percent of the dry weight of the cells, thus the dry weight may continue to increase without corresponding cell growth. Yet, for many organisms the determination of dry weight is an accurate and reliable way to measure growth and is widely used in research.

Measurement of a specific chemical change produced on a constituent of the medium

As an example of this method of estimating cell mass, we may take a species that produces an organic acid from glucose fermentation. The assumption is that the amount of acid produced, under specific conditions and during a fixed period of time, is proportional to the magnitude of the bacterial population. Admittedly, the measurement of acid or any other end product is a very indirect approach to the measurement of growth and is applicable only in special circumstances.

The Selection of a Procedure to measure Growth

Table 1 summarizes the methods described above for measuring bacterial growth. Each has its particular advantages and limitations, and no one method can be recommended universally. The best procedure for your work can be selected only after these factors are considered in relation to the problem at hand. The colony count is the most widely used procedure for general microbiological work, and complete familiarity with this technique, both in principle and practice, is essential. It should be emphasized that the colony count is theoretically the only technique that reflects the viable population. Furthermore, it is not at all unlikely that discrepancies may occur in results of growth of a bacterial population when measured by two different methods. For example, a microscopic count of a culture in the stationary phase would include all cells, viable and nonviable, whereas the colony count would reveal only the viable population.

Importance of Quantitative Measurement of Growth

Before we can evaluate or interpret growth responses of bacteria in different media or under various conditions, growth must be expressed in quantitative terms. In microbiology the term growth is used in several ways. For example, we may judge a certain set of conditions as being good because the bacteria grow rapidly, but the final total cell crop may not be as large as under another set of conditions where growth proceeds at a slower rate but continues to increase over a longer time period. Such a situation is shown schematically in Fig.9, where the growth of the same bacterial species is compared in two different media. If we measured growth at time A, we should conclude that growth is best in medium II; measured at time B, growth would be equally good in both media; and at time C, growth would be better in medium I. If we were primarily interested in a large cell crop, we should select medium I. In any event, we must have knowledge of growth in quantitative terms to make the correct choice.

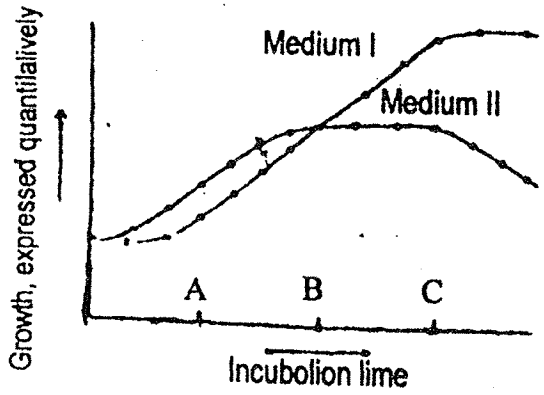


Fig.9. *Quantitative measurement of growth is significant for interpretation of various growth responses. Hypothetical growth response of same bacterium in medium of two different compositions. Compared the cell crops, or amount of growth, at times A, B and C.*

Effect of Environmental Factors on Growth

Activities of microorganisms are greatly affected by the chemical and physical conditions of their environments. Understanding environmental influences helps us to explain the distribution of microorganisms in nature and makes it possible for us to devise methods for controlling microbial activities and destroying undesirable organisms. Not all organism respond equally to a given environmental factor. In fact, an environmental condition may be harmful to one organism and actually beneficial to another. Regardless of whether organisms are interacting with other organisms in natural communities or with each other in pure culture in the laboratory, the environment can significantly affect their ability to carry out metabolic reactions and grow. Many environmental factors could be considered in this connection, however, four main factors have been identified that clearly play major roles in controlling microbial growth, temperature, pH, water ability, and oxygen. We consider each of these factors in detail here.

Effect of Temperature on Microbial Growth

Temperature is one of the most important environmental factors influencing the growth and survival of organism. It can affect living organisms in either of two opposing ways. As the temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates and growth becomes faster. However, *above* a certain temperature, proteins, nucleic acids, and other cellular components may be irreversible damaged. Thus as the temperature is increased within a given range, growth and metabolic function increase up to a point where inactivation reactions set in. Above this point, cell functions fall sharply to zero. Thus, we find that for every organism there is a **minimum temperature** below which growth no longer occurs, an **optimum temperature** at which growth is most rapid, and a **maximum temperature** above which growth is not possible (Figure 10). The optimum temperature is nearer the *maximum* than the *minimum*. These three temperatures, after called the **cardinal temperatures**, are generally characteristic of each type of organism but are not completely fixed, as they can be modified slightly by other factors of the environment – in particular, the composition of the growth medium.

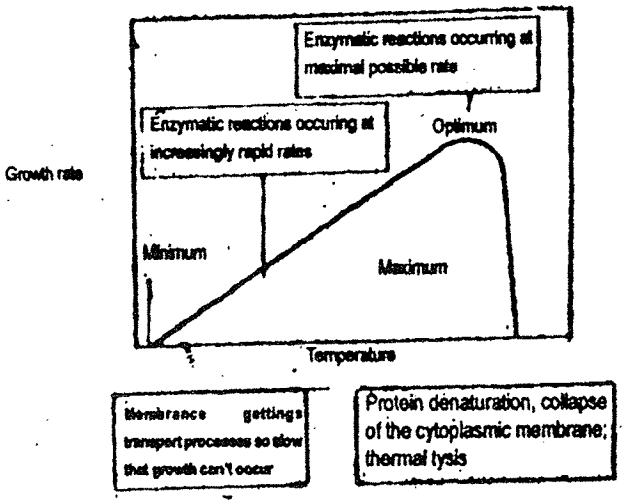


Fig.10. *Effect of temperature on growth rate and the molecular consequences for the cell.*

Temperature classes of organisms

Although there is a continum of organisms, from those with very low temperature optima to those with high temperature optima, it is possible to broadly distinguish *four groups* of microorganisms in relation to their temperature optima: **psychrophiles**, with low temperature optima, **mesophiles**, with midrange temperature optima, **thermophiles**, with high temperature optima, and **hyperthermophiles**, with very high temperature optima (Figure 11). Mesophiles are found in warm-blooded animals and in terrestrial and aquatic environments in temperature and tropical latitudes. Psychrophiles and thermophiles are found in unusually cold and unusually hot environments, respectively. Hyperthermophiles are found in extremely hot habitats such as hot springs, geysers, and deep-sea hydrothermal vents.

Microbial Growth at Temperature Extremes

Because humans live and work on the surface of the earth where temperatures are generally moderate, it is natural to consider very hot and very cold environment as being "extreme". And they are extreme for human habitation because humans would die quickly if immersed in boiling of freezing water. However, the natural habitats of many microorganisms can be either extremely hot or extremely cold, and the organisms that live there have evolved to grow optimally under these conditions.

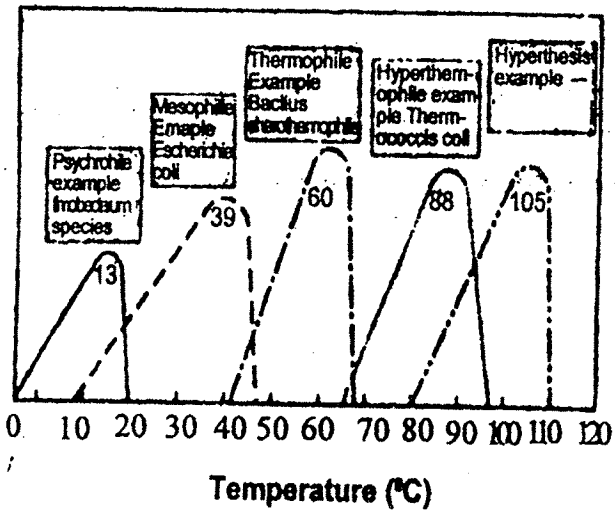


Fig.11. *Relation of temperature to growth rates of a typical psychrophile, a typical mesophile, a typical thermophile, and two different hyperthermophiles. The temperature optima of the example organisms are shown on the graph.*

Organisms with low temperature optima are called **psychrophiles.** A psychrophile can be defined as an organism with an optimal temperature for growth of 15°C or lower, a maximum growth temperature below 20°C and a minimal temperature for growth at 0°C or a lower. Organisms that grow at 0°C bit have optima of 20-40°C are called *psychrotolearnt.*

Molecular adaptations to psychrophily

Psychrophiles produce enzymes that function optimally in the cold and that are often denatured or otherwise inactivated at even very moderate temperatures. Another feature of psychrophiles is that compared

to mesophiles, active transport occurs well at low temperature, an indication that the cytoplasmic membranes of psychrophiles are constructed in such a way that low temperatures do not inhibit membrane phenomena. Studies on the composition of cytoplasmic membranes from psychrophiles have shown them to contain a higher content of *unsaturated* fatty acids, which help to maintain a semifluid state of the membrane at low temperatures (membranes composed of predominantly saturated fatty acids would become waxy and nonfunctional at low temperatures). The lipids of some psychrophilic bacteria also contain *polyunsaturated* fatty acids and long chain hydrocarbons with multiple double bonds. In the latter connection, a hydrocarbon with nine double bonds ($C_{31:9}$) has been identified from the lipids of several Antarctic bacteria.

Freezing

Despite the ability of some organisms to grow at low temperatures, there is a lower limit below which reproduction is impossible. Pure water freezes at 0°C and seawater at –2.5°C, but freezing is not continuous and microscopic pockets of water continue to exist at much lower temperatures. Although freezing prevents microbial growth, it does not always cause microbial death. In addition, the medium in which the cells are suspended considerably affects sensitivity to freezing. Water-miscible liquids such as glycerol and dimethylsulfoxide (DMSO), when added at about 10% (final concentration) to the suspending medium, penetrate the cells and protect by reducing the severity of dehydration effects and preventing ice crystal formation. In fact, the addition of such agents, called cryoprotectants, is a common way of preserving microbial cultures at a very low temperatures (usually –70°C to –196°C).

Table-2. Presently known upper temperature limits for growth of living organisms

Group	Upper temperature limits (°C)
Animals	
Fish and other aquatic vertebrates	38
Insects	45 – 50
Ostracods (crustaceans)	49 – 50
Plants	
Vascular plants	45
Mosses	50
Eukaryotic microorganisms	
Protozoa	56
Algae	55 – 60
Fungi	60 – 62
Prokaryotes	
Bacteria	
Cyanobacteria	70 – 74
Anoxygenic phototrophic bacteria	70 – 73
Chemoorganotrophic bacteria	90
Archaea	
Hyperthermophilic methanogens	110
Sulfer-dependent hyperthermophiles	113

High temperature environments and thermophiles and hyperthermophiles

Organisms whose growth temperature optimum is above 45°C are called **thermophiles**, and those whose optimum is above 80°C are called **hyperthermophiles**. Temperatures as high as these are found in nature only in certain restricted areas. For example, soils subject to full sunlight are often heated to temperatures above 50°C at midday, and some soils may become warmed to even 70°C, although a few centimeters under the surface the temperature is much lower. Fermenting materials such as compost piles and silage usually reach temperatures of 60-65°C. However, the most extensive and extreme high temperature environments are found in nature in association with volcanic phenomena. Many hot springs have temperatures near boiling and steam vents (fumaroles) may reach 150-500°C. Hyperthermal vents in the bottom of the ocean have temperatures of 350°C or greater.

Many hot springs are at the boiling point for the altitude (92-93°C at Yellowstone, 99-100°C at locations where the springs are close to sea level). As the water overflows the edges of the spring and flows away from the source, it gradually cools, setting up a *thermal gradient*. Along this gradient, various microorganisms grow with different species growing in the different temperature ranges. By studying the species distribution along such thermal gradients and by examining hot springs and other thermal habitats at different temperatures around the world, it is possible to determine the upper temperature limits for each kind of organism (Table 2). From this information we can condlude that (I) prokaryotic organisms in general are able to grow at temperatures higher than those at which eukaryotes can grow; (2) the most thermophilic of all prokaryotes are certain species of Archaea; and (3) nonphototrophic organisms are able to grow at higher temperatures than can phototropic forms. However, it should be emphasized that not all organisms from a group are able to grow near the upper limits for that group. Usually only a relatively few species or genera are able to function successfully near the upper temperature limit.

Molecular adaptations to thermophily

How can thermophiles and hyperthermophiles thrive at high temperatures? First, their enzymes and other proteins are much more stable to heat than are those of mesophiles/and these macromolecules actually function *optimally* at high temperatures. How is heat stability achieved? Studies of thermophilic enzymes have shown that they often differ very little in amino acid sequence from an enzyme that catalyzes the same reaction in a mesophile. It appears that a critical amino acid substitution in one or a few locations in the enzyme allows it to fold in a different way and thereby withstand the denaturing effects of heat.

Heat stability of proteins from hyperthermophiles is also improved as a result of the increased

number of *salt bridges* (bridging of changes on amino acids by Na^+ or other cations) present and the density packed highly hydrophobic interiors of the proteins, which naturally resist unfolding in the aqueous milieu. In addition to enzymes and other proteins in the cell, the protein-synthesizing machinery (that is, ribosomes and other constituents) of thermophiles and hyperthermophiles, as well as the cytoplasmic membrane, are likewise heat-stable. It has been found earlier that psychrophiles have membrane lipids rich in *unsaturated* fatty acids, thus making the membranes fluid and functional at low temperatures. Conversely, thermophiles have membrane lipids rich in *saturated* fatty acids, thus allowing the membranes to remain stable and functional at high temperatures. Saturated fatty acids form much stronger hydrophobic bonds than do unsaturated fatty acids, which accounts for the membrane stability. Hyperthermophiles, virtually all of which are Archaea, do not contain fatty acids in the lipids of their membranes but instead have hydrocarbons of various lengths composed of repeating units of the five-carbon compound phytane bonded by ether linkage to glycerolphosphate.

Why are eukaryotes absent from environments with temperatures above about 60°C (Table – 2)? This most likely involves the stability of organeller membranes, which must remain fairly porous to permit passage of large molecules like ATP and RNA. It is likely that porous membranes such as these would be more temperature-liable than the typical lipid bilayers of prokaryotes (or lipid monolayers of some hyperthermophiles). Thus, above 60°C, the organelles of eukaryotes cannot survive and the only life forms observed are prokaryotes.

Acidity and Alkanity (pH)

Acidity or alkanity of a solution is expressed by its pH on a scale on which neutrality is pH 7 (Figure 12). Those pH values that are less than 7 are said to be *acidic*, and those greater than 7 are *alkaline* (or *basic*). It is important to remember that pH is *logarithmic function*; a change of 1 pH unit represents a *10-fold* change in hydrogen ion concentration. Thus vinegar (pH near 2) and household ammonia (pH near 11) differ in hydrogen ion concentration by a billionfold.

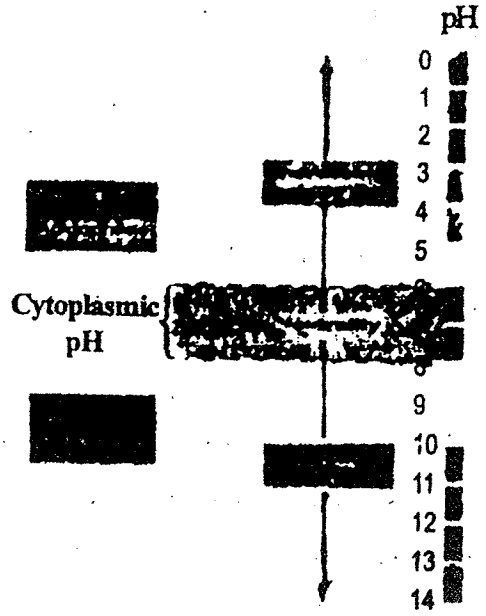
pH and microbial growth

Each organism has a pH range within which growth is possible and usually has a well-defined pH optimum. Most natural environments have pH values between 5 and 9, organisms with optima in this range are most common. Only a few species can grow at pH values less than 2 or greater than 10. Organisms that live at low pH are called **acidophiles**. Fungi as a group tend to be more acid-tolerant than bacteria. Many fungi grow optimally at pH 5 or below, and a few grow well at pH values as low as 2. Several bacteria are

also acidophilic. In fact, some of these bacteria are *obligate* acidophiles, unable to grow at all at neutral pH. Obligately acidophilic bacteria include several species of *Thiobacillus* and several genera of Archaea, including *Sulfolobus* and *Thermoplasma*.

A few organisms have high pH optima for growth, sometimes as high as pH 10-11, and are known as alkaphiles. Alkaphilic microorganisms are usually found in highly basic habitats such as soda lakes and high carbonate soils. Most alkaliphilic prokaryotes studied have been aerobic nonmarine bacteria, and many are Bacillus species. Some extremely alkaliphilic bacteria are also halophilic (salt-loving), and most of these are Archaea. Some alkaliphiles have found industrial uses because they produce hydrolytic enzymes, such as proteases, which function well at alkaline pH, and are used as supplements for household detergents.

Finally, concerning pH and microbial growth, it should be emphasized that despite the requirements of a particular organism for a specific pH for growth, the optimal growth pH represents the pH of the *extracellular* environment only; the *intracellular* pH must remain near neutrality in order to prevent destruction of acid – or alkali-labile macromolecules in the cell. In extreme acidophiles or extreme alkaliphiles the intracellular pH may vary by 1 – 1.5 units from neutrality, but for the majority of macroorganisms, whose pH optimum for growth is between pH 6 and 8 (referred to as **neutrophiles**), the cytoplasm remains neutral or very nearly so **(Figure 12).**



Example	Moles per liter H^+	OH^-
	1	10^{-14}
Volcanic soils, waters	10^{-1}	10^{-13}
Gastric fluids		
Lemon juice		
Acid mine drainage	10^{-2}	10^{-12}
Vinegar		
Rhubarb	10^{-3}	10^{-11}
Peaches		
Acid soil	10^{-4}	10^{-10}
Tomatoes		
American cheese	10^{-5}	10^{-9}
Cabbage		
Peas	10^{-6}	10^{-8}
Corn, salmon, shrimp		
Pure water	10^{-7}	10^{-7}
Seawater	10^{-8}	10^{-6}
Very alkaline natural soil	10^{-9}	10^{-5}
Alkaline lakes	10^{-10}	10^{-4}
Soap solutions		
Household ammonia	10^{-11}	10^{-3}
Lime (saturated solution)	10^{-12}	10^{-2}
	10^{-13}	10^{-1}
	10^{-14}	1

Fig.12. *The pH scale. Not that although microorganisms can live at very low or high pH, the cell's internal pH remains near neutrality.*

Buffers

In a batch culture the pH can change during growth as the result of metabolic reactions that consume or produce acidic or basic substances. Thus, chemicals called *buffers* are frequently added to microbial culture media to keep the pH relatively constant, such pH buffer generally work over only a narrow pH range; hence different buffers much be used to buffer at different pH values. For near neutral pH ranges (pH 6 – 7.5), phosphate, usually supplied as KH_2PO_4, is an excellent buffer. Many other buffers for use in microbial growth media or for the assay of enzymes extracted from microbial cells are available, and the best buffering system for one organism or enzyme may be considerably different from that of another. Thus, the optimal buffer for use in a particular situation must usually be determined empirically, although for assaying enzymes in vitro, a certain buffer that works well in an assay for enzyme from one organism will usually work for assaying this same enzyme from other organisms.

Water Availability

Water is the solvent of life. All organisms require water, and water availability is an important factor affecting the growth of microorganisms in nature. Water availability not only depends on the water content of an environment, that is, how moist or dry a solid microbial habitat may be, but is also a function of the concentration of solutes such as salts, sugars, or other substances that are dissolved in water. This is because dissolved substances have an affinity for water, which makes the water associated with solutes unavailable to organisms.

Oxygen

(Discussed in Module-1).

Chapter - 2
Bacterial Morphology

Contents

* Surface structures of the Procaryotic Cell
* Cell Membrane
* Cell Wall (Peptidoglycan component)
* Location of Peptidoglycan
* The Outer Membrane
* The Periplasm
* Peptodoglycan in gram-positive bacteria
* Protoplasts & Spheroplasts
* Capsule
* Flagella & Pili
* Special Procaryotic Organelles
* Reserve Materials
* The Nucleus and Chromosome
* Endospore

Surface Structures of the procaryotic cell

The outcome of the Gram reaction (Christion Gram 1884) is able to correlates with major differences in the chemical composition ultrastructure of eubacterial cell walls. It distinguishes the gram positive form the gram negative bacteria. These two groups along with the molecules, which completely lack cell walls, constitute the eubacteria. The Gram-negative group is larger and more diverse than the Gram-positive group; the molecules group is quite small.

Electron micrographs of thin section of the two types of cell wall reveal the wall of a Gram-positive bacterium to be a structure of almost uniform appearance. Some 10 to 80 nm width, whereas the wall of a Gram-negative bacterium is revealed to be composed of two readily distinguishable layers, both considerably thinner than the wall of a Gram-positive bacterium (Fig.1).

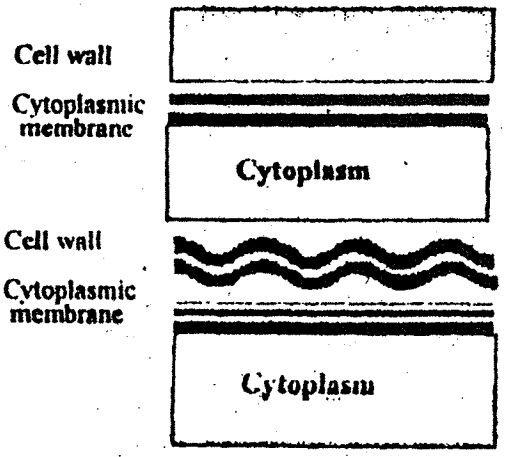


Fig.1. *Schematic interpretation of cell wall of eubacteria from electron-microscope observation. (A) Gram-positive bacteria, showing thick consisting mainly of peptidoglycan. Although the wall is often nomogenous in appearance, in some bacteria, it may consist of several layers. (B) Gram-negative bacteria, showing outer membrane and thin peptidoglycan layer.*

Chemical analyses show that the walls of Gram-positive bacteria contain peptidoglycan as a major component (generally accounting for 40 to 90 percent of the dry weight) with which are associated polysaccharides and a special class of polymers, the teichoic acids.

The inner layer of the walls of Gram-negative bacteria is very thin layer of peptidoglycan; the outer one is a membrane, termed the *outer membrane*.

Table 1. **Surface Structures of the Procaryotic Cell**

Structure	Location	Structure and Dimensions	Chemical Composition
Membrane	Bounding layer of protoplast	Unit membrane, 7.5-8 mm wide	20-30% phospholipid, remainder mostly protein
Wall	Layer immediately	Gram-negative eubacteria:	Peptidoglycan (murein),

Structure	Location	Structure and Dimensions	Chemical Composition
	External to Membrane	Inner single layer 2-3mm wide	Phospolipids, proteins, lipopolysaccharides
		Gram-positive eubacteria: Homogeneous layer 10-80 mm wide	Peptidoglycan(murein); teichoic acids; polysaccarides
		Archaebacteria: Variable	variable.
Capsule or slime layer	Diffuse layer external to wall	Homogeneous structure of low density and very variable width	Diverse; usually a polysaccharides, rarely a polypeptide
Flagella	Anchored in protoplast, traversing Membrane and wall	Helical threads, 12,18 mm wide	Protein
Pili	Anchored in Protoplast Traversing Membrane and wall	Straight threads, 4-35mm vide	Protein

In addition to the wall and the membrane; procaryotic cells maybe enclosed by a loose outer layer known as a *capsule* or *slime layer*. Also, two class of thread-shaped organelles, *flagella* and *pili*, occur on the cell surface of many bacteria. Table-1 summarizes the distinguishing properties of the various surface structures with the procaryotic cell.

The Surface Structure Of Archaebacteria

The chemical composition of the surface structures of Archaebacteria is quite different from that of other bacteria. Their walls are composition of protein or a form of peptidoglycan termed *pseudomurein* rather than the typical *murein* that is found in all other bacterial walls.

A significant difference exists between the phospholipids of eubacteria and those of archaeobacteria. In eubacteria the phospholipids are phosphoglycerides, in which straight-chain fatty acids are ester-linked to glycerol in archaeobacteria, the lipids are plolyisopreoid branched-chain lipids, in which long-chain beanched alcohol (phytanols) are ether-linked to glycerol.

The Cell Membrane

The eubacterial cell membrane, is made up of a bilayer of phopholipids which are the major lipids of bacterial cell membranes and which account for about 20 to 30 percent of their dry weight. The polar "head" regions of the phospholipids are located at the two outer surfaces of the bilayer, while the hydrophobic

fatty acid chains extend into the centre of the membrane, perpendicular to its plane (Fig. 2). The membrane proteins, which account for more than one-half of the dry weight of the membrane, are intercalculated into this phospholipid bilayer.

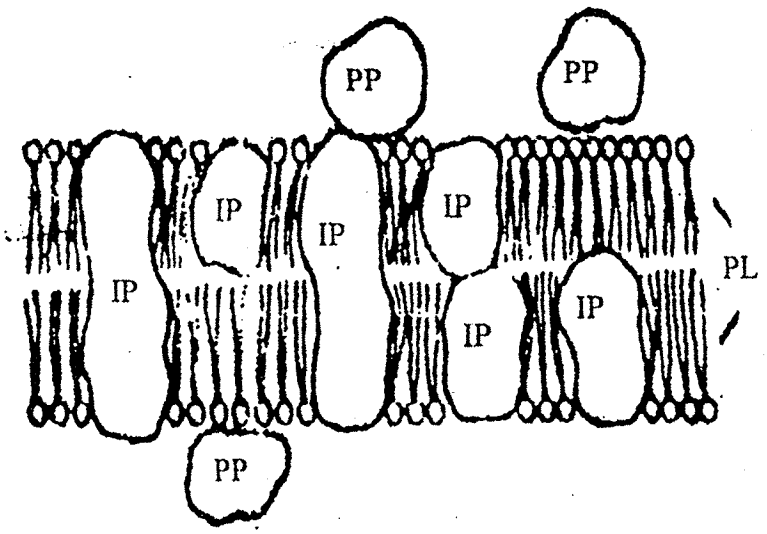


Fig.2. *Schematic interpretation of the structure of the cytoplasmic membrane. Phospholipids (PL) are arranged in a bilayer such that the pollar portions (circles) face outward and the nonpolar portions (filaments) face inward. IP=integral portion; PP=peripheral portions, are believed to span the membrane.*

The bacterial cell membrane is an important centre of metabolic activity; it contains many different kinds of proteins, each of which probably has a specific catalytic function. Most of these proteins are tightly integrated into the hydrophobic region of the membrane. Major classes of proteins known to be localized in the membrane include (1) the permeases responsible for the transport of many organic and inorganic nutrients into the cell; (2) biosynthetic enzymes that mediate terminal steps in the synthesis of the membrane lipids, and of the various classes of macromolecules that compose the bacterial cell wall (peptidoglycans), teichoic acids, lipopolysaccharides and simple polysaccharides; (3) the proteins that participate in generation of ATP in those bacteria that do so by electron transport. In respiratory bacteria the components of the electron transport chain and the ATP phosphotransferase are located in the membrane. In purple bacteria these, as well as the other components of the complete photosynthetic apparatus (antenna pigments and reaction centres) are also located in the membrane. Lastly procaryotic cell membrane contains specific attachment sites for the chromosome and for plasmids, and that it plays an active role in the partitioning of these genetic elements to daughter cells. The bacterial membrane contains from 10 to 20 percent of the total cell protein.

Complex, localized infoldings known as *mesosomes* occur in many bacteria, often at or near the site of cell division (Figure 3), and probably participate in the formation of the transverse septum.

Fig.3 *Electron micrograph of a thin section of a dividing cell of Bacillus megaterium, containing three mesosomes(m). One is located in association with the nearly formed transverse septum and wall.*

Membrane infoldings of a different type occur in purple bacteria and in many nonphotosynthetic bacteria that possess a high level of respiratory activity, such as the nitrogen fixers of the Azotobacter group and the nitrifying bacteria the greatly enlarged total area of the membrane produced by such intrusions serves to accommodate more centres of respiratory (or photosynthetic) activity than could be housed in a membrane of simple contour. The most convincing evidence in support of this interpretation has come from studies on the membrane structure of certain purple bacteria, where the photosynthetic pigment content (and hence photosynthetic activity) can very widely in response to environmental factors (light intensity, presence or absence of oxygen). Here, the extent of the membrane intrusions is directly related to the pigment content and photosynthetic activity of the cells.

In most procaryotes, there is a physical continuity between membrane intrusions and the surface region of the membrane. This may not be true, however, of the cyanobacteria. In these organisms, the photosynthetic apparatus is contained in a system of flattened membranous sacs (thylakoids), which have been very rarely observed in connection with the cell membrane, and may be in large part physically distinct from it.

The Bacterial Cell Wall : *Its peptideglycan Component*:

Of the many classes of macromolecules that may be associated with procaryotic cell walls, only one-the *peptidoglycans* – is of universal occurrence. The only procaryotes that possess walls devoid of peptidoglycans are certain *archaebacteria* and the *planctomyces* group of eubacteria.

Peptidoglycans are heteropolymers of substituted sugars and amino acid synthesized uniquely by procaryotes. The form of peptidoglycan (termed *murein*) found in eubacteria is always composed of two

acetylated amino sugars, N-acetylglucosamine, and N-acetylmuramic acid and a small number of amino acid, the particular representatives of which very amoung different groups. Indeed, on the basis of these variations, over 100 different chemotypes of mureins are now known. Some of the amino acids in murein are "unnatural" in the sense that they never occur in proteins. The form of peptidoglycan, *pseudomurein*, found in archaebacteria differs from murein in two respects; N-acetylmuramic acid is never present (in its place is another amino sugar acid, ***N-acetyltalosamimuronicacid***), nor are "unnatural" amino acids present.

Two amino sugars of muein form glycan strands composed of alternating residues of N-acetylglucosamine (g) and N-acetylmuramic acid (M) in beta-1,4 linkage (Figure 4). Each strand contain from 10 to 65 disaccharide units. Muramic acid, being a lactyl ether of glycosamine, provides a carboxy group to which a peptide chain is attached.

Adjacent peptide chains projecting from different glycan strains may be *cross linked* by formation of a peptide bond between the carboxyl group of a terminal D-alanine in one chain and the free a-amino group of *meso*-diaminopimemelie acid in another. Not all peptide chains participate in cross linkage, but enough to do from a molecular mesh or fabric (Figure-5). Infact, the peptidoglycan layer of the bacterial cell wall is made up of a single giant macromolecule that completely encloses the protoplast. In Grame-negative bacteria it occurs as a single layer; in Grame-positive bacteria there are many layers.

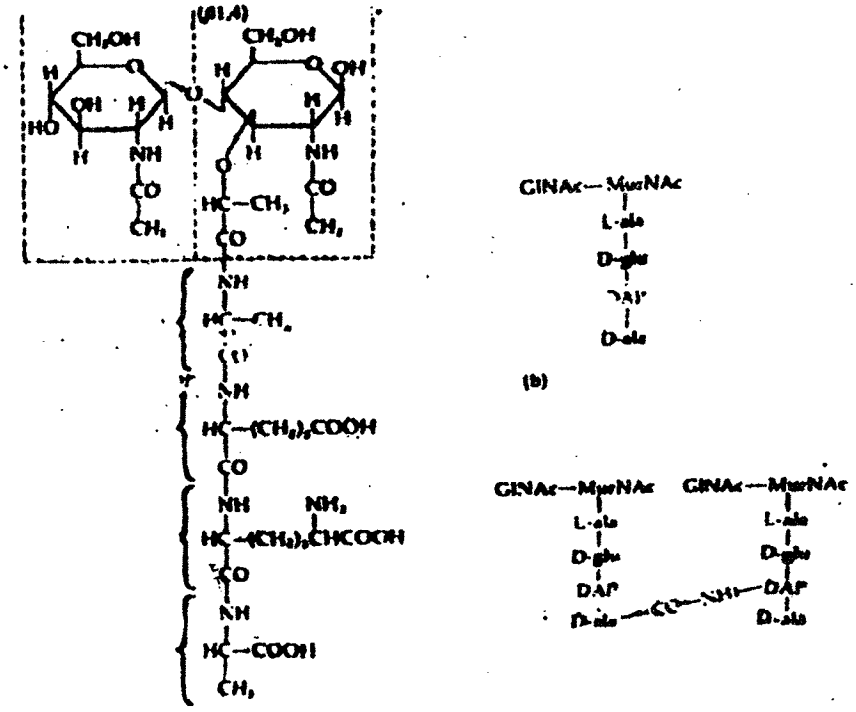


Figure 4 . *General structure of a peptidoglycan (mDpm-direct type). (a) Completer structure of a single subunit, showing the linkage between the two amino sugars that make up the glycanstrand, and between muramic acid and the four amino acids in the short peptide chain. (b) Schematic, simplified representation of structure shown in (a). (c) Representation of the mode of cross-linking between the terminal carboxyl group ao D-alanine on one subunit and the free amono group of the diamino acid (diaminopimelic acid) on an adjacent subunit).*

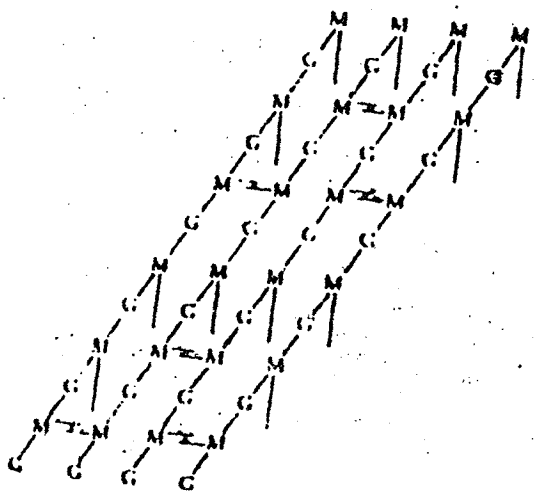


Fig.5. *A schematic representation of the intact peptidoglycan sac of E. coli; G and M designate residues of N-acetylglucosamine and N-acetylmuramic acid, respectively, joined (diagonal lines) by 1,4-glycosidic bonds. The vertical lines represent free tetrapeptide side chain, attached to muramic acid residues. The symbol represents cross-linked tetrapeptide side chains.*

The murein structure shown in Figure 4 sometimes termed m*Dpm-direct* is the most wide spread one; it occurs in the walls of nearly all Gram-negative bacteria and many Gram-positive ones. But there are many variation on this general pattern (Read Stanier P-153).

The Location of Peptidoglycan in the Walls of Grame-Negative Bacteria

The walls of Grame-negative bacteria have a comparatively low peptidoglycan content, seldom exceeding 5 to 10 percent of the weight of the wall. The location of the peptidoglycan layer in this type of wall was first established by W. Weidel and his collaborators, for walls of *Escheichia coli*. They showed that peptidoglycan constitutes the innermost layer of the multilavered wall and can be isolated as a very thin sac that retains the form and shape of the original cell, after other wall components have been stripped off it by appropriate treatments (see Figure-6).

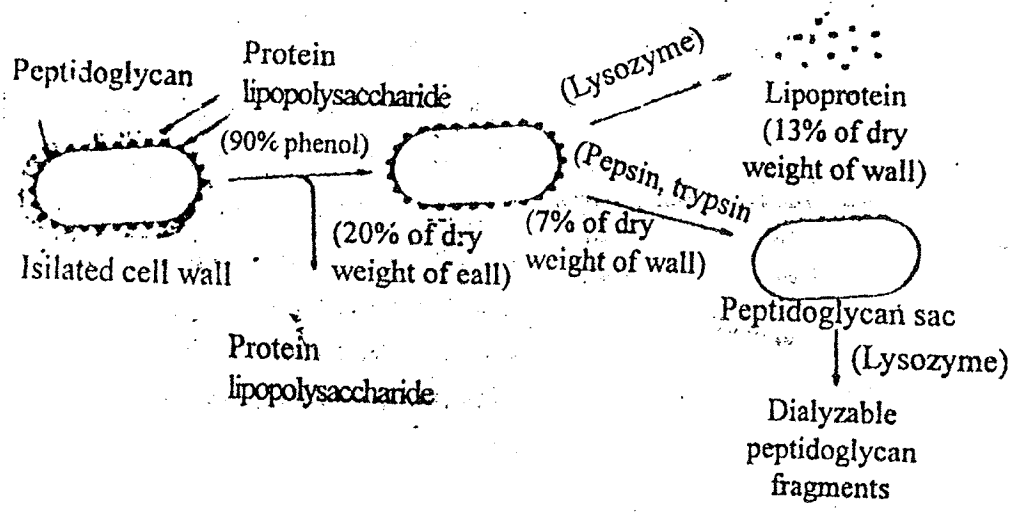


Fig.6. *A diagrammatic representation of successive steps in the fraction of the cell wall of E.coli.*

The peptidoglycans of Gram-negative bacteria characteristically display a rather low degree of cross-linkage between the glycan strands; many of the peptide chains are not cross-linked (see Figure-5). The thickness of the petidoglycan layer of the wall varies somewhat in different group of Gram-negative bacteria. Calculations suggest that in many Gram-negative organisms it is a monomolecular (or at most bimolecular) layer.

The Outer Membrane

Superimposed on the thin murien sac characteristic of Gram-negative bacteria is an outer layer that has the width and fine structure typical of a unit membrane. This layer, the *outer membrane*, has some chemical and physical properties in common with the cell membrane, and others that are quite different. Like the cell membrane, it is a lipid bilayer containing phospholipids and proteins, but in addition it contains large amounts of a unique lipid, lipopolysaccharide (LPS), which replaces, probably completely, phospholipids in the outer leaf of this unique structure. Although chemically quite different from a phospholipid, LPS has physical properties that are sufficiently similar so that it can participate in forming a membrane; one end of the molecule is hydrophobic and the other is hydrophilic, the hydrophobic end becomes inserted in the membrane's hydrophobic core and the hydrophilic end is on the outer surface. The structure of LPS is shown in Figure 7 and its orientation in the outer membrane is shown in Figure 8A.

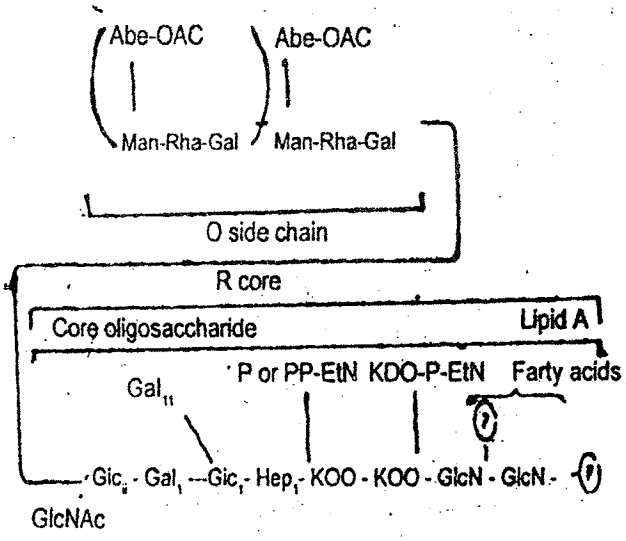


Fig.7. *Structure of the lipopolysacchari of Salmonell typhimurium. Abe, abequose; Man, D-mannose; Rha, Lose; Gal, D-galactose; GlcNAc, N-acetyl-D-glycosamine; Gle, D-glucose; Hep, Heptose; KDO, 2-keto-3 deoxyoctonic acid; EtN, ethanolamine: Ac, acetyl, Biosynthesis starts at the lipid A end, and the molecule is progressively elongated by the addition of sugar residue.*

LPS is composed of three distinct regions : *lipid A*, the *R core region*, and the *side chain*. Lipid A, the hydrophobic membrane anchoring region of LPS, rather than carrying the two fatty acid residues typical of a phospholipid has six or seven attached to a phosphorylated glucosmine diner. Unlike those in phospholipids, all the fatty acids in lipid A are saturated. Some are attached directly to the glucosamine dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. Attached to the opposition of one one glucosamine residue in lipid A is the R core oligosaccharide : a short chain of

sugars, which include two unusual ones, 2-keto-3-deoxyoctonoic acid (KDO) and heptose. The R core, in turn bears the hydrophilic Oside chain, likewise composed of sugar. It is much longer than the R core, being composed of many repeating tetra-or pentasaccharide units. The elucidation of structure depended heavily on the availability of mutants, each blocked at a particular point in LPS biosynthesis (Figure 7). Biosynthesis of LPS is strictly sequential, starting with lipid A from which the oligosaccharide is built by successive sugar additikon, theoside chain being added last. The innermost region, consisting of lipid A and three residue of KDO, appears to be essential, but the rest of the molecule is dispensable.

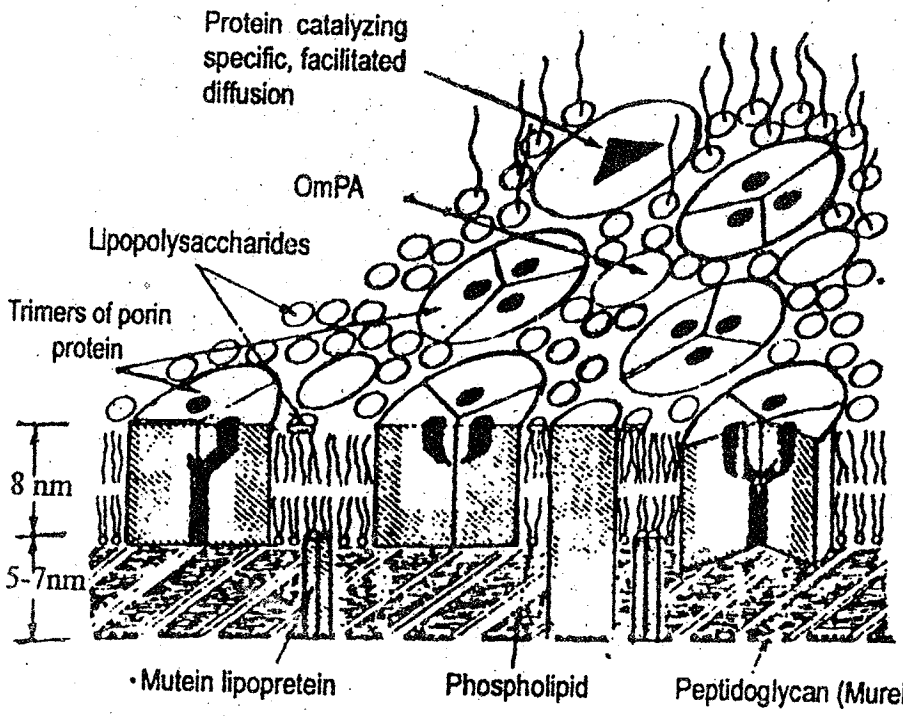


Fig.8. *Schematic model of the outer membrane of Eschericha coli and Salmonella tyhimurium showing the presumed arrangement of its components and its attachment to the murien layer.*

The peptidoglycan layer of the wall bears a small (MW = 7,200) specific type of lipoprotein termed *murein lipoprotein*, which forms an anchoring bridge to the outer membrane. The C-terminus of this protein is a lysine residue which is peptidebonded to an amine group of a *meso*-diaminopimelic acid residue that is not cross-linked in the peptidoglycan layer. At the other end (the N-terminus) of the protein is a cysteine residue to which fatty acids are attached; one is attached in an amide linkage to the terminal amino group, and two more are esterfied to a glycerol residue which is attached by sulfur ether linkage to the cysteine.

The resulting brushlike structure composed of the hydrophobic chains of three fatty acids becomes inserted into the inner leaf of the outer membrane, thereby anchoring it to the peptidoglycan layer.

Lipoprotein is quite abundant, there being 7×10^5 molecules of it in each cell, about half of which are bonded to peptidoglycan. In spite of its abundance and clear structural role, murein lipoprotein is apparently not essential to survival, atleast under conditions of laboratory cultivation, because munate strains have been isolated that grow well despite lacking this protein.

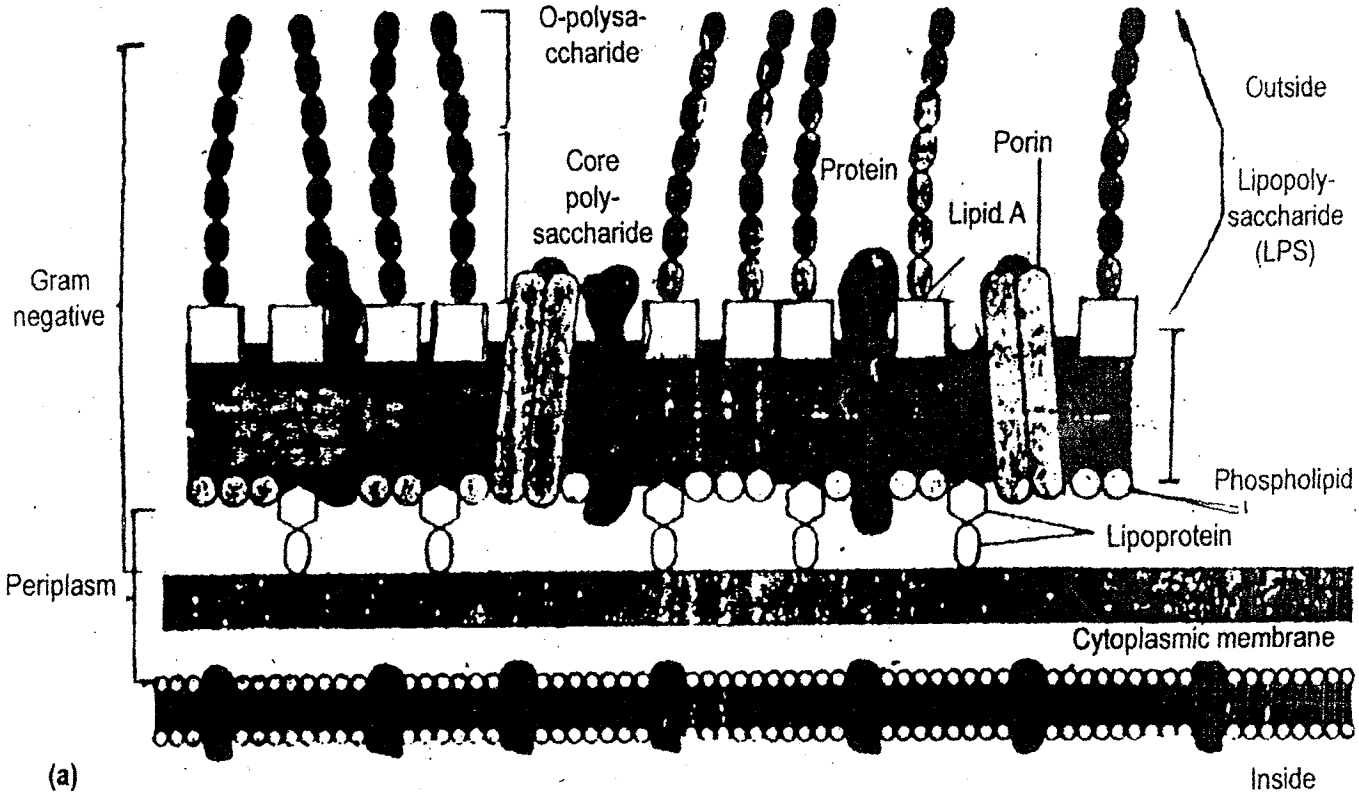


Fig.8A. *Bacterial LPS layer (a) Arrangement of lipopolysaccharide, lipid A, phospholipid, porins, and lipoprotein in gram-negative Bacteria.*

The physiological significance of the outer membrane is threefold : (1) it forms the outer limit of the *periplasm*, the region between the two membrane that contains a set of digestive enzymes (2) it presents an outer surface with strong negative charge which is important in evading phagocytosis and the action of complement and (3) it provides a permeability barrier and thereby increased resistance to a number of toxic agents. Among these are host defense agents like lysozyme, beta-lysin, and leucocyteprotein, which are quite toxic to grame-positive bacteria which lack an outer membrane.

Of course the outer membrane cannot present a barrier to all substances in the environment because all cellular nutrients must pass through it. Permeability of the outer membrane to nutrients is provided in part by proteins collectively termed *porins* which, in aggregates generally of three, form cross-membrane channels through which certain small molecules can diffuse (Figure 8). A variety of different porins are presents in the outer membrane (Table 2).

Table 2. Channel Proteins in the Outer Membrane of *E. coli* and *S. typhimurium.*

Protein	Physiological Role
PORINS	
OmpC	Forms small (1.1 nm) pores
OmpD	Present in *S. typhimurium* only
OmpF	Forms of larger pore (1.2 nm) than OmPC; repressed by elevated temperature and higher osmatic pressure
PhoE	Formed in response to restricted supply of phosphate
SPECIFIC CHANNEL PROTEINS	
LamB	Specific for the diffusional entrance of maltose and maltodextrins induced by maltose; site of adsorption of phage lambda
Tsx	Specific for the diffusional entrance of nucleosides; Side of adsorption of phage T6
TonA	Specific for diffusional entrance of ferrichrome; site of adsorption of phages T1 and T6.

In addition to the nonspecific channels formed by porins, the outer membrane contains a variety of channels formed by other proteins that exhibit a remarkable specificity. For example, the channel sometimes called the *maltoporin*, formed by the inducible LamB protein. Specifically allows the diffusional entrance of the disaccharide maltose and maltodextrans into the cell.

In addition to the channel-forming proteins, aprotein termed OmpA is quite abundant in the outer membrane. Its specific role has not been clearly defined, but mutant strains that lack it produce a more fragile outer membrane, so we assume that OmpA contributes in some way to the membrane's structural integrity.

The Periplasm

The region, termed *the periplasm*, between the cell membrane and the outer membrane of Gram-negative bacteria has been defined by ultrastructural and biochemical studies. Electron micrographs of the walls of Gram-negative bacteria typically reveal an open region on either side of the peptidoglycan layer that is identified as the periplasm. Biochemical studies have shown that a unique set of proteins (*periplasmic proteins*) are released from Gram-negative bacteria by treatments that disrupt the outer membrane while maintaining the cell membrane intact. Such studies establish the periplasmic location of this set of proteins.

Electron micrographs of Gram-negative walls have appeared that indicate that the peptidoglycan layer is, indeed, in intimate contact with both the cell membrane and outer membrane. These micrographs were made of material that was specially prepared to prevent damage to the cell envgelope, and they suggest that the appearance of the periplasm in conventional micrographs might be artifactual. The scientists who made these new micrographs suggest that the region between the two membranes of a Gram-negative bacterium is filled with highly hydrated peptidoglycan, only the outer portion of which is sufficiently cross-linked for it to be isolated as an intact structure; the inner portion of peptidoglycan consists of largely uncross-linked strands which detach from the intact saculus when the cvell is disrupted. Thus, these authors believe that the intermembrane region ought more properly to be termed a *periplasmic gel.*

Peptidoglycan In Walls of Gram-Positive Bacteria

In most Gram-positive bacteria, peptidoglycan account for some 40 to 90 percent of the dry weight of the cell wall. The wall is homogeneous in fine structure, and considerably thicker (10 to 80 nm) that the peptidoglycan wall layer in Gram-negative groups. As already mentioned, there is considerable diversity with respect to peptidoglycan structure and composition among Gram-positive bacteria. The peptidoglycan matrix of the wall is covalently linked to other macromolecular wall constituents which may include a wide variety of polysaccharides and polyolphosphate polymers known as teichoic acids. the teichoic acids are water-soluble polymers, containing ribitol or glycerol residues joined through phosphodiester linkages (Figure 9).

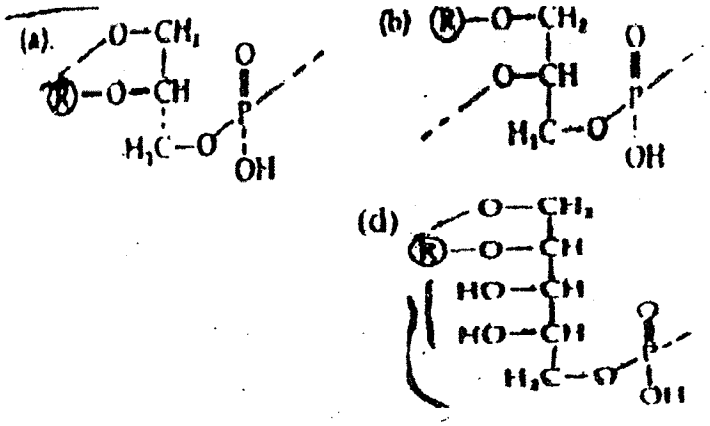


Fig.9. Repeats units of some teichoic acid. (a) Glycerol teichoic acid of *Lactobacillus casei* 7469 (R=D-alanine). (b) Glycerol teichoic acid of Actinomyces antibioticus (R=D-alanine). (d) Ribitol teichoic acids of Bacillus subtilis (R=glucose) and Actinomyces streptomycini (R=succinate). (The D-alanine is attached to position 3 or 4 ribitol).

Their exact location in the cell envelope is not certain; most of the teichoic acid remains associated with cell wall material during cell fractionation, and covalent linkage to muramic acid has been demonstrated. However, a small percentage (consisting entirely of glycerol teichoic acids) remains associated with the cell membrane. This material, called *membrane teichoic acid or lipoteichoic acid*, has been found to be convalently linked to membrane glycolipid. Much teichoic acid may lie within the peptidoglycan layer (Figure 10).

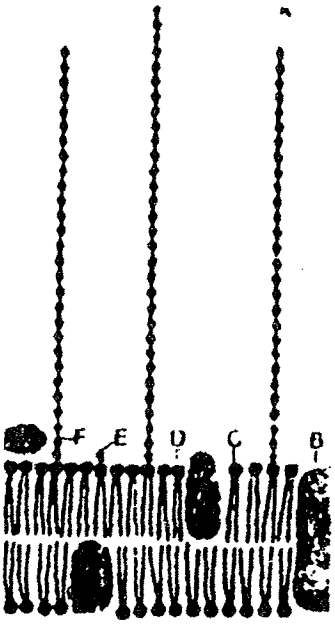


Fig.10. *A model of the cell wall and membrane of a Gram-positive bacterium, showing lipoteichoic acid molecules extending through the cell wall. The wall teichoic acids, covakently linked to muramic acid residues of the peptidoglycan layer, are not shown. (A) cell wall, (B) protein, (C) phospholipid (D) glycolipid, (E) phosphatidyl glycolipid, (F) lipoteichoic acid.*

The repet units of some teichoic are shown in Figure 9. The repeat units may be glycerol, joined by 1, 3 or 1, 2-linkages; ribitol, joined by 1,5-linkages; or more complex units in which glycerol or ribitol is joined to a sugar residue such as glucose, galactose, or N-acetyl glucosamine. The chains may be 30 or more repeat units in length, although chain lengths of 10 or less are common.

The function of the teichoic acids is unknown, but they do provide a high density of regularly oriented charges to the cell envelope, and these must certainly affect the passge of ions through the outer surface layers.

Function of The peptidoglycan Layer

The peptidoglycan layer appears to be the primary determinant of cell shape, as well as the wall component largely responsible for counteracting turgor pressure, and hence preventing osmotic lysis.

Protoplasts, Spheroplasts

Protoplasts

A protoplast is that portion of a bacterial cell consisting of the cytoplasmic membrane and the cell material bounded by it. Protoplasts can be prepared from Gram-positive bacteria by treating the cell with an enzyme such as lysozyme, which selectively dissolves the cell wall, or by culturing the bacteria in the presence of an antibiotic such as penicillin, which prevents the formation of the cell wall. some bacteria, the mycoplasms, never have cell walls and are bounded by only a cytoplasmic membrane; therefore, they have many of the properties of protoplasts, yet they manage to thrive nonetheless.

Spheroplasts

Round, osmotically fragile forms of Gram-negative bacteria can be prevented by procedures similar to those used for the protoplasts of Gram-positive bacteria. However, the cell walls of Gram-negative bacteria differ from those of Gram-positive bacteria by possessing an outer membrane. Although the peptidoglycan of the cell wall may be destroyed by lysozyme or its synthesis inhibited by antibiotics, the flexible outer membrane of the cell wall remains. Because the treated cell has two membranes, the cytoplasmic membrane of the protoplast plus the outer membrane of the cell wall, the cell is called a **spheroplast** rather than a protoplast.

Capsules

Some bacterial cells are surrounded by a viscous substance forming a covering layer or envelope around the cell wall. If this layer can be visualised by light microscopy using special staining methods it is termed a **capsule**. If the layer is too thin to be seen by light microscopy it is termed a **microcapsule**; if it is so abundant that many cells are embedded in a common matrix, the material is called **slime**.

In many instances capsular material is not highly water-soluble and therefore does not readily diffuse away from the cells that produce it. In other instances the material is highly water-soluble and dissolves in the medium, sometimes dramatically increasing the viscosity of the broth in which the organisms are cultured.

Capsules (Fig.11) can serve a number of functions, depending on the bacterial species. (1) They may provide protection against temporary drying by binding water molecules. (2) they may block attachment of bacteriophages. (3) They may be antiphagocytic; i.e., they inhibit the engulfment of pathogenic bacteria by white blood cells and thus contribute to invasive or infective ability (virulence). (4) They may promote attachment of bacteria to surfaces; for example, streptococcus mutans, a bacterium associated with producing dental caries, firmly adheres to the smooth surfaces of teeth because of its secretion of a water-insoluble capsular glucan. (5) if capsules are composed of compounds having an electrical charge, such as sugar-uronic acids they may promote the stability of bacterial suspension by preventing the cell from aggregating and setting out, because cell bearing similarly charged surfaces tend to repel one another;

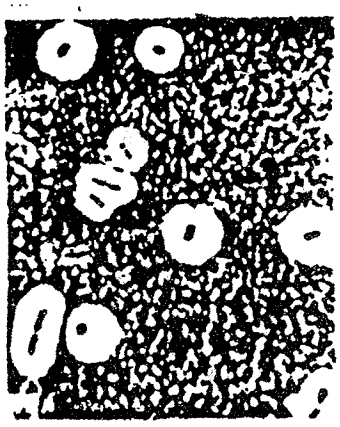


Fig.11. *Bacterial capsules as seen by light microscopy.*

Most bacterial capsules are composed of polysaccharides Capsules composed of a single kind of sugar are termed **homopolysaccharides**; are usually synthesized outside the cell from disaccharides by exocellular enzymes. The synthesis of glucan (apolymer of glucose) from sucrose by S. mutansis an example. Other capsules are composed of several kinds of sugar and are termed **heteropolysaccharides;** these are usually synthesized from sugar precursors that are activated (energized) within the cell, attach to a lipid carrier molecule, transported acoss the cytoplasmic membrane and polymerized outside the cell the capsule of *Klebsiella* pneumoniae is an example.

A few capsules are polypeptides. For example, the capsule of the anthrax organism, *B. anthracis*, is composed entirely of a polymer of glutamic acid. Moreover, this peptide in an unusual one because the glutamic acid is the rare D optical isomer rather than the usual isomer commonly found in nature.

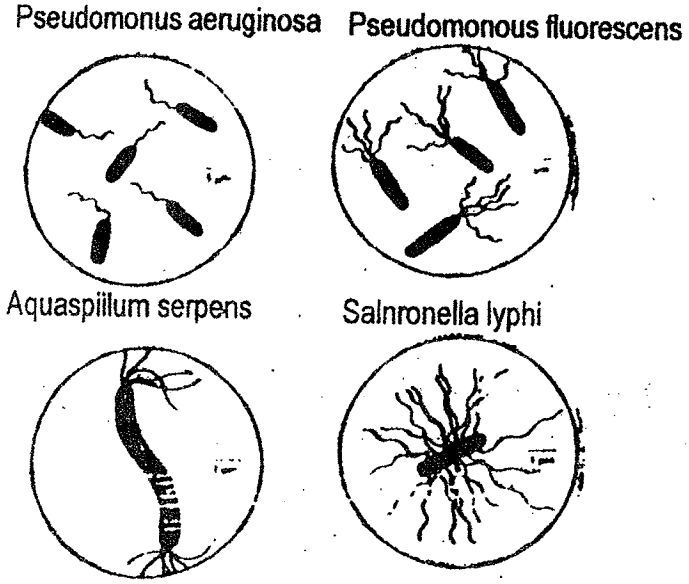


Fig.12. *Drawings of various arrangements of bacterial flagella. (A) Monotrichous; a single polar flagellum, (B) Lophotrichous; a cluster of polar flagella. (C) Amphitrichous; flagella, either single or clusters, at both cell poles, (D) Peritrichous; surrounded by lateral flagella. (Erwin F. Lessel, illustrator).*

THE MOLECULAR STRUCTURE OF FLAGELLA AND PILI

Although they differ both in function and in gross from, the two classes of filiform bacterial surface appendages, flagella and pili, share many common structural features. Both originate from the cell membrane and extend outward through the wall to a distance that may be as much as 10 times the diameter of the cell. The external part of these organelles can be detached from the cell by mechanical means (e.g. shearing in a blender), and subsequently isolated and purified. The filaments of flagella and pili are made up of specific proteins known as *flagellins* and *pilins*. Studies of isolated flagella and pili by electron microscopy and x-ray diffraction have shown that the protein monomers are assembled in helical chains, wound around a central hollow core. The structure of the filament is consequently a reflection of the properties of the specific type of protein subunit from which it is built; it is determined by the size of the subunit and by the number and pitch of the helical chains into which they aggregate. The flagella of different bacteria differ

slightly both in diameter (12 to 18 nm), and in form (i.e., height and wavelength of the helical curvature). Different types of pili differ greatly in width (4 to 35 nm). Theseminor variation within each class of organelle evidently reflect differences in the assembly properties of different flagellins and pilins. It has been shown that a single mutational change in the amino acid sequence of a flagellin can cause a change in the height any wavelength of the flagellum formed from it.

Fig.13. *Electron micrograph of a Vibrio with mixed polar-peritrichous flagellation. The cell bears a single sheathed polar flagellum, together with numerous laterally inserted unsheathed flagella (x 16,600).*

Some bacteria bear flagella that are notably thickend. These *sheathed flagella* are surrounded by an extension of the cytoplasmic membrane. Under certain condition of growth, some bacteria produce polar sheathed flagella along with many peritrichously arranged unsheathed flagella (Figure 13); such flagellation is termed *mixed flagellation.*

The probable ultrastructure of two filaments that have been studied in some detail, the flagellum of *Salmonella typhimurium* and the type I pilus of *Escherichia coli*, are shown schematically in Figure 13A.

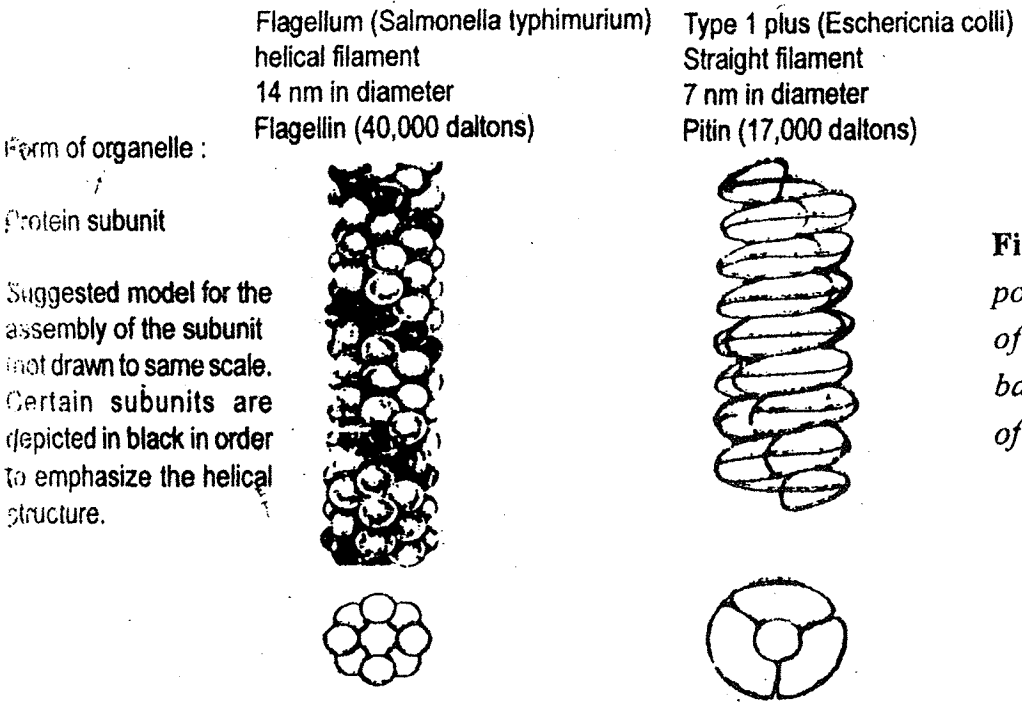


Fig.13A. *Models showing the portable helical arrangement of the protein subunits of a bacterial flagellum (left) and of a pilus (right).*

One function of type I pili is adhesion to surfaces including those of eucaryotic cells, and hence these pili are sometimes essential to the first step in infection. In these cases the tip of pilus attaches to specific receptor sites on the surface of the eucaryotic cell.

Another type of pili, termed *sex pili*, which are morphologically similar to type I pili but which are composed of different pilin monomers, are synthesized by cells that contain plasmids that determined the cell's capacity to carry out conjugative genetic exchanged with other cells. These pili also piay an attachment role, which in this case is the first step in the process of conjugation.

The basal Structure of the Flagellum

The entire flagellar apparatus is made up of three distinct regions. The outermost region is the helical flagellar filament, of constant width, made up of flagellin. Near the cell surface this is attached to a slightly wider *hook*, about 45 mm long, made up of a different kind of protein which is in turn attached to a *basal body*, located within the cell envelop.

The basal body consists of a small central rod, inserted into a system of rings. In gram-negative bacteria the basal body typically bears two pairs of rings (Figure 14). The outer pair (Land P rings) are situated at the level of the outer membrane; apparently, their function is to serve as bushings for the insertion of the body through this layer. The inner pair (S and M rings) are located near the level of the cell membrane: the M ring is embedded either in it, or just below it, while the S ring lies just above, possibly attached to the inner surface of the peptidoglycan layer. On flagella of Gram-positive bacteria, only the lower (S and M) rings are present; apparently, the upper pair is not required to support the rod as it passes through the relatively thick and homogeneous Gram-positive wall. this difference is significant, since it implies that only the S and M rings are essential for flagellar function.

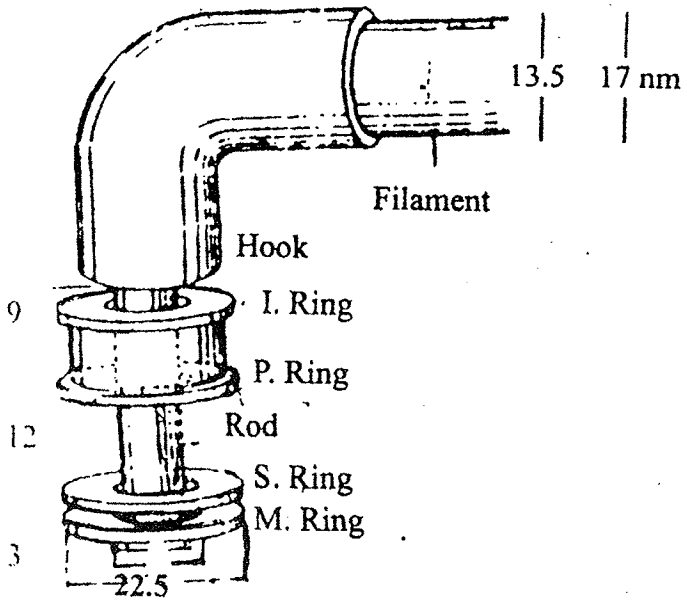


Fig.14. *Diagrammatic model of the basal end of the flagellum of E. Coli based on electron micrographs of the isolated organelle. From M.L. Pamphilis and J. Adler. "Fine Structure and Isolation of the Hook-Basal Body Complex of Flagella from Eschericha coli and Bacillus subtilis". J. Bacteriol. 105. 384 (1971).*

Hydrodynamics of Flagella

Large motile bodies such as boats and fish make use of the inertia of water for their propulsion. When pushed against with, for example, an our, a propeller blade, or fins, the water temporarily acts as a solid, thereby enabling the boat or fish to generate a forward propulsive force. However, the small size of bacteria probhibits their use of the intertia of water to gain propulsive force, because the drag forces due to the viscosity of water become thousands of times greater than any forces that can be generated from inertia. The difficulty would be similar to what we would encounter if we attempted to row a boat on a lake filled with thick molasses. However, bacteria can swim many times their own length per second under analogous conditions.

Bacteria propel themselves by rotating their helical flagella. The principle involved can be illustrated by imaging the penetration of a piece of cork by a corkscrew. If one tries to ram the corkscrew directly through the cork, great force will probably be needed. On the other hand if one merely rotates the corkscrew, the cork can be easily penetrated. In the case of bacteria, the cork is analogous to the viscous medium and the corkscrew to the helical flagellum. It is apparent from this analogy that a mutant bacterium having straight rather than helical flagella would be unable to swim. The nature of the rotary motor that spins each corkscrew-shaped flagellum is still not understood, but the rings found in the basal body are probably involved, it is known that the flagellar motor is driven by the protonmotive force, i.e. the force derived from the electrical potential and the hydrogen-ion gradien across the cytoplasmic membrane (see Chap.10). Moreover recent studies suggest that the concentration of cGMP (guanosine 3',5'-cyclic phosphoric acid) within the cell governs the direction in which the rotation occurs.

Bacteria having polar flagella swim in a back-and-forth fashion; they reverse their direction of swimming by reversing the direction of flagellar rotation. Bacteria having lateral flagella swim in a more complicated manner. Their flagella operate in synchrony to form a bundle that extends behind the cell (Figure 15). However when the flagellar motors reverse, conformation changes occur along the flagella, the bundle files apart, and the cell tumbles widely. Finally the flagellar motors resume their normal direction, the flagellar bundle again forms and the cell begins to swim but now in a different direction. This sequence of event occurs repeatedly, so that the motility becomes a series of periods of swimming (runs) punctuated by periods of tumbling (twiddles), with a change in direction after each tumble.

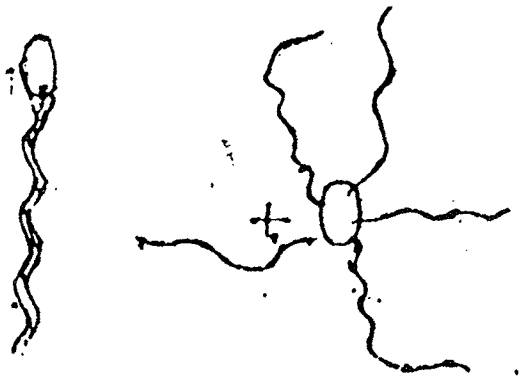


Fig.15. *Diagram of the configuration and arrangement of petrichous flagella during swimming and tumbling. The small arrows indicate the direction of propagation of helical waves along the flagella. (A) During swimming the flagella are in the form of left-handed helicles and rotate counterclockwise in synchrony to form a bundle. The large arrow indicates the direction of swimming. (B) During tumbling the flagella reverse their rotation, portions of the flagella acquire a short wavelength and right-handed configuration, and the bundle Mles apart. The cell cannot swim under these conditions and instead exhibits a chotic motion, as symbolized by the large crossed arrows.*

Swimming Motility Without Flagella

Certain helical bacteria (spirochetes) exhibit swimming motility, particularly in highly viscous media, yet they lack external flagella, however they have flagella. However they have flagella like structures located within the cell, just beneath the outer cell envelop. These are called **periplasmic flagella**; they have also been termed axial **fibrils** or **endoflagella**. They are responsible for the motility of spirochetes, but how they accomplish this is not yet clear. Other helical bacteria called **spiroplasmas** are able to swim in viscous media, yet lack any apparent organless for motility, even periplasmic flagella. The mechanism for their motility is completely unknown.

Gliding Motility

Some bacteria, e.g. Cytophaga species, are motile only when they are in contact with a solid surface. As they glide they exhibit a sinuous, flexing motion. This kind of movement is comparatively slow, only a few p.m. per second. The mechanism of gliding motility is unknown no organelles responsible for motility have been observed.

Bacterial Chemotaxis

Many, perhaps most, motile bacteria are capable of directed swimming toward or away from various chemical compounds – a phenomenon called **bacteria chemotaxis**. Swimming towards a chemical is termed **positive chemotaxis**; swimming away is **negative chemotaxis**. Although chemicals may act as attractants or repellents, the stimulus is in fact not the chemical itself but rather a change in the concentration of the chemical with time i.e., a **temporal gradient**. Such gradient are sensed by means of protein **chemorecepters** which are located on the cytoplasmic membrane and are specific for various attractants and repellents.

By means of its chemoreceptors, a bacterium continually compares its immediate environment with the environment it had experienced a few moments earlier. To illustrate this, suppose we are observing the behaviour of a bacterium that has peritrichous flagella and for which glucose is an attractant. If the cell is placed in a homogeneous glucose broth, the glucose concentration remains constant regardless of the direction of the bacterium's swimming and the glucose-specific chemoreceptors can sense no change in glucose concentration. Consequently, the cell exhibits a normal swimming pattern-periods of swimming with intermittent periods of tumbling. Suppose that the cell is now placed in a long capillary tube with a higher concentration of glucose at one end than at other. If the cell happens to swim toward the higher concentration of glucose (i.e., in the "right" direction), the chemoreceptors sense that the glucose concentration is increasing with time. This results in suppression of normal tumbling, causing the cell to swing smoothly ahead for a long period before it tumbles. On the other hand, if the cell happens to swim toward the end of the tube where there is less glucose (i.e. in the "wrong" direction), the chemoreceptors sense that the glucose concentration is decreasing with time, and no suppression of tumbling occurs. Therefore, the cell soon tumbles, changes direction, and tries again until finally the "right" direction is

achieved. (In a gradient of a repellent compound) the right direction would be *down* the gradient, i.e., towards a *decreasing* concentration, and the wrong direction would be up the gradient).

Tactic responses are not limited to chemical gradients. For instance, phototrophic bacteria exhibit positive **phototaxis** toward increasing light intensities and are repelled by decreasing light intensities, still another type of taxis is exhibited directed swimming in response to the earth's magnetic field or to local magnetic fields (magnets placed near the culture). This is attributed to a chain of magnetite inclusions (**magnetosomes**) within the cell, which allows the cell to become oriented as a magnetic dipol (Figure 16). Because of the downward inclination of the Earth's magnetic field in the regions where these bacteria have been found, **magnetotaxis** may serve to direct the cell downward in aquatic environments toward oxygen-deficient areas more favourable for growth (For Details see magnetosome in the next page).

SPECIAL PROCARYOTIC ORGANELLES

As already mentioned, most procaryotic do not form intracellular organelles bounded by unit membranes, the only possible exceptions are the thylakoids, which house the photosynthetic apparatus of cyanobacteria. However, three classes of procaryotic organelles are bounded by *nommit membranes,* made up of protein, at least in part, in all cases. These are *gas vesicles, chlorosome,* and *carboxysomes (polyhedral bodies).*

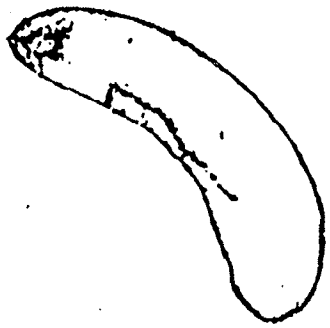


Fig.16. *Transmission electron micrograph of a magnetotactic bacterium and the single string of magenetosomes that it contains.*

Gas Vesicles and Gas Vacuoles

Since most cells have a slightly hogher density than water, they tend to skin in an aqueous medium at a rate that is a function of cell size. This effect can be counteracted by swimming against the gravitational pull. However, many aquatic procaryotes have developed another device to counteract gravitational pull, their cells contain gas-filled structures known as *gas vacuoles.* By light microscopy, gas vacuoles appear densely refractile, and have an irregular contour. If such cells are subjected to a sudden sharp increase in hydrostatic pressure, the gas vacuoles collapse, and the cells simultaneously lose their buoyancy and become much less refractile. Electron microscopy shows that gas vacuoles are compound organelles made up of a variable number of individual *gas vesicles.* Gas vacuoles occur in a wide variety of procaryotes including

archaebacteria purple bacteria, cyanobacteria, green bacteria, and various groups of chemoheterotrophic eubacteria the only common denominator of all these organism is ecological they occur in acquatic habitats, there can be little doubt accordingly, that the function of gas vacuoles is to enable their possessors to regulate the buoyancy of the cell in order to occupy a position in the water column that is optimal for their metabolic activity with respect to light intensity, dissolved oxygen concentration, or the concentration of other nutrients.

Chlorosomes

In the green bacteria, part of the photosynthetic apparatus has a distinctive intracellular location: the antenna pigments are housed in a series of cigar-shaped vesicles, arranged in a cortical layer that immediately underlies, but is physically distinct from, the cell membrane. These structures, detectable only by electron microscopy. The photosynthetic pigments are entirely contained within them; these are probably the sites of the photosynthetic apparatus.

Carboxysomes (Polyhedral Bodies)

A number of photosynthetic bacteria (cyanobacteria, certain purple bacteria) and chemoautotrophic bacteria (nitrifying bacteria, thiobacilli) contain structures termed *polyhedral bodies*. These structures contain most of the cellular content of ribulose bisphosphate carboxylase (carboxydismutase), the key enzyme in the fixation of CO_2 associated with the operation of the Calvin-Benson cycle. They have been termed *carboxysomes,* and evidently represent the principal site of CO_2 fixation in these autotrophic procaryotes.

Magnetosomes

In 1975 a remarkable group of bacteria were described by R P Blakemore that are magnetotactive, i.e., when they are placed in a magnetic field as weak as 0.2 gauss they orient and swim towards one or another of the magnetic poles. The sensing organelles, termed magnetosomes, within these cells are uniformly shaped enveloped crystals of magnetite (Fe_3O_4), a ferrimagnetic meneral. These are often arranged in a string (Fig. 16) the essentiality of the preence of magnetosomes to magnetotaxis can be clearly established by the simple experiment of growing these bacteria in amedium that contains a restricted concentration of iron; such cells do not contain magnetosomes and they are not magnetotactic.

Magnetosomes appear merely to orient the cells in a magnetic field and thereby determine the direction in which they swim; they are not an object that is pulled by a magnetic field as can be shown by the fact that dead cells are not magnetotactic.

The selective advantage of magnetotaxis seems to lie in the relationship of these bacteria to oxygen. All are acquatic organisms, found in either freshwater or marine environments and they are either microaerophiles or strict anerobes. In northern latitudes, the earth's magnetic field is included downward; in southern latitudes it is directed upward. Thus, magnetotactic bacteria that happened to be suspended in

the water column by some disturbance of the bottom layer could, by swimming along the magnetic field lines, return to the bottom where microaerophilic conditions are favourable for their survival. This hypothesis is materially strengthened by the observation that magnetotactic bacteria from the Northern Hemisphere are almost exclusively, north-seeking and those from the Southern Hemisphere are almost exclusively south-seeking. In both cases, magnetotaxis directs cells in a downward direction. Curiously, populations of magnetotactic bacteria collected at the geomagnetic equator where the magnetic field has no vertical component are composed of equal mixtures of north-seeking and south-seeking cells.

THE PROCARYOTIC CELLULAR RESERVE MATERIALS

A variety of cellular reserve materials may occur in procaryotic organism; they are frequently detectable as granular cytoplasmic inclusions. Distribution of Nonnitrogeneous Organic Reserve Materials has been shown in Table 3.

Table – 3

The distribution of nonnitrogenous organic reserve materials among procaryotes

A. GLUCANS
   Cyanobacteria (most reprsentative)
   Enteric bacteria (most genera, except those listed under B).
   Sporeformers: many *Bacillus* and *Clodtridium* species.
B. POLY-β-HYDROXYBUTYRATE
   Enteric bacteria genera Vibrio and Photobacterium Pseudomonous (many species)
   Azotobacter group (Azotobacter, Beijerinckia, Derxia) Rhizobium
   Moraxella (some species)
   Spirillum
   Sphaerotilus
   Bacillus (some species)
C. BOTH GLUCANS AND POLY-β-HYDROXYBUTYRATE
   Cyanobacteria (a few species)
   Purple bacteria.
D. NO DEMONSTRATABLE RESERVE MATERIAL
   Green bacteria
   Pseudomonous (many species)
   Acinetobactor

Note : This list is partial and includes only groups in which the nature of reserve materials have been systematically investigated. [For details read Gen. Microbiology by Stainer et al].

Nitrogeneous Reserve Materials

As a rule, procaryotes do not produce intracellular nitrogenous organic reserve materials. However, many of the cyanobacteria accumulate a nitrogenous reserve material known as cyanophycin when cultures approach the stationary phase. It is a copolymer of arginine and aspartic acid.

Polyphosphate Granules

Many microorganisms, both procaryotic and eucaryotic, many accumulate *polyphosphate granules*, which are stainable with basic dyes such as methylene blue, these bodies are also sometimes termed *volutin* or *metachromatic granules*, because they exhibit a *metachromatic effect*, appearing red when stained with a blue dye. In electron micrographs of bacteria they appear as extremely electron-dense bodies the polyphosphates are linear polymers of orthophosphate, of varying chain lengths. The polyphosphate granules therefore appear to function primarily as an intracellular phosphate reserve, formed under a variety of conditions when nucleic acid synthesis is impeded.

Sulfur Inclusions

Inclusions of inorganic sulfur may occur in two physiological groups; the purple sulfur bacteria, which use H_2S as a photosynthetic electron doner, and the filamentous, nonphotosynthetic organism, such as *Beggiatoa* and *Thiothrix*, which use H_2S as an oxydizable energy source. In both these groups, the accumulation of sulfur is transitory and takes place when the medium contains sulfide after the sulfide in the medium has been completely utilized, the stored sulfur is further oxidized to sulfate.

THE NUCLEUS

Recognition and Cytological Demonstration of Bacterial Nuclei

Basic dyes, which selectively stain the chromatin of the eucaryotic nucleus, stain most bacterial cells densely and evenly. The basophilic property of the bacterial cell is caused by the abundance of ribosomes, which confers as usually high nucleic acid content on the cytoplasmic region. Hence, in order to stain selectively the bacterial nucleus, the fixed cells must first be treated with ribonuclease or with dilute HCl, which hydrolyzes the ribosomal RNA subsequent staining with a basic dye then reveals the bacterial nuclei as dense, centrally located bodies of irregular outline; two to four are present in an exponentially growing cell, the growth and division of bacterial nuclei in living cells can be observed by the phase-contrast microscopy, provided that the cells are suspended in a medium (e.g., a concentrated protein solution) that enhances the slight difference in contrast between nucleoplasm and cytoplasm.

In Electron micrograph it appears as a region closely packed with fine fibrils of DNA. This region is not separated from the cytoplasm by a membrane, and contains no evident structures, apart from the DNA fibrils.

The Bacterial Chromosome

By 1960 the cytological information about the structure of the bacterial nucleus had been complemented by genetic studies of E. coli, which suggested the presence of a single circular linkage group. This in turn implied that each nucleus should contain a single circular chromosome. If such were indeed the case, the fibrils of DNA revealed in the nuclear region by electron microscopy should represent sections of an extremely long circular molecule of DNA, highly folded to form a compact mass.

In 1963, J. Cairns succeeded in extracting DNA from E. coli under conditions that minimize its shearing (Radioautography).

Examination of the developed radioautographs showed that the DNA was present as extremely long threads, the longest of which were slightly more than 1 mm in length. Furthermore, a few of the threads were circular.

Actually folded chromosomes are associated with a considerable amount of RNA and protein the protein is largely RNA polymerase and the RNA is newly transcribed single RNA strands RNAse treatment causes a rapid increase in viscosity, which indicates that some of the associated RNA is responsible for holding the DNA into a compact form. The DNA in these structures is folded into a number (between 12 and 80) of supercoiled loops. In the light of these facts, the folded chromosome can be represented schematically as shown in Figure 17.

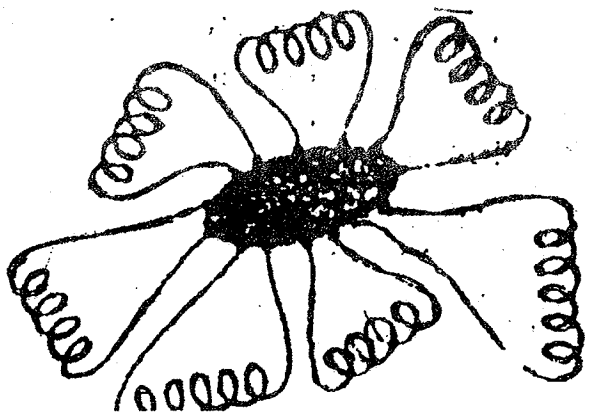


Fig.17. *A schematic drawing illustrating the proposed structure of the folded chromosome of E. coli the chromosome is shown as seven loops, each twisted into a superhelix (the actual number is much greater) and hold together by a core of RNA (shaded area).*

SPORES AND CYSTS

Certain species of bacteria produce spores, either within the cell (endospores) or external to the cell (exospores). The spore is a metabolically dormant form which under appropriate conditions can undergo germination and outgrowth to form a vegetative cell.

Endospores

These structures are unique to bacteria. They are thick-walled, highly refractile bodies that are produced (one per cell) by *Bacillus clostridium, Sporosarcina, Thermoactinomyces* and few other genera. The shapes of endospores and also their location within the vegetative cell vary depending on the species (Fig. 18). Endospores are usually produced by cells growing in rich media but which are approaching the end of active growth. Various factors such as aging or heat treatment are needed to activate the dormant spores (i.e. permit them to be able to undergo germination and outgrowth when they are placed in a suitable medium).

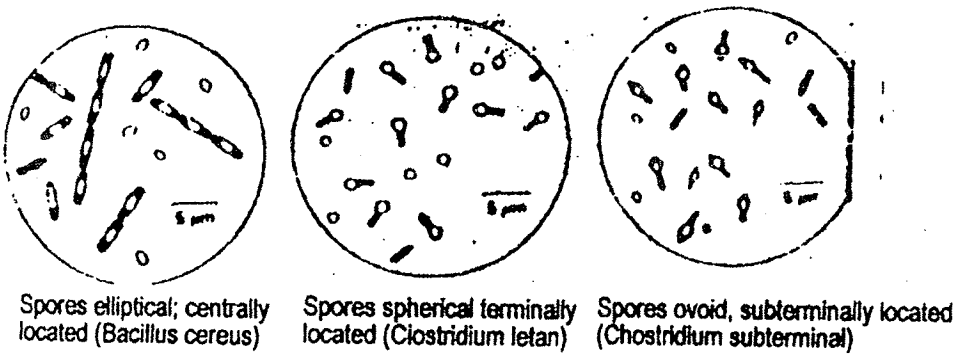


Fig.18. *Drawings showing the location, size and shape of endospores in cells of various species of Bacillus and Clostridium.*

Endospores are extremely resistant in dislocation, staining, disinfecting chemicals, radiation and heat. For example, the endospore of *Clastridium botulinum* type A have been reported to resist boiling for several hours. The degree of heat resistance of endospores varies with amounts of calcium and is probably located in the core i.e. in the central part of the spore. The calcium-DPA complex may possibly play a role in the heat resistance of endospores.

Endospore Formation

The structural events associated with spore formation have been elucidated by a combination of light and electron microscopic observations. A synthesis based on both types of observations is presented in Figure 19.

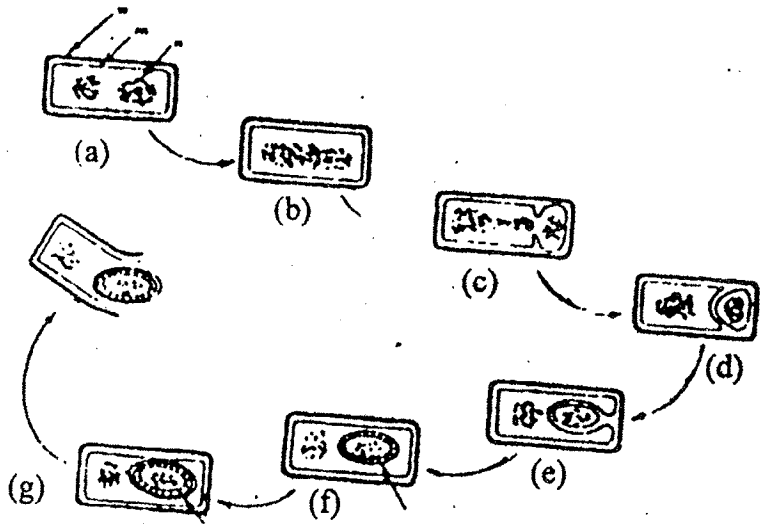


Fig.19. *A diagrammatic representation of the cytological changes accompanying endospore formation in Bacillus cercus. (a) Vegetative cell, containing two nuclear bodies (stippled area). (b) Condensation of the nuclear material. (c) Beginning of transverse wall; the forespore with its nuclear material is now out off from the vegetative cell. (e) Engulfment of the forespore. (f) Synthesis of the spore cortex. (g) Synthesis of the spore coat, (h) Liberated spore w. cell wall m cell membrane; n nuclear area; c cortex, sc spore coat.*

At the end of exponential growth, each cell contains two nuclear bodies. These coalesee to form an axial xhromatin thread, the first definite sign of the onset of sporulation. A transverse septum is then formed near one cell pole, which separates the cytoplasm and the DNA of the smaller cell (destined to become the spore) from the rest of the cell contents. Septum formation is not accompanied, as in normal cell division, by the development of a transverse wall, instead, the membrane of the larger cell rapidly grows around the small cell, which thus becomes completely engulfed within the cytoplasm of the larger cell, to produce a so-called forespore. In effect, the forespore is a protoplast, enclosed by two concentric sets of unit membrane, and the membrane of the mother cell which has grown around it. At this stage, the development process becomes irreversible; the cell is said to be "committed" to undergo sporulation. By phase contract microscopy, the forespore appears as a dark, nonrefractile area that is free of granular inclusions.

Once the forespore has been engulfed by the mother cell, there is a rapid synthesis and deposition of new structures that enclose it. The first to appear is the cortex, which develops between the two membranes surrounding the forespore. Shortly afterward, a more electrondense layer, the spore coat, begins to form exterior to both membranes surrounding the cortex. In the B cereus group an additional, looser and thinner layer, the exosporium, forms outside the spore coat. Once the spore coat is synthesized, the maturing spore begins to become refractile, although it is not yet heat-resistant. The development of heat resistance closely follows two major chemical changes; a massive uptake of Ca2+ ions by the sporulating cell, and the synthesis in large amount of dipicolinic acid, a compound absent from vegetative cells.

The cortex is largely composed of a unique peptidoglycan, containing three repeating N-acetyl-glycosamine-muramic lactum subunit, without any attached amino acids; an alanine subunit, without any attached amino acids; an alanine subunit, bearing only an L-alanyl residue; and a tetrapeptide subunit, bearing the sequence L-ala-D-glu-meso-DAP-D-ala (Figure 20). These subunits represent, respectively, approximately 55, 15 and 30 percent of the total. There is very little cross-linking between tetrapeptide chains.

The outer spore coat, which represents 30 to 60 percent of the dry weight of the spore, is largely composed of protein and accounts for about 80 percent of the total spore protein. The spore coat proteins have an unusually high content of crysteine and hydrophobic amino acids, and are highly resistant to treatments that solubilize most proteins.

After the completion of spore development, the spore protoplast, accordingly, contains a high content of Ca dipicolinate and is enclosed by newly synthesized outer layers of unique chemical structure (the cortex and the spore coat, sometimes also an exosporium), which accounts for a large fraction of the spore dry weight. When liberated by autolysis of the mother cell, the mature endospore is highly dehydrated, show no detectable metabolic activity.

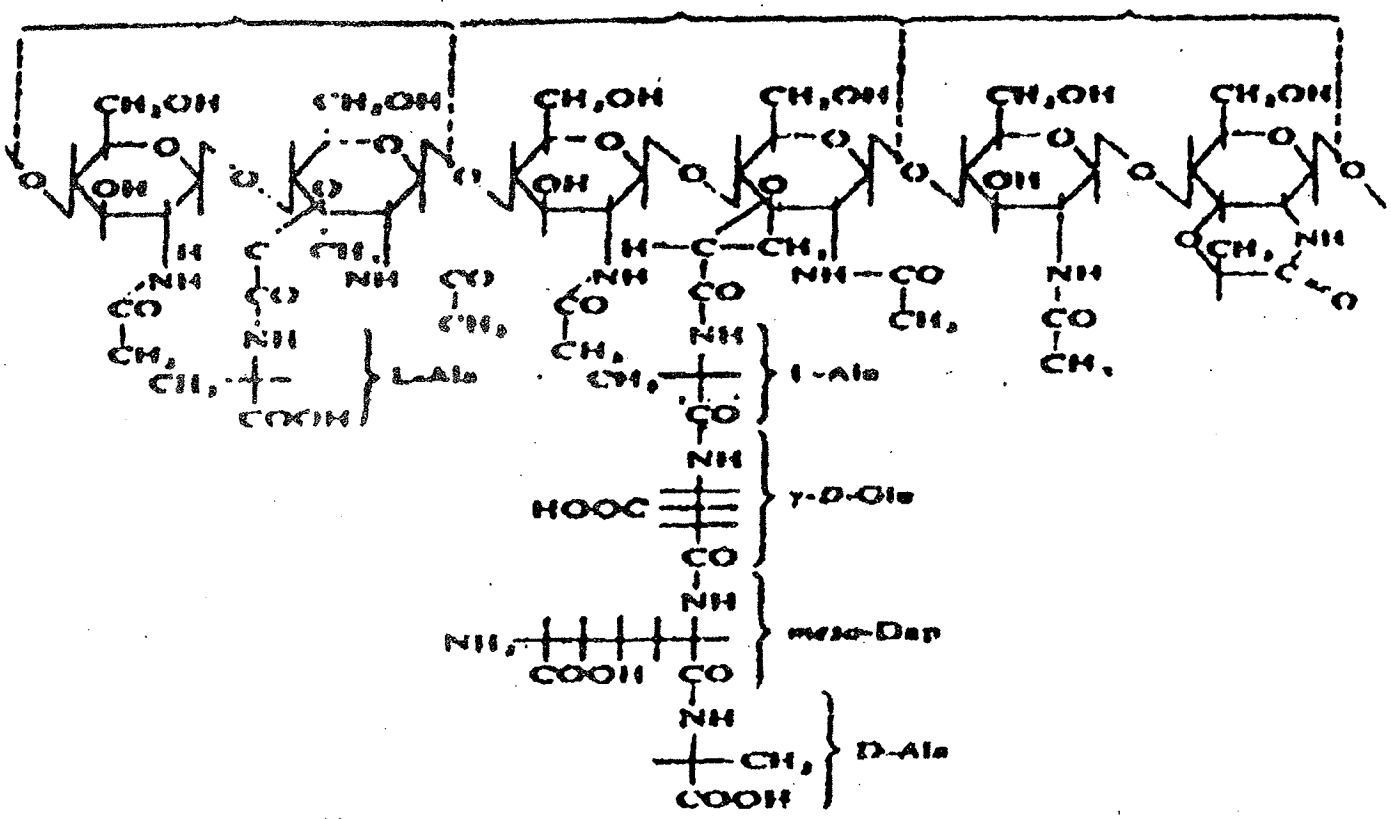


Fig.20. *Structures of the repeating subunit of the spore cortex peptidoglycan.*

Other Biochemical Events Related to Sporulation

Although the synthesis of an endospore is the main enterprise of a aporulating cell, it is by no means the only one. one striking concomittant event, characteristic of Bacillus thuringenisis, is the formation of a bipyramidal parasporal protein crystal adjascent to each endospore. Various non-crystalline parasporal structures of defined form have been described in other species of Bacillus and Clostridium.

In many sporoformers, both aerobic and anaerobic, the onset of sporulation is accompanied by the synthesis of a distinctive class of antimicrobial substances; peptides with molecular weights of approximately 1,400 daltons. Many of these peptide antibiotics have been characterized chemically and functionally. They can be assigned to three classes edeines, linear basic peptides that inhibit DNA synthesis; bacitracins, cyclic peptides that inhibit cell wall synthesis; and the gramicidin-polymyxin-tyrocidin-type peptides, which are linear or cyclic and modify membrane structure of function.

Suggested Questions

Write Notes on :

(a) Synchronous Growth; (b) Exponential Phase; (c) Chemostat; (d) Endospore; (e) Bacterial flagella; (f) Cell wall of gram negative bacteria; (g) Periplasms; (h) Capsule or slime layer; (i) Cell membrane.

In the log phase of growth the number of bacterium remains constant. Does this mean the cells are dormant and inert? Explain.

During log phase of growth of bacterial culture, a sample is taken at 8.00 am and found to contain 1000 cells per millimeter. A Second sample is taken at 5.54 pm and is found to contain 1,000,000 cells per millimeter. What is the generation time in hours.

How is growth rate controlled in a chemostat?

Compare the direct and indirect methods for estimating bacterial population on the basis of (a) Practical application (b) advantages (c) limitations of use.

Or

Compare the structure and chemistry of the cell wall of gram positive eubacteria & those of gram negative eubacteria.

Botany
Chapter - 3 , Paper - I (1st Half)
Microbial Metabolism

CONTENTS

Bacterial fermentation :-

Befinition between Substate level phosphorylation and oxidative Phosphorylation

Major pathways of carbohydrate breakdown

Pathways of utilization of Pyruvate by Aerobes.

Glyoxylate Cycle

Special pathways for Primary attack on organic compounds by microorganisms

The fueling reactions of anaerobic chemotroph

Anaerebic respiration

Fermentation.

CHAPTER – 3

(BACTERIAL FERMENTATION)

The pathways for the oxidation of organic compounds and conservation of energy in ATP can be divided into two major groups : (1) **fermentation,** in which the O-R process occurs in the absence of any added terminal electron acceptors; and (2) **respiration,** in which molecular oxygen or some other oxidant serves as the terminal electron acceptor.

In the absence of externally supplied electron acceptors, many organisms perform in*ternally balanced* oxidation – reduction reactions of organic compounds with the release of energy, a process called fermentation. There are many different types of fermentation's but under fermentative conditions only partial oxidation of the carbon atoms of the organic compound occurs and therefore only a small amount of the potential energy available is released. The oxidation in a fermentation is coupled to the subsequent reduction of an organic compound generated from catabolism of the initial fermentable substrate, thus, no externally supplied electron acceptor is required (Figure-1).

Fermentation and substrate-Level Phosphorylation :

ATP is produced in fermentations by a process called substrate-level phosphorylation. In substrate level phosphorylatio, ATP is synthesized during specific enzymatic steps in the catabolism of the organic compound. This is in contrast to oxidative (or electron transport) phosphorylation, where ATP is produced via membrane-mediated events not connected directly to the metabolism of specific substrates.

An example of fermentation is the catabolism of glucose by a lactic acid bacterium :

Glucose $\rightarrow$2 lactate$^-$ + 2H$^+$

($C_6H_{12}O_6 \rightarrow 2C_3H_4O_3^- + 2H^+$)

Note that this is a balanced reaction and that the products, lactate plus protons, have the same proportion of hydrogen and oxygen atoms as glucose. Likewise, a similar situation exists in the catabolism of glucose by yeast in the absence of oxygen:

Glucose $\rightarrow$2 ethanol + 2 carbon dioxide

($C_6H_{12}O_6 \rightarrow 2C_3H_4O_3^- + 2H^+$)

Note that in this reaction some of the carbon atoms end up in CO_2 a more *oxidized* form than the carbon atoms in the starting molecule, whereas other carbon atoms end up in ethanol, which is more *reduced* (that is, it has more hydrogens and electrons per carbon atom) than glucose. In each fermentation, oxidation-reduction is internally balanced.

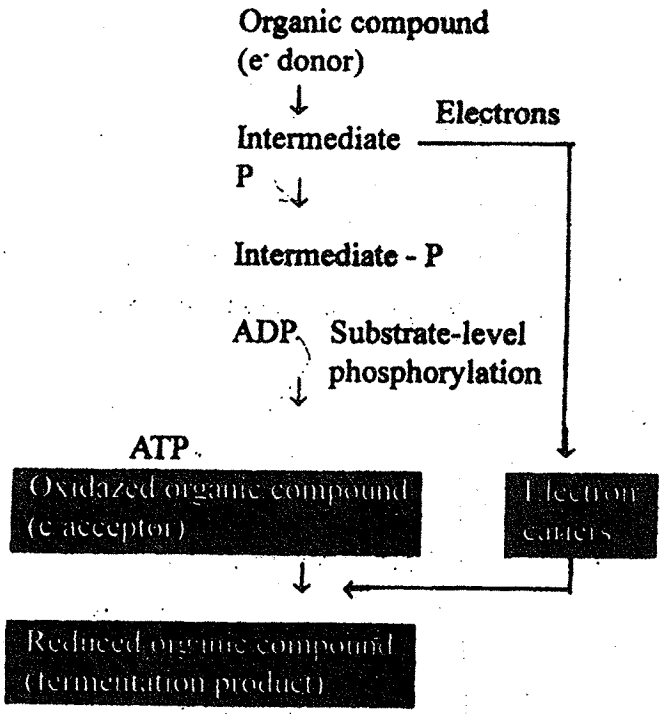


Figure 1 *Carbon and electron flow in fermention. Note that there is no externally supplied electron acceptor and that ATP is produced by donation of a high energy phosphate group from a phosphorylated intermediate directly to ADP.*

MAJOR PATHWAYS OF CARBOHYDRATE METABOLISM :-

A. PATHWAYS LEADING TO PYRUVATE FORMATION
   - I. Glycolysis : Embden-Meyerhof Parnas (EMP) scheme.
   - II. Hexose monophosphate (HMP) pathway (pentose phosphate or Warburg-Dickens pathway)
   - III. Entner-Duodoroff (ED) pathway.
   - IV. Phosphoketolase pathways.
     1. Pentose phosphoketolase pathway.
     2. Hexose phosphoketolase pathway.

B. AEROBIC PATHWAYS OF PYRUVATE METABOLISM
   - I. Krebs tricarboxylic acid (TCA) cycle or citric acid cycle.
   - II. Electron transport.
   - III. Glyoxylate cycle (special modification of the TCA cycle).

C. FERMENTATION
   - I. Alcoholic fermentation

II. Lactic acid fermentation
   1. Homolactic fermentation
   2. Heterolactic fermentation.

III. Propionic acid fermentation
   1. Propionic acid fermentation from glucose.
   2. Propionic acid fermentation from lactate.

IV. Formic acid fermentation
   1. Mixed acid producers.
   2. Butanediol producers.
   3. Butyric acid producers.

There are four major pathway of carbohydrate breakdown in micro-organisms. These pathways result in the breakdown of sugars to the key metabolite *pruvate.* The pathways are :

I. Glycolysis : the Embden-Meyerhof-Parnas (EMP) pathway. (Fig. 2).

II. The hexose monophosphate (HMP) pathway (pentose phosphate pathway or Warburg-Dickens pathway). (Fig. 3)

III. The Entner-Duodoroff (ED) pathway. (Fig. 4)

IV. The phosphoketolase pathways (Fig. 5 & 6)

The EMP and the HMP pathways occur in both prokaryotes and eukaryotes. The ED and phosphoketolase pathway are restricted to prokaryotes. The Enterobacteriaceae as facultative anaerobes mainly use the EMP and HMP pathways, while the Pseudomonadaceae prefer the ED pathway.

The major pathway for the aerobic metabolism of pyruvate is the *Krebs tricarboxylicacid* (TCA) cycle or the citric acid cycle. This pathway is the major aerobic pathway for aerobic generation of ATP, which is produced by oxidative phosphorylation through the *electron transport system.* A special modification of the TCA cycle, the glyoxylate cycle, is present in many bacteria, algae and some higher plants.

(A) PATHWAYS LEADING TO PYRUVATE FORMATION

I. Glycolysis (Embded-Meyerhof-Parnas Pathway)

The sequence of reactions which converts *glucose acid* along with the production of ATP is known as *glycolysis.* The most common pathway in glycolysis is the *Embden-Meyerhof-Parnas (EMP) pathway.* It

is found in many organisms, both prokaryotes and eukaryotes. Mammalian biochemists use the term 'glycolysia' for the pathway by which *glycogen or glucose* is converted via *pyruvic acid to lactic acid under anaerobic* conditions. Among the micro-organisms this complete pathway from glucose to lactic acid is found only in the homofermentative lactic acid bacilli. The term ' glycolysis' is therefore best restricted to its mammaalian usage. In aerobic organisms glycolysis leads to the Krebs citric acid cycle and the electron transport chain, which release most of the energy contained in glucose. Under aerobic conditions pyruvate *entere* the *mitochondria* where it is completely oxidized to CO_2 and H_2O. In actively contracting muscie, where there is insufficient oxygen supply, pyruvate is converted to *lactate*. In the fermentation of certain anaerobic organisms, e.g. yeast, pyruvate is converted into ethylalcohol (ethanol). The formation of lactate is known as lactic acid fermentation and that of ethanol, *alcoholic fermentation.*

The EMP pathway is found in many microorganisms e.g., Enterobacteriaceae, Lactobacillaceae, saccharolytic clostridia and yeasts.

In glycolysis, as shown in Fig.2 fructose –1, 6-diphosphate formed from glucose is split into two 3-carbon units (dihydroxyacetone phosphate and glyceraldehyde-3 phosphate) and they are subsequently oxidized to pyruvic acid. At the step where glyceraldehyde-3 phosphate is oxidized, a pair of electrons (two hydrogen atoms) is removed in the absence of oxygen, this pair of electrons may be used to reduce pyruvic acid to lactic acid or ethanol. In the presence of oxygen, this pair of electrons may enter the respiratory chain.

Many of the reactions of the glycolytic pathway are freely reversible and can be used for the synthesis of glucose as well as for its breakdown. Only three of the reactions are not reversible by common enzymes; but the presence of other enzymes can reverse them for glucose synthesis to occur. Thus phosphoenol-pyruvate is synthesized from ppyruvate by the action of phosphoenolpyruvate synthase and specific phsphatases hydrolyze fructose-1, 6-diphosphate and glucose-6 phosphate in the biosynthetic direction. The enzymes at these steps in the degradative direction are kinases and require ATP (See Fig. –2). For each molecule of glucose metabolized, two molecules of ATP are used up and four molecules of ATP are formed. Therefore for each molecule of glucose metabolized by glycolysis, there is a net yield of two ATP molecules. This is shown in Fig.

The overall reaction of glycolysis can be summarized as follows :

$$C_6H_{12}O_6 + 2NAD+2ADP+2P_i \rightarrow 2CH_3COCOOH+2NADH_2 + 2ATP$$

Glucose Inorganic Phosphate Pyruvic acid

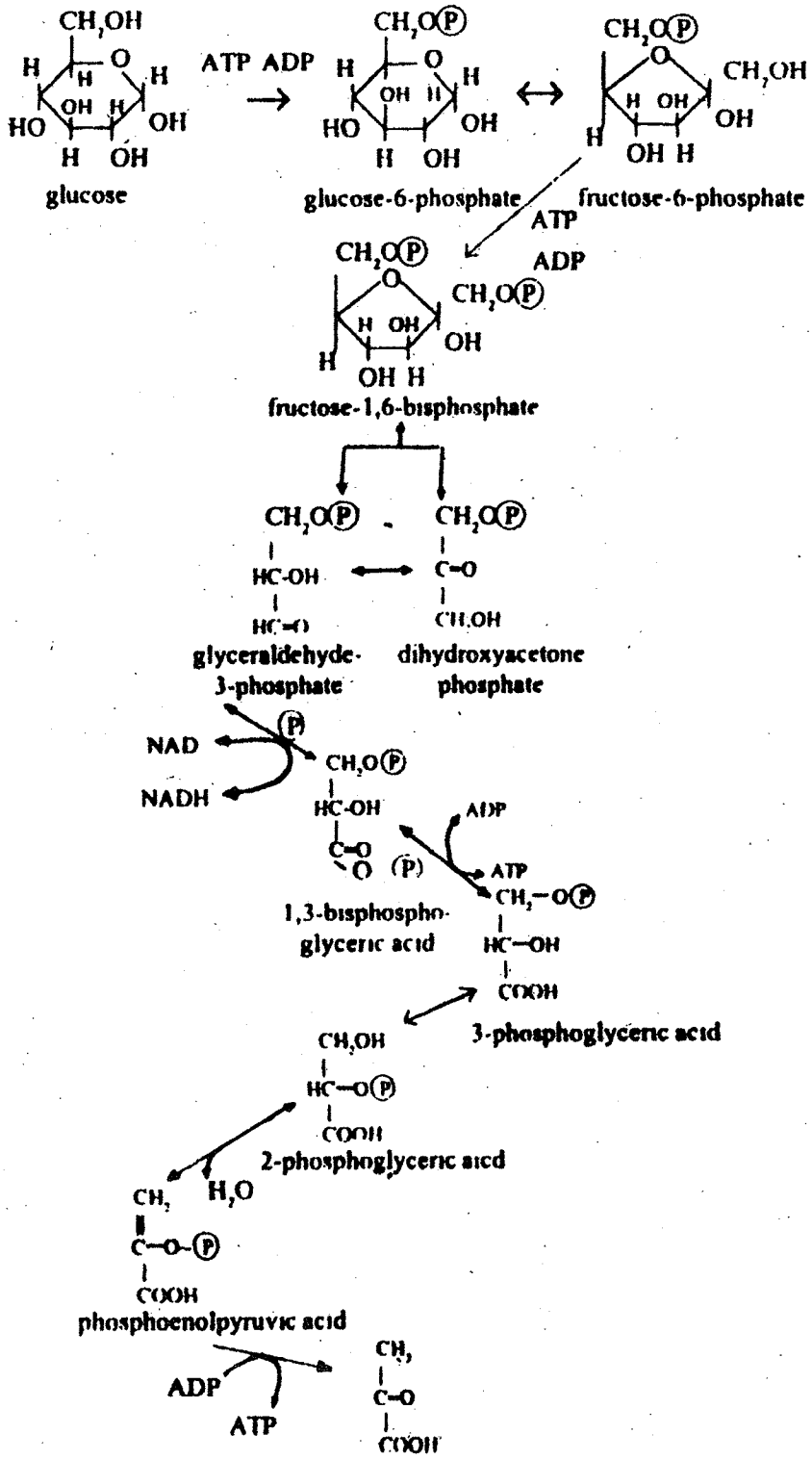


Figure –2 *The Embden-Meyerhof (glycolytic) pathway of conversion of glucose to pyruvic acid. The six precursor metabolities formed as intermediates are shown in color.*

II. The Pentose Phosphate Pathway:

The pentose pathway, like the glycolytic one, is another catabolic reaction pathway that exists in both procaryotic and eucaryotic fells. Since it involves some reactions of the glycolytic pathway, it has been viewed as a "shunt" of glycolysis; hence it may also be called the hexose monophosphate shunt. Its other synonym is the phosphogluconate pathway.

Glucose can be oxidized by the pentose phosphate pathway with the liberation of electron pairs, which may enter the respiratory chain. However, this cycle is not generally considered a major energy-yielding pathway in most micro-organisms. It provides reducing power in the form of NADPH + H^+, which is required in many biosynthetic reactions of the cell, and it provides pentose phosphates for use in nucleotide synthesis. Although it can produce energy for the cell as an alternate pathway for the oxidation of glucose, it is also a mechanism for obtaining energy from 5-carbon sugars.

As seen in Fig.3 the pentose phosphate pathway involves the initial phosphorylation of glucose to form glucose-6 phosphate; the latter is oxidized to 6-phosphogluconic acid with the simultaneous producetion of NADPH. Decarboxylation of 6-phosphogluconic acid, together with a yield of NADPH, produces ribulose 5 phosphate. Epimerization reactions yield xylulose-5 phosphate and ribose-5 phosphate. These two compounds are the starting point for a series of transketolase reactions and transaldolase reactions leading

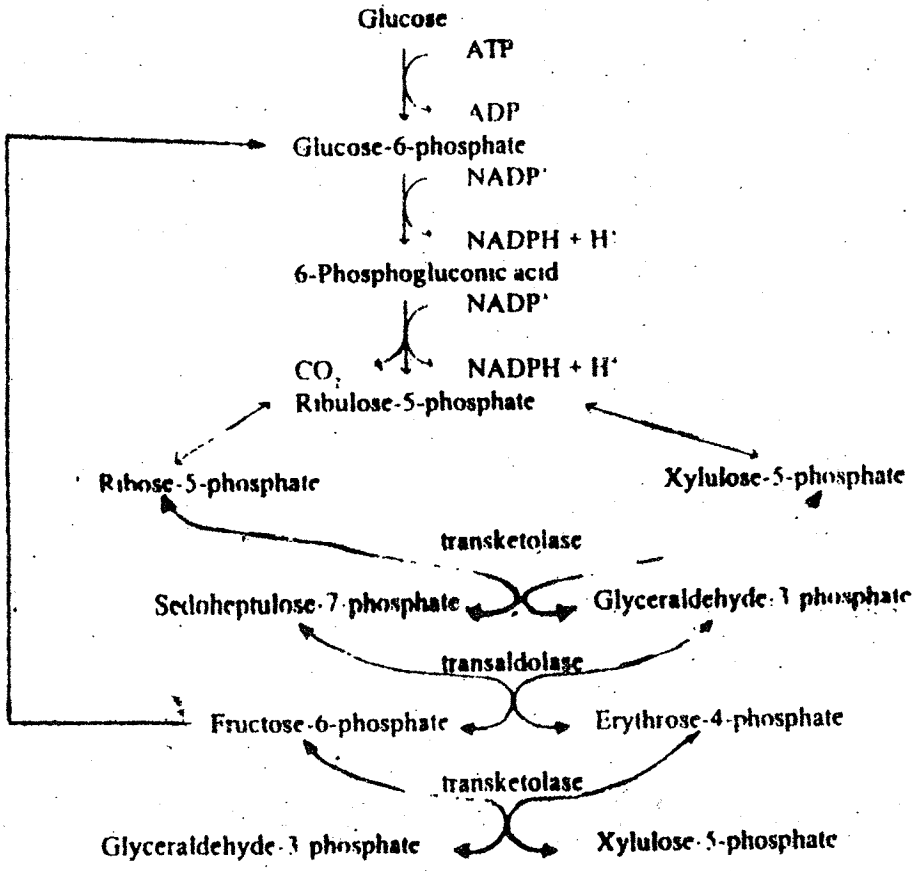


Table 3 *The pentose phosphate pathway of glucose catabolism yielding ribose-5-phosphate and NADPH+H^+*

subsequently to the initial compound of the pathway, 6-phosphogluconic acid, thus completing the cycle. Note that two intermediates of glycolysis-fructorse-6-phosphate and glyceraldehyde-3-are generated. Theoretically, by means of this cycle, the cell can carry out the complete oxidation of glucose-6-phosphate to CO_2. Specifically, six molecules of glucose-6-phosphate are then regenerated from the six molecules of ribulose-5-phosphate. The overall equation is as follows:

6 Glucose-6-phosphate +12NADP$^+$ → 5 glucose-6-phosphate+6CO_2+12NADPH+12H$^+$+Pi

The net equation is therefore :

Glucose-6-phosphate +12NADP$^+$ → 6CO_2+12NADPH+12H$^+$+Pi

In the real situation, it is more probable that the pentose phosphate pathway feeds into the glycolytic pathway by means of fructose-6-phosphate and glyceraldehyde-3-phosphate.

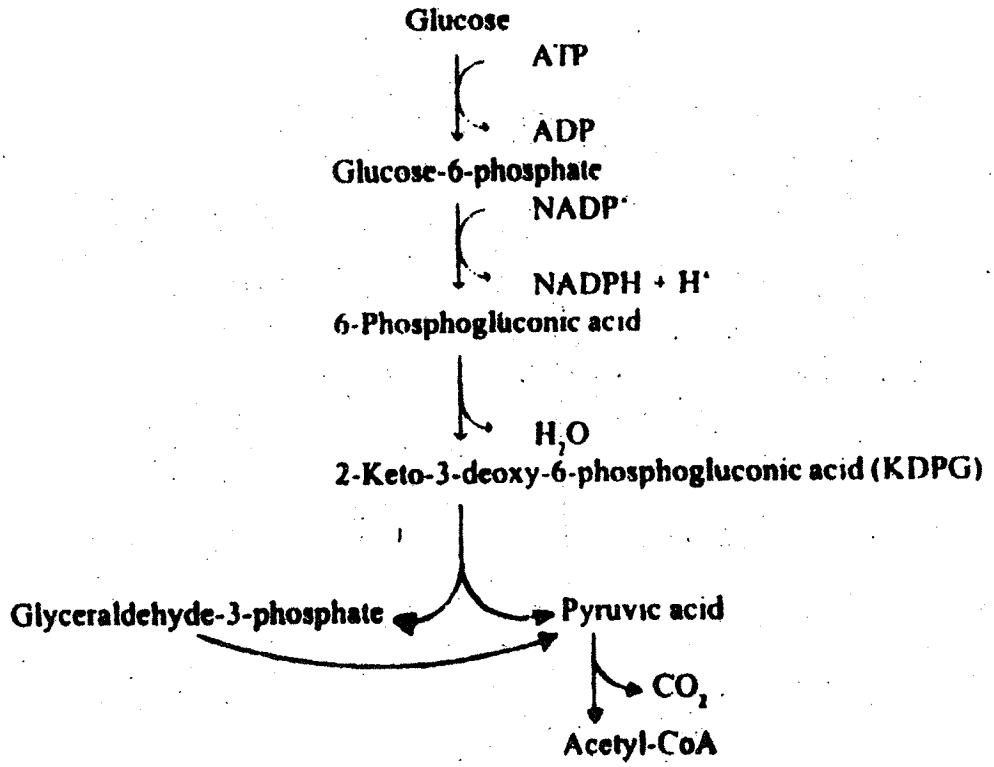


Figure 4 *The Enter-Doudoroff pathway of glucose catabolism for some bacteria.*

III. The Enter-Doudoroff Pathway :

Another pathway of glucose catabolism is called the Entner-Doudoroff pathway. It is found in both aerobic and anaerobic procaryotes but not in eucaryots. It is fairly widespread, particularly among Gram-

negative bacteria. As shown in Fig.-4, glucose is phosphorylated to glucose-6-pihosphate. It is then oxidized to 6-phosphogluconic acid. A dehydration step follows to yield 2-keto-3-thoxy-6-phosphogluconic acid (KDPG); the latter is cleaved to pyruvic acid and glyceraldehyde-3-phosphate, which is metabolized via some Embden-Meyerhof pathway enzymes to produce a second molecule of pyruvic and in the arobic pseudomonads the catabolism is completed via acetyl-CoA and the tricarboxylic acid cycle.

IV. Posphoketolase pathways:

. The heterofermentative lactobacilli and the Bifidobacteria possess the *phosphoketolase* pathway, which is a variation of the HMP pathway. There are two types of phosphoketolase pathways, the *pentose phosphoketolase pathway* and the *hexose phosphoketolase pathway.*

1. Pentose phosphoketolase pathway (Fig.-5)

The carbon source is ribose as well as other pentoses. Ribose –5-phosphate or xylulose-5-phosphate are formed via the HMP pathway. The pentose phosphoketolase pathway is found in Leuconostoc mesenteroides and Leuconostic plantarum, in which the EMP, HPM and ED pathways are absent.

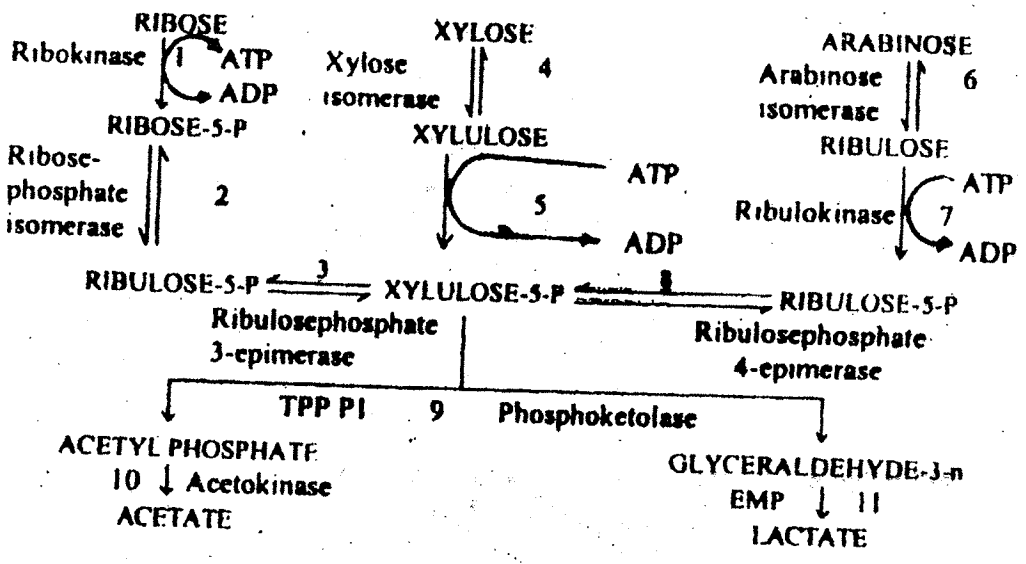


Fig. 5 *The pentose phosphoketolase pathway.*

2. Hexose phosphoketolase pathway (Fig. –6)

This pathway is found in the genus Bifidobacterium (Lactobacillus bifidus) which lacks glucose-6-phosphate dehydrogenase and fructosediphosphate aldolase. Because of this, the EMP, HMP, ED or pentose phosphoketolase pathways cannot operate.

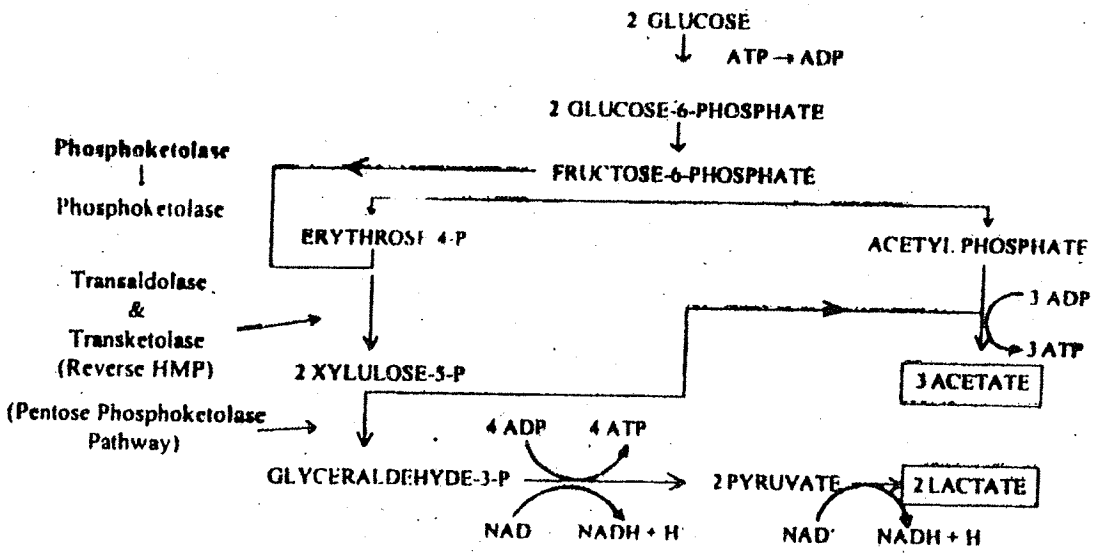


Figure 6. *The hexose photoketolase pathway.*

Pathways of Utilization of Pyruvate by Aerobes

In most aerobes, pyruvate is oxidatively decarboxylated by an elaborate enzyme system termed the pyruvate dehydrogenase complex producing acetylcoenzyme A (acetyl-CoA) as a product according to the following reaction :

$$CH_3 - CO - COOH + CoA + NAD^+ \rightarrow CH_3 - CO - CoA + CO_2 + NADH + H^+$$

Acetyl-CoA, being a precursor metabolite, enters into biosynthetic pathways; alternatively, it can be completely oxidized in a cyclic manner through a pathway known as the tricarboxylic acid (TCA) cycle (Figure –7). This cycle is the major route of ATP generation in aerobic heterotrophs (by passage of electrons from reduced pyridine nucleotides through an electron transport chain). The TCA cycle also generates three of the precursor metabolites, α- ketoglutarate, succinyl-CoA, and oxaloacetate, so even strict anaerobes possess most of the enzymes of the cycle, lacking only the step between α –ketoglutarate and succinate (the α –ketoglutarate dehydrogenase complex). Thus, by reverse flow from oxaloacetate to succinyl-CoA and for ward flow from citric acid to α –ketoglutarate, anaerobes are able to synthesize all precursor metabolites, even under anaerobic conditions. The TCA cycle effects the complete oxidation of one molecule of pyruvic acid to CO_2 and generates three molecules of reduced pyridine nucleotides, one molecule of ATP, and one molecule of reduced FAD which donates electrons to a transport chain independent of pyridine mucleotides.

Carbon from pyruvate enters the cycle by two routes : via acetyl-CoA and via pyruvate or phosphoenolpyruvate

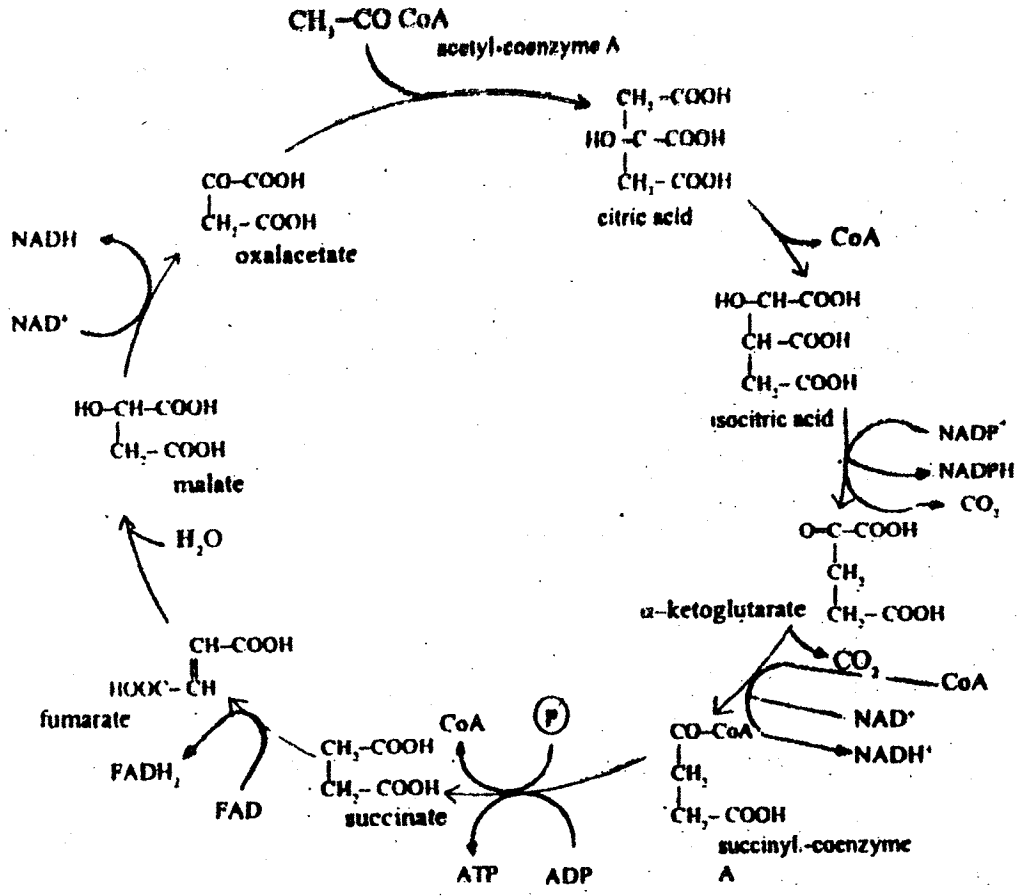


Fig. 7 *The tricarboxylic acid (TCA) cycle by which acetyl-CoA is oxidized. Precursor metabolities are shown in color.*

The Role of the Glyoxylate Cycle in Acetic Acid Oxidation

The special modification of the TCA cycle, known as the *glyoxylate cycle,* comes into play during oxidation of acetic acid or substrates (such as fatty acids) that are converted to acetyl-CoA without the intermediate formation of pyruvate. Under these circumstances, oxalacetate cannot be generated from pyruvate or phosphoenolpyruvate, because in aerobes there is no mechanism for synthesizing pyruvate from acetate : the oxidation of pyruvate by the pyruvate dehydrogenase complex is completely irreversible.

The supply of oxalacetate required for oxidtion of acetate is replenshed by the oxidation of succinate and malate, which are produced through a sequence of two reactions. In the first reaction, isocitrate, which is a normal intermediate of the TCA cycle,is cleaved to yield succinate and glyoxylate:

In the second reaction, acetyl-CoA is condensed with glyoxylate to yield malate :

$$\begin{array}{l} \text{COOH} \\ | \\ \text{CHO} \end{array} + \text{acetyl} + \text{CoA} \rightarrow \text{HO} - \begin{array}{l} \text{CH} \\ | \\ \text{CH}_2 - \text{COOH} \end{array} + \text{CoA}$$

In combination, these two reactions constitute a bypass whereby two carbon atoms lost from the TCA cycle as CO_2 are preserved in the glyoxylate cycle (Figure-8) as glyoxylate, which can then combine with acetyl-CoA to form malate, the immediate precursor of oxalacetate. Thus the glyoxsylate cycle acts as an anaplerotic sequence allowing the normal TCA cycle to function.

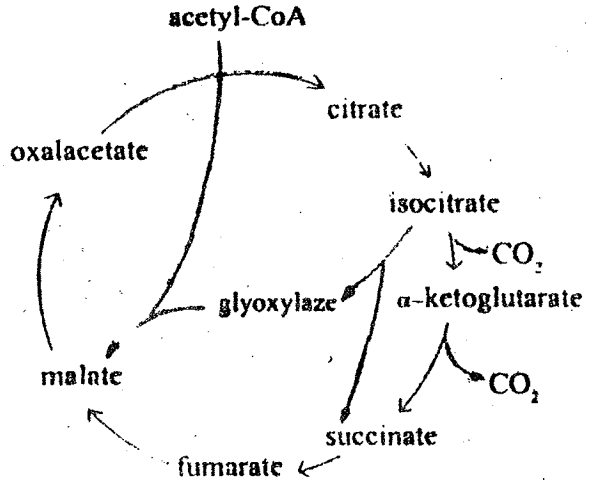


Figure – 8 *The glyoxylate bypass (bold) and its relation in the reactions of the citric acid cycle.*

Special Pathways for Primary Attack on Organic Compounds by Micro-organisms.

In the preceding sections, we have emphasized the pathways by which glucose is metabolized. Sometimes these are termed *central metabolism,* because of the vital roles they play in the synthesis of precusor metabolites and forming part of the pathways by which a variety of other substrates are metabolized. Owing to their essential nature, they are functionally reversible. For example, when an aerobe such as E. coli is grown in a medium that contains a compound such as succinate as a total source of carbon, the Embden- Meyerhof pathway must operate in a reverse direction in order to synthesize several precursor metabolites. It will be noted that all reactions linking glucose-6-phosphate and pyruvate are reversible with the exception of the conversion of fructorse-6-phosphine to fructose-1, 6-bisphosphate. In order to overcome this apparent blockade, a second enzymes (fructose-1, 6-bisphosphatase) is present that catalyze and ATP – independent conversion of the diphosphate to the monophosphate form of fructose.

As stated earlier, there is probably no naturally occurring organic compound that cannot be used as a substrate for metabolism by some micro-organism. However complex the structure of the substrate may be, its utilization always involves the same basic principle; a stepwise degradation to yield eventually one or more fragments capable of entering central metabolism. As examples of the many such specialized microbial pathways, we shall describe those involved in the utilization of the disaccharide lactose, the metabolic products of which enter the Embden-Meyerhof pathway, and certain aromatic compounds whose products enter the TCA cycle.

Lactose is hydrolytically split (Figure-9) yielding glucose, which enters central metabolism directly,

and galactose, which is phosphorylated and then converted to another intermediate of central metabolism, glucose-1-phosphate, by a cyclic pathway. The galactose and glucose moieties of galactose-1-phosphate and UDP-glucose are exchanged, yielding glucose-1-phosphate and UDP-galactose, which can undergo epimerization to UDP-glucose. The catalytic amount of UDP-glucose required for the cycle to function can be supplied by a reaction between UTP and glucose-1-phosphate, yielding pyrophosphate as the other product.

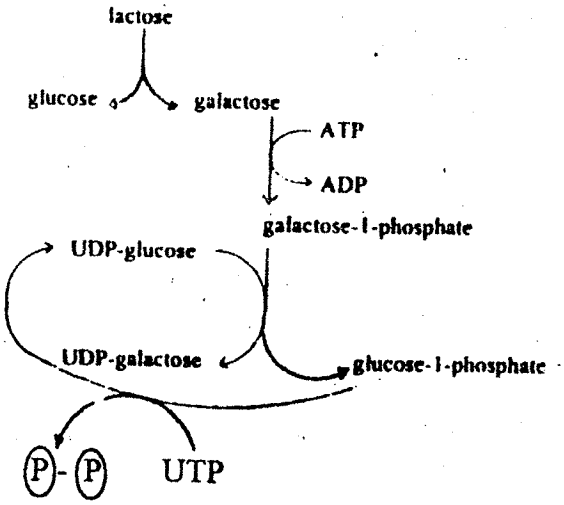


Figure-9 *The pathway of utilization of the disaccharide lactose, Compounds that enter central metabolic pathways are bold.*

One route by which aromatic compounds are attacked by those bacteria that have this capacity is through one or another of two convergent branches of the β-ketoadipate pathway (Figure-10). Through these reactions, the six carbon atoms of the aromatic nucleus are converted to those of an aliphatic acid; this is in turn cleaved to acetyl-CoA and succinate, both of which enter the TCA cycle.

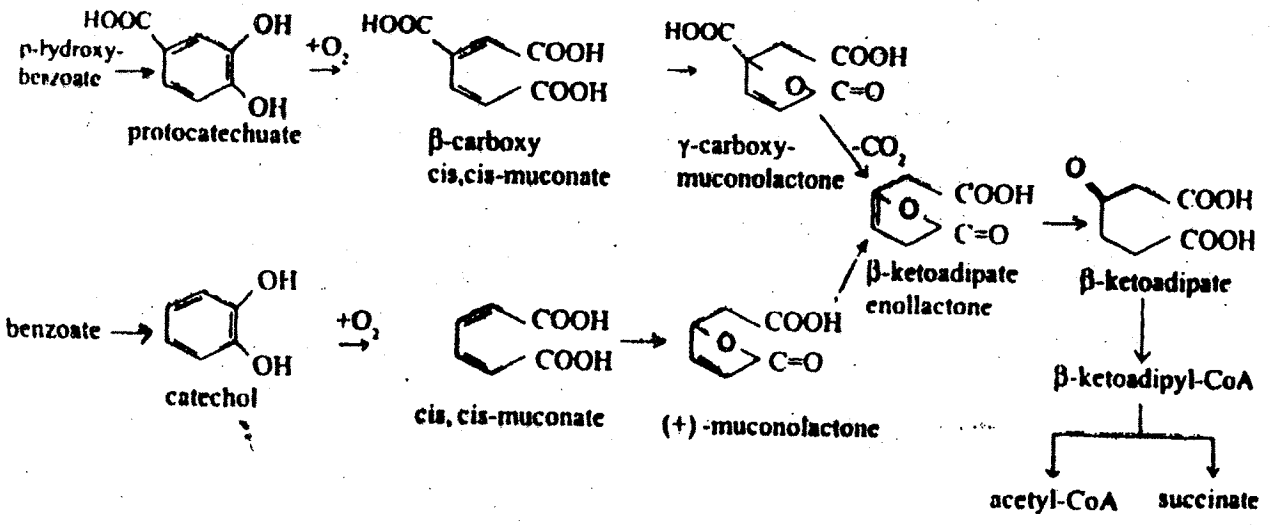


Figure 10 *The chemistry of the β – ketoadipate pathway. Compounds that enter the TCA cycle are shown in bold.*

A number of other structurally related compounds are metabolized to intermediates of the β – ketoadipate pathway and flow through it to intermediates of the TCA cycle. Some of these compounds and their points of convergence with the β – ketoadipate pathway are shown in Figure-11.

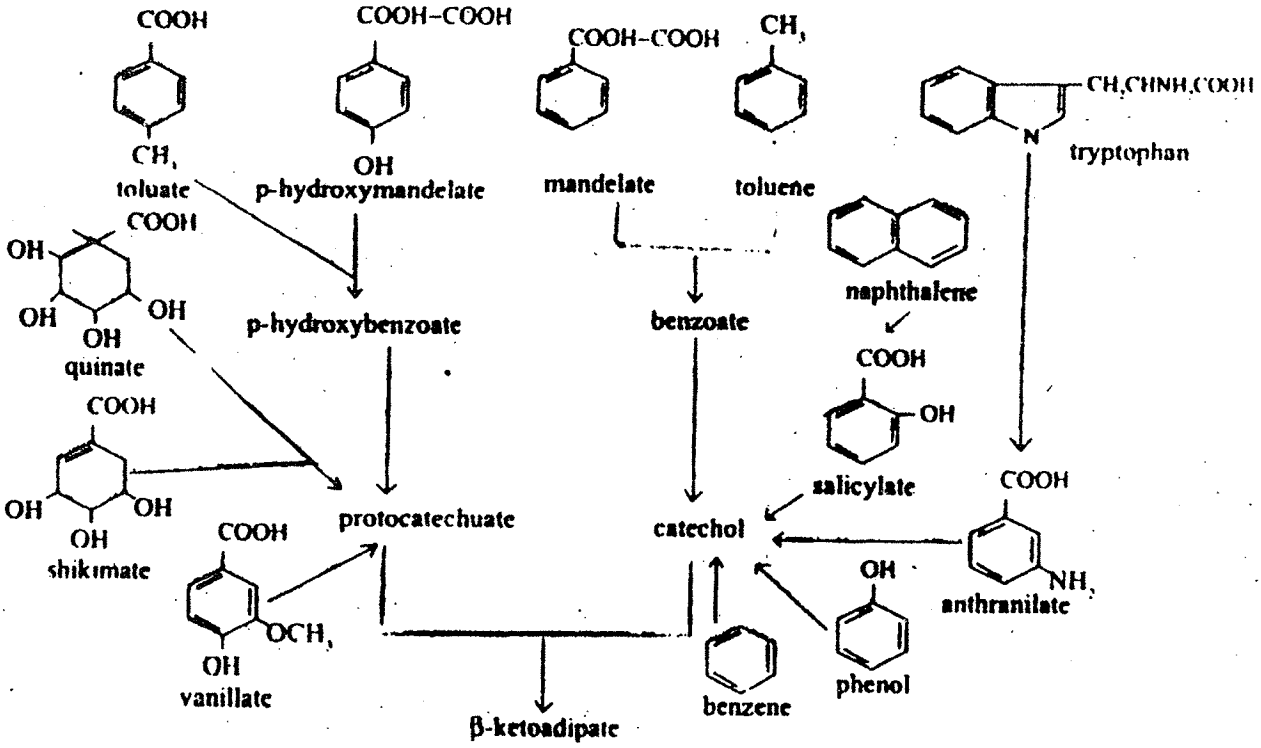


Figure 11 *Certain compounds which are metabolized through the β – ketoadipate pathway: their structures and points of convergence with the pathway. Intermediates of the β – ketoadipate pathway (see Figure10) are shown here in boldface type.*

THE FUELING REACTIONS OF ANAEROBIC CHEMOTRAPHS

Anaerobic and facultatively anaerobic chemohetertrophs have one or both of two patterns of fueling reactions, termed *anaerobic respiration and fermentation*

Araerobic Respiration

Anaerobic respiration involves essentially the same biochemical pathways as the aerobic metabolism (or aerobic respiration) heterotrophs, differing principally in the compound that serves as the terminal electron acceptor of the electron transport chain. Rather than molecular oxygen being the terminal acceptor, nitrate (NO_3^-), sulfate (SO_4^{2-}), fumarate, or trimethylamine oxide may serve the corresponding role. In the case of SO_4^{2-} or NO_3^-, the products of their reduction are also capable of being terminal electron acceptors, thus forming a cascade of anaerobic respirations. In the case of the reduction of NO_3^-, some bacteria, by a

set of three anaerobic respirations, produce dinitrogen as a final product; NO_3^- is the terminal electron acceptor of the first chain; its reduction product, nitrite (NO_2^-), is the acceptor for the next; and its reduction product, nitrous oxide (N_2O), is the acceptor for the final one. This process is termed *denitrification* because it converts a nonvolatile form of nitrogen to a volatile one, thereby depleting an aqueous or terrestrial environment of fixed nitrogen which is essential for the growth and development of most organisms.

Fermentation

Fermentations are fueling reactions that do not require the participation of an electron transport chain, although these do play minor roles in certain cases. As a consequence of the lack of participation of an external electron acceptor, the organic substrate undergoes a balanced series of oxidative and reductive reactions; pyridine nucleotides reduced in one step of the process are oxidized in another. This general principle is illustrated by two fermentations: alcoholic fermentation (typical of the anaerobic metabolism of glucose by yeasts), and the homolactic acid fermentation (typical of the metabolism of certain lactic acid bacteria). Both of these fermentative processes (Figure – 12) utilize the Embden-Meyerhof pathway : the two molecules of NAD reduced by this pathway are reoxidized in reactions involving the subsequent metabolism of pyruvate. In the case of the homolactic acid fermentation, this oxidation occurs as a direct consequence of the reduction of pyruvic acid to lactic acid. In the case of the alcoholic fermentation, pyruvic acid is first decarboxylated to form acetaldehyde; the reoxidatiion of NADH occurs concomitantly with the reduction of acetaldehyde to form ethanol.

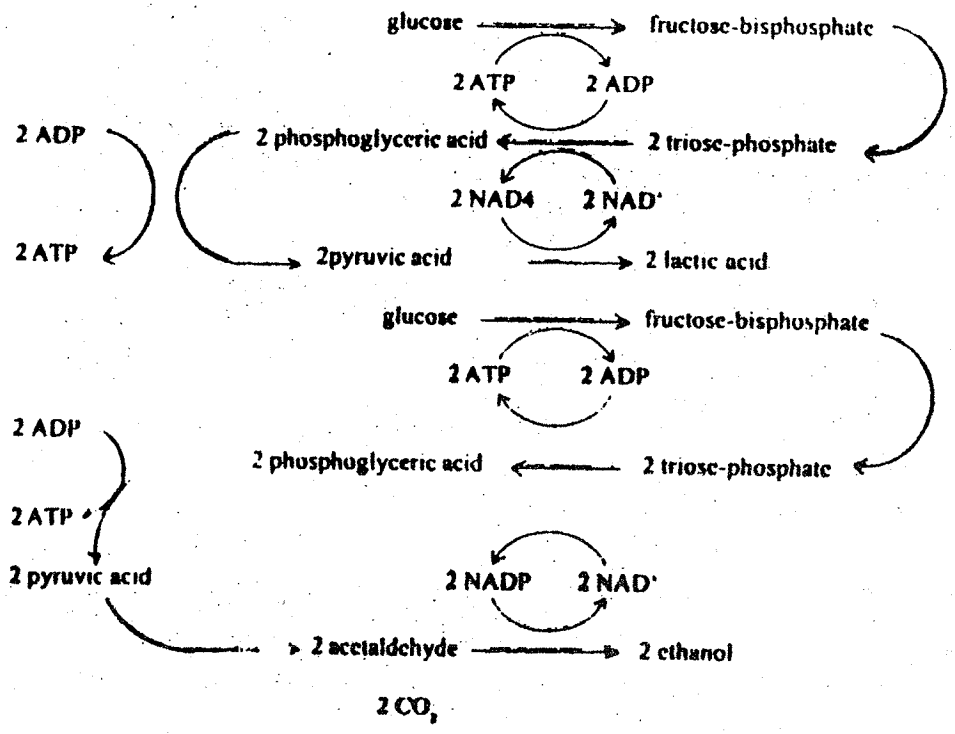


Figure – 12 *A comparison between (a) lactic acid and (b) alcoholic fermentations.*

The Embden-Meyerhof pathway is the most widespread one for the fermentative conversion of glucose to pyruvic acid, and employed by many bacteria that produce fermentative end products other than lactic acid and ethanol. These differences exclusively reflect differences with respect to metabolism of pyruvic acid. The various patterns of pyruvic acid metabolism are summarized in Fugure-13. Most bacterial fermentations may produce several end products; however, no single fermentation produces all of the end products shown in Figure –13.

Not all fermentative mechanisms follow the Embden-Meyerhof pathway. Certain fermentations of glucose follow the pentose phosphate pathway, and others follow the Enter-Doudoroff pathway Fermentations of substrates other than sugars (e.g., amino acids) involve highly specific pathways..

The end products of fermentation and the pathway by which they are formed are group specific.

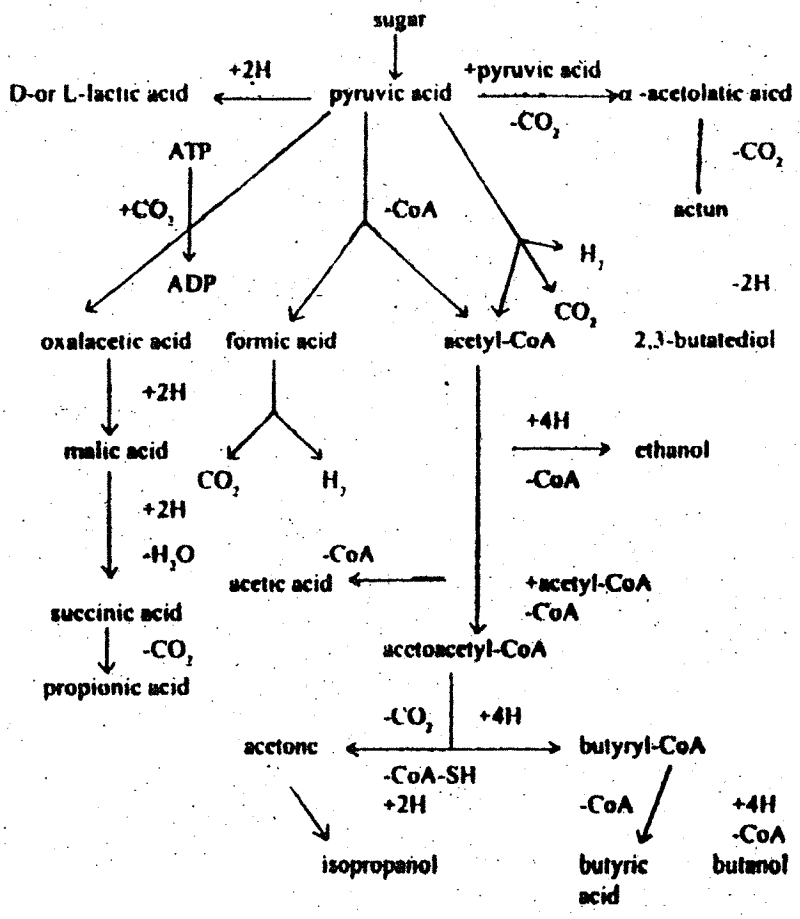


Figure 13 *Derivations of some major and products of the bacterial fermentations of sugars from pyruvic acid. The end products are shown in boldface type.*

The principle substrates of fermentation are carbohydrates. Bacteria can also utilize compounds like organic acids, amino acids, purines and pyrimidines.

The process of fermentation take place in two stages :

1) *Glucose* is broken down to pyruvate with the release of two pairs of hydrogen atoms.

2) Pyruvate or compounds derived from pyruvate are reduced by the hydrogens released in the first state.

1. Alcoholic fermentation

In alcoholic fermentation (Figure-14) pyruvate is converted to *ethanol* and *carbon dioxide*. This process is characteritic of yeasts, particularly strains of *Saccharomyces cerevisiae*. It is also foundation some moulds and in the Mucorales, but is comparatively rare in bacteria. In the *bacterium Pseudomonas*, Pyruvate is produced through the Entner-Duodoroff pathway. It is then metabolized to *ethanol* through *acetaldehyde*.

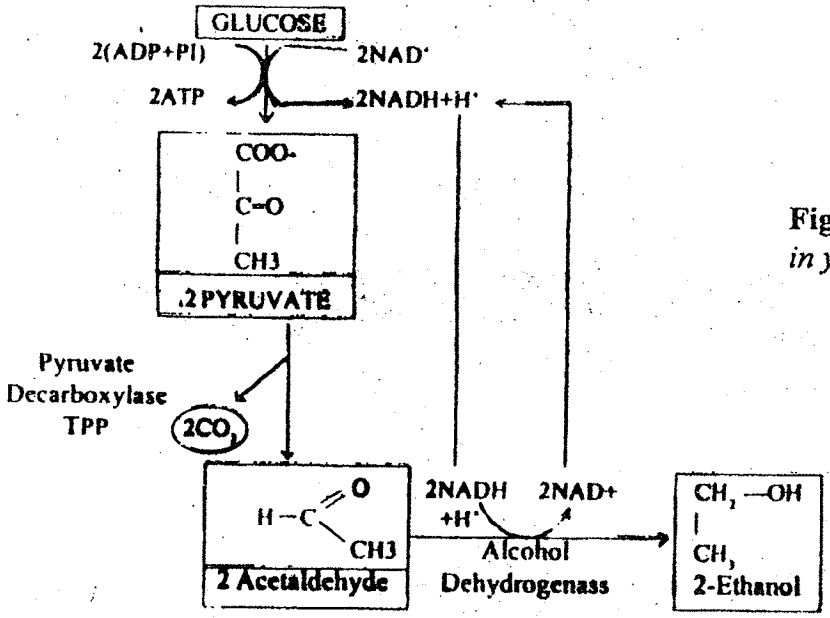


Figure 14 : *Alcoholic fermentation in yeasts*

II. Lactic acid fermentation

Lactic acid fermentation is a one-step reaction similar to glycolysis of mammalian cells. *Pyruvic acid* is reduced to lactic acid, the reaction being catalysed by *pyruvate reductase*. Lactic acid fermentation is characteristic of the lactic acid bacteria (lactobacillaceae) which cause spoilage of food. Although morphologically heterogeneous, the bacteria are characterized by the fact that they produce lactic acid as the end product.

The *lactobacilli* are divided into two groups, *homofermentative* and *heterofermentative strains*. The demarcation between the two groups is indefinite in some cases.

1. Homolactic fermentation

Homolactic fermentation is found in members of the genera *Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus*. It is also found in muscle cells of animals. In this type of fermentation the predominant product is lactic acid. Other products are found only in traces.

Glucose yields two molecules of *pyruvate* through the EMP pathway. Pyruvate is reduced to *lactate* by the enzyme lactate dehydrogenase (*pyruvate reductase*). $NADH_2$ is the hydrogen donor and is oxidized to NAD.

$$\text{Glucose} \xrightarrow{\text{EMP}} \underset{\text{Pyruvic acid}}{CH_3\text{- CO-COOH}} \xrightarrow[NADH_2 \rightarrow NAD]{} \underset{\text{Lactic acid}}{CH_3 - CHOH - COOH}$$

In addition to glucose, homolactic micro organisms also ferment other monosaccharides like fructorse, mannose, and galactose and disaccharides like lactose, maltose and sucrose. These sugars are apparently converted into intermediates of the EMP pathway by inducible ezymes.

2. Heterolactic fermentation (Figure – 15 & 16)

This type of fermentation is found in some strains of Leuconostoc, Lactobacillus, the anaerobic peptostreptococci and the anaerobic species of *Eubacterium, Ramibacterium. Bifidobacterium and Catenabacterium.* In heterolactic fermentation like *ethanol, glycerol acetate, propionic acid, CO_2 and butyric* acid are found in addition to lactic acid. Among the heterofermenters are included organisms that produce less than 1,8 moles of lactic acid per mole of glucose and in addition, some of the compounds mentioned above. In *Leuconostoctype heterofermentation, acetate, ethanol* and *glycerol* are formed in addition to lactic acid. In *Peptostreptococcus-type* heterofermentation, *lactic* and *propionic* acids are the end products of glucose fermentation. In a third type of heterofermentation, *lactic* and *butyric* acids are the end products.

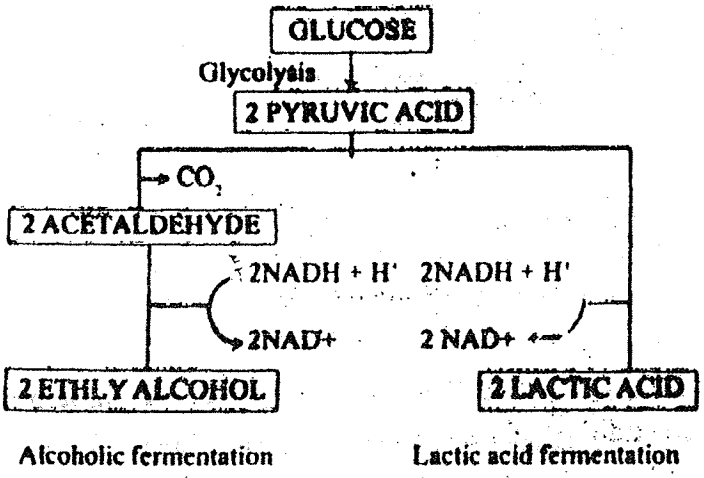


Figure 15 *Alcoholic and lactic and lactic acid fermentation.*

In *Lactobacillus brevis* and *Leuconostoc* species, the EMP, HMP and ED pathway are absent, and these organisms possess the *phosphoketolase pathway.* The overall reaction is :

Glucose → Lactic acid + Ethanol + CO_2

Glucose is first metabolized to pyruvic acid, acetic acid and CO

Glucose → Pyruvic acid + Acetic acid + CO_2 + 6H

Pyruvic acid is then reduced to acid, and acetic acid to ethanol through acetaldehyde

$$\text{Glucose} \xrightarrow{2H} \text{Pyruvic acid} \rightarrow \text{Lactic acid}$$

$$\text{Acetic acid} \xrightarrow{2H} \text{Acetaldehyde} \xrightarrow{2H} \text{Ethanol}$$

For every mole of glucose fermentation there is a net yield of one more of ATP. Thus heterolactic fermentation produces only half the energy produced by homolactic fermentaion.

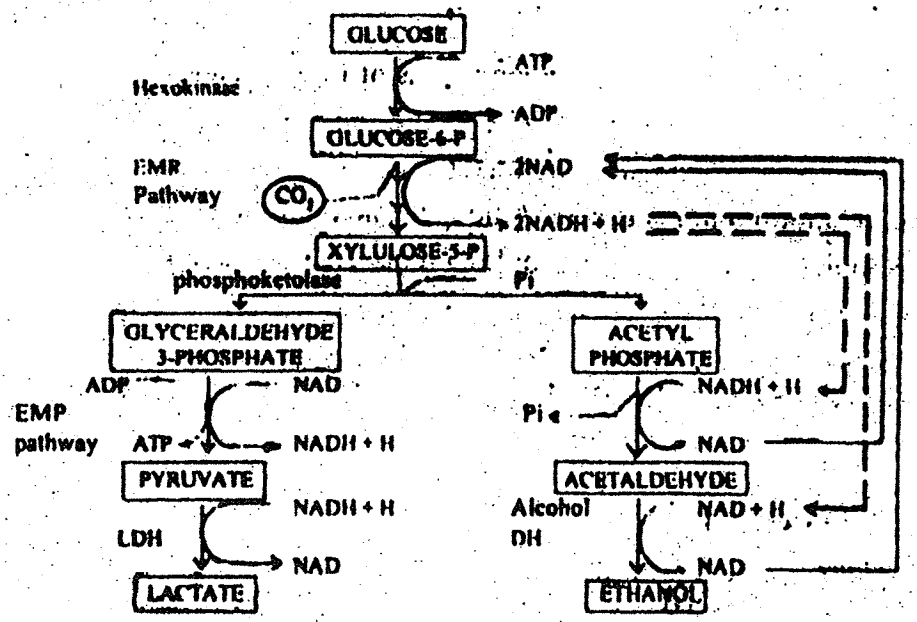


Figure – 16 *Heterolactic fermentation*

III. Propionic acid fermentation (Figure-17)

The propionic acid bacteria belong to the genus *Propionibacterium.* These bacteria can ferment *glucose of lactate to propionic acid* under anaerobic conditions. Propionic acid bacteria are especially numerous in the digestive tract of ruminants. The cellulose digesting bacteria in the rumen can digest

cellulose to form glucose, which is then converted to lactate. The propionic acid bacteria can then convert the glucose or lactate into propionic and acetic acids, along with some succinic acid. The propionic and acetic acids are absorbed into the blood stream of the host.

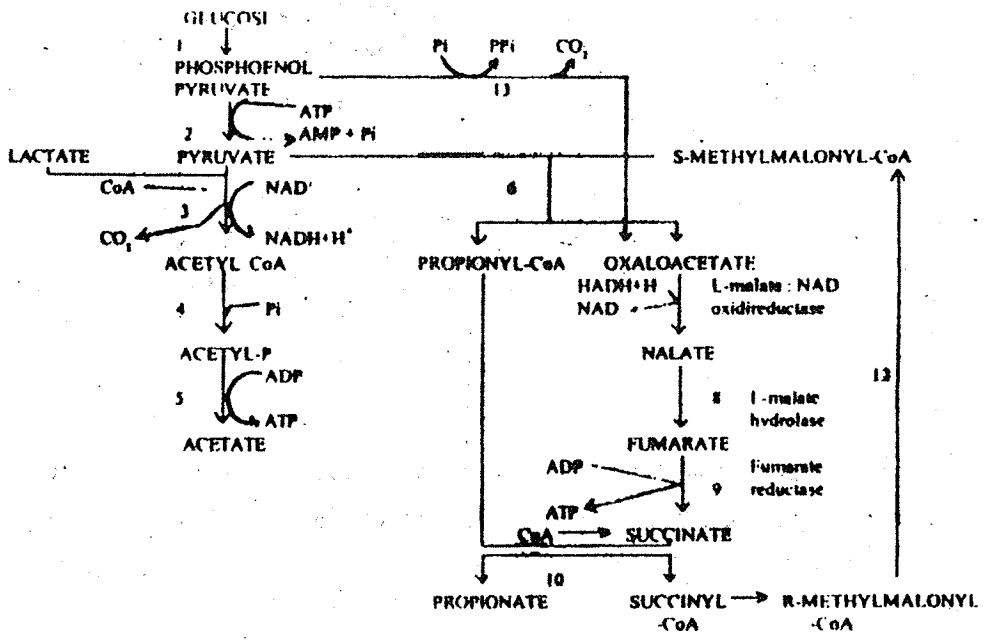


Figure-17 *Propionic acid fermentation*

Propionic acid fermentation from lactate.

In *Clostridium propionicum* and *Bacteriodes ruminocola* there is an entirely different mechanism for production of propionic acid. The route from lactate is :

Lactate → Acrylate → Propionate

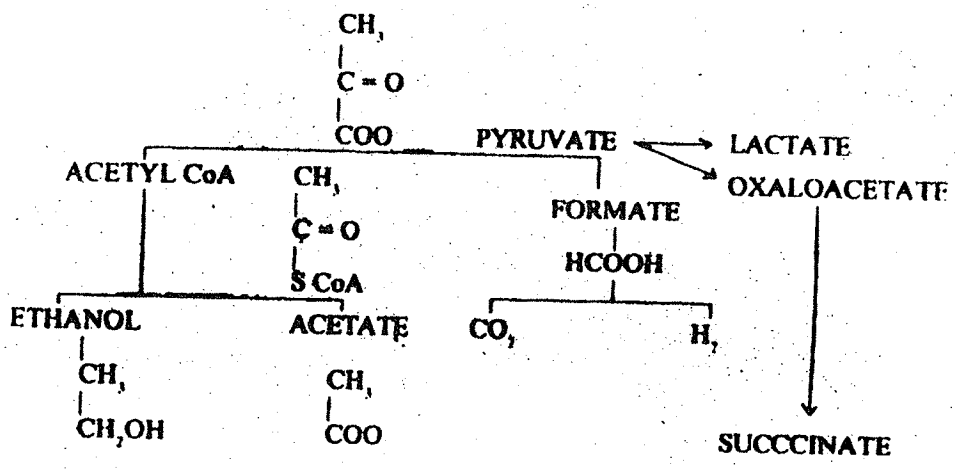
It has been suggested that the conversion of lactate propionate takes place via the CoA esters of lactate, acrylate and propionate (*lactolyl-CoA and propionic-CoA,* respectively). It is, however, not certain whether this pathway is applicable to *C-propionicum and B-ruminicola.*

IV. Formic acid fermentation.

Formic acid fermentation is found in a number of bacteria, especially members of theEnterobacteriaceae. Metabolism of pyruvate produces several different products which vary in different organisms, but formate is always one of them. Bacteria carrying out formic acid fermentation can be divided into three groups : (1) mixed acid producers, (2) butaeedio producers and (3) butyric acid producers.

1. Mixed acid producers

E. coli is the most characteristic member of this group. Glucose is converted into ethanol and acetate, and either format or its derived products CO_2 and H_2. Some glucose is also converted into *lactate* and *succinate.*



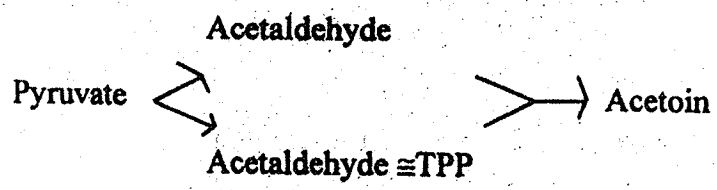
2. Butanediol producers.

In this group are included such genera as *Aerobacter,.Serratia* and *Bacillus spp.* Some of the pyruvate is metabolized, as in mixed acid fermentation, but most of it is condensed with decarboxylation to yield α -acetolactate. This in turn is decarboxylated to aceto in (acetylmethyl-carbionol), which is reduced with $NADH_2$ to 2,3-butanediol.

$$2\text{Pyruvate} \xrightarrow{TTP,\ Mg^{++}} \alpha\text{ - Acetolactate} + CO_2 \xrightarrow{} \text{Acetoin} + CO_2 \xrightarrow{NADH_2} \text{Butanediol} + NAD$$

Formation of acetoin Acetoin can be formed from acetaldehyde, acetolactate and from diacetyl. The postulated schemes are follows:

(i) In yeasts there is direct formation of acetoin from pyruvate.



3. Butyric acid producers (Figure – 18).

Butyric acid fermentation is carried out by saccharolytic clostridia. Several products may be formed, but butyric acid is always one of them, either in the intermediate or terminal stage. Different species of Clostridium produce different products :

Species	Products
Cl. butyricum	Acetate, butyrate, CO_2, H_2
C1. Acetobutylicum	Acetone and butanol + above
C1. Butylicum	Isopropanol from acetone.

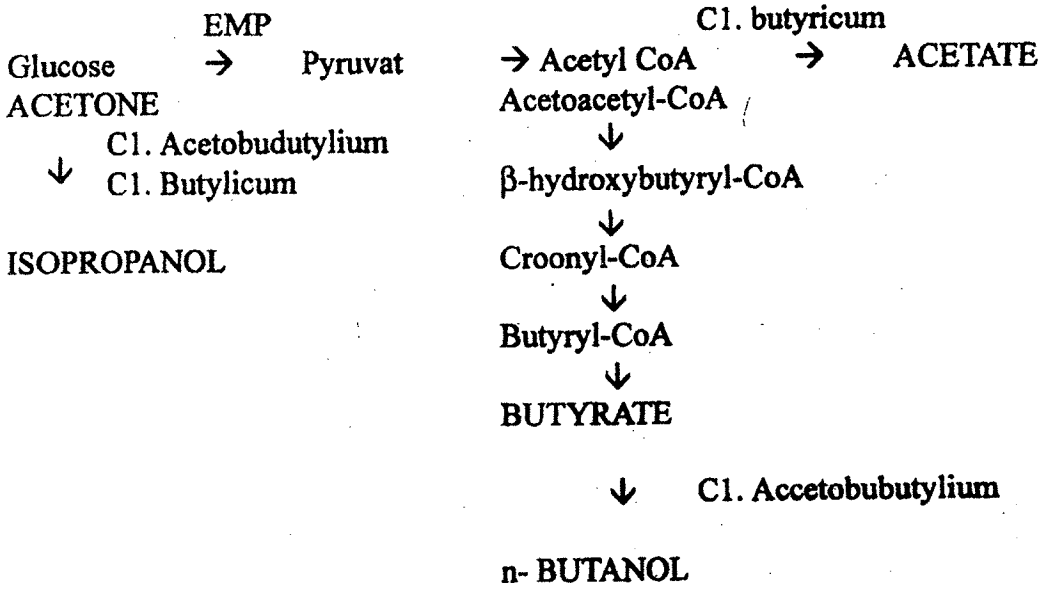


Figure – 18 Pathways in the fermentation of glucose by clostridia

Diversity of fermentations

Table 2 summarizes some of the main types of fermentations as classified on the basis of products formed. Note some of the broad categories such as alcohol, lactic acid, propionic acid, mixed acid, butyric acid, and homoacetic acid.

Many unusual fermentations are carried out by only a very restricted group of anaerobes and, in some cases by only a single known bacterium. Some examples are listed in Table 3.

TABLE-2 Examples of common bacterial fermentations and some of the organisms carrying them out

Type	Overall reaction[a]	Organisms
Alcoholic fermentation	Hexoses → 2 Ethanol + $2CO_2$	Yeast
		Zymomonas
Homolactic fermentation	Hexose → 2 Lactate⁻	Streptococcus
		Some Lactobacillus
Heterolactic fermentation	Hexose → Lactate⁻ + Ethanol + CO_2	Leuconostoc
		Some Lactobacillus

Type	Overall reaction[a]	Organisms
Propionic acid	Lactate$^-$ → Propionat$^-$ + Acetate$^-$ CO_2	Propionibacterium Clostridium propionicum
Mixed acid	Hexoses → Ethanol+ 2,3-Butanediol + Succinate^{2-}+ Lactate$^-$ Acetate$^-$ + Formate$^-$+ H_2 + CO_2	Enteric bacteria Escherichia Salmonella Shigella Klebsiella Enterobacter
Butyric acid	Hexoses → Butyrate$^-$Acetate$^-$+H_2+CO_2	Clostridium butyricum
Butanol	Hexoses → Butanol + Acetate$^-$+ Acetone + Ethanol + H_2 +CO_2	Clostridium acetobutylicum
Caproate	Ethanol + Acetate$^-$ + CO_2 → Caproate + Butyrate + H_2	Clostridium kluyveri Clostridium aceticum
Homoacetogenic	Fructose → 3 Acetate$^-$ +3H 4H_2 +2CO_2+H^+ → Acetate$^-$ 2H_2O	Acetobacterium
Methanogenic	Acetate$^-$ + H_2O → CH_4 + HCO_3^-	Methanothrix Methanosarcina

Reactions are intended as an overview of the process and are not necessarily balanced.

TABLE 3 Some unusual bacterial fermentations

Type	Overall balanced reaction	Organisms
Acetylene	$2C_2H_2$→ethanol+acetate$^-$+H^+	Pelobacter acetylenices
Glycerol	4 Glycerol +2HCO_3^-→ 7 acetate$^-$5H^++4H_2O	Acetobacterium spp.
Resorcinol (an aromatic compound)	$2C_6H_4(OH)_2$+6H_2O → 4acetate$^-$+butyrate$^-$5H^+	Clostridum spp.
Cinnamate (an aromatic compound)	$2C_9H_7O_8$+2H_2O → $C_9H_9O_2$ + benzoate$^-$+acetate$^-$	Acetivibric multivorus
Phloroglucinol (an aromatic compound)	$C_6H_6O_3$+3H_2O → 3acetate$^-$ + 3H^+	Pelobacter massilienss
Putrescine	10$C_4H_{14}M_2$ +26H_2O → 6acetate$^-$+7butyrate$^-$ + 10NH_4^++ 16H_2 +13H^+	Unclassified gram-positive nonsporing anaerobes
Citrate	Citrate^{3-}+2H_2O → formate$^-$ 2 acetate$^-$+HCO_3^-+H^+	Bacteroides sp.
	4Glyoxylate$^-$+3H++3H_2O→ 6CO_2+5H_2+Glycolate$^-$	Unclassfied gram-negative bacterium
Succinate	Succinate^{2-}+H_2O → propionate$^-$+HCO_3^-	Propionigenium modistum
Oxalate	Oxalate^{-2}+H_2O→ formate$^-$+HCO_3^-	Oxalobacter formigeus
Malonate	Malonate^{-2}+H_2O→acetate-+HCO_3^-	Malonomonas rubrs Sporomusa malonica

Botany
Module No.- 3,
Part - I , Paper -I (1st Half)
Chapter - 1

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CHAPTER - 1

MUTATIONS:-

As a consequence of normal chromosomal replication, or exposure to certain chemical or physical agents termed *mutagens*, the sequence of basis in the bacterial genome occasionally changes, any such change is called a *mutation*. Under normal conditions of cultivation, mutations occur only rarely – a population typically contains about one cell in 10^8 that carries a detectable mutation in any given gene, but exposure to certain powerful mutagens increases dramatically the frequency of cells that carry mutations.

Table 1
Classification of Mutations Based on Genotype

I. MACROLESION
   - A. Deletions
   - B. Duplications
   - C. Inversions
   - D. Insertions
   - E. Translocations

II. MICROLESIONS
   - A. Base-pair substitutions (translations and transversions)
     1. Neutral
     2. Missense
     3. Nonsense
   - B. Frame shift
     1. +1 or +2
     2. –1 or –2

Mutations may involve a change in only a single base pair (*microlesions*) of the cell's DNA or they may produce a change that extends over a number of base pairs or even a number genes (*macrolesions*) (Table 1). If a microlesion involves the loss or gain of a base pair it is called a frame-shift mutation because it changes the reading frame of all codons of the gene or operon distal to the point of the mutation. If a microlesion involves the change of one base pair [e.g., adeninethymine (A-T) pair] to another [for example, a guaninecytosine (G-C) pair] it is called a *base-pair substitution mutation*.

Because of the fundamental differences in the mechanism of their formation, base substitution mutations are subdivided into *transition and transversion mutations* depending on whether the purine base of a pair is changed to another purine base (transition) or to a pyrimidine base (transversion) (Figure 2).

Macrolesions include a variety of changes in DNA : the complete elimination of a segment of DNA (*deletion*), the inverting of a segment of DNA (*inversion*), the introduction of a new segment of DNA within an existing sequence (*insertion*), and the movement of a segment of DNA to another site in the genome (*translocation*).

The base pair substitution may result in one of three kinds of mutation affecting the translation process;

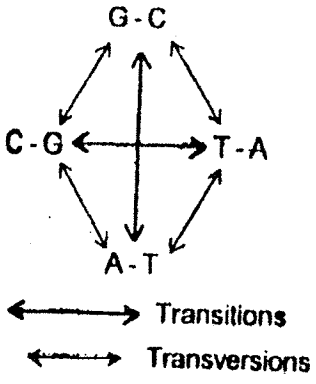


Figure 2. *Base-pair substitutiions. Those in which a purine is replaced by a different purine and a pyrimidine is replaced by a different pyrimidine are called transitions. Those in which a purine, or vice versa, are called transversions.*

1. The altered gene triplet produces a condon in the mRNA which specifies an amino acid different from the one present in the normal protein. This mutation is called a **missense** mutation. Such a protein may be functionally inactive or less active than the normal one. A good example of a missense mutation in humans is the disease sickle cell anemia. A single base substitution in the codon for the sixth amino acid of normal hemoglobin A changes the sixth amino acid from glutamic acid to valine, thus forming the charcteristic hemoglobin S or sickle cell anemia. That is, GAG, which codes for glutamic acid, has changed to GUG for valine. Under low oxygen concentration the altered hemoglobin S molecules stack into crystals, giving the red blood cells a sickle shape.

2. The altered gene triplet produces a chain terminating condon in mRNA, resulting in premature termination of protein formation during translation. This is called **nonsense mutation.** The result is an incomplete polypeptide which is nonfunctional.

3. The altered gene triplet produces a mRNA codon which specifies the same amino acid because the codon resulting from mutation is a synonym for the original codon. This is a **neutral-mutation.**

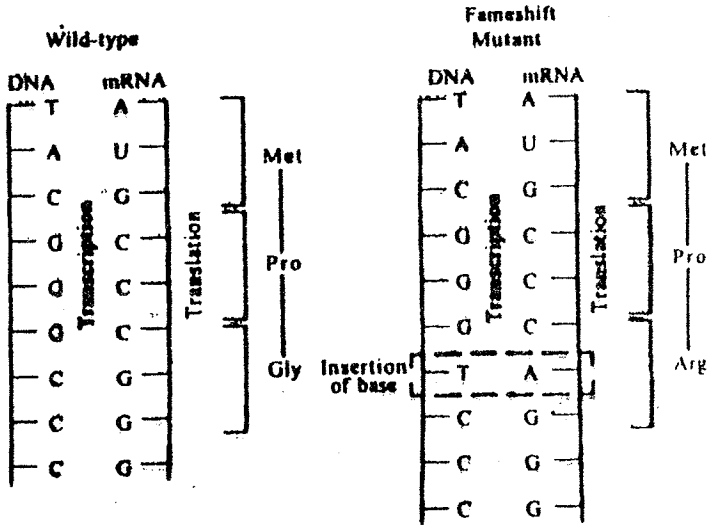


Figure 3. *Frameshift mutation, as a result of insertion of a nucleotide in a gene results in the transcription of an additional nucleotide in Mrna. This results in a frameshift when codons are read during translation, so all codons following the insertion are altered and all amino acids coded for are changed. A frameshift mutation as a result of delection of a nucleotide would have essentially the same effect.*

Frameshift Mutations :

These mutations result from an addition or loss of one or more nucleotides in a gene and are termed **insertion or deletion mutations, respectively. This results in a shift of the reading frame.** We saw earlier that during protein synthesis the reading of the genetic code starts from one end of the protein template, mRNA and is read in consecutive blocks of three bases. Frameshift mutations, therefore, generally lead to nonfunctional proteins, because an entirely new sequence off amino acids is synthesized from a frameshift reading of the nucleotide sequences of mRNA. This type of mutation is illustrated in Fig. 3 & 3A.

The consequences of various types of macrolesions are quite variable. Deletions that remove a gene, of course, always cause a complete loss of the function of that gene; and deletions that remove a portion of a gene almost always cause the loss of function of the gene. ON the other hand, duplications cause only the relatively subtle effects deriving from genes being present in two copies : generally more gene product is produced. Invertions that cover several genes (not in the same operon) cause, with high probability, loss of both genes in which the inverted segment ends but the intervening genes remain functional. Insertions almost always cause loss of function of the gene where they occur. The consequence of a translocation is normally restricted to the gene into which the translocated fragment is inserted and is usually the same as an insertion. Most translocations occur as a consequence of *replicative translocation*, after which the translocated segment is found in its original site as well as the new one.

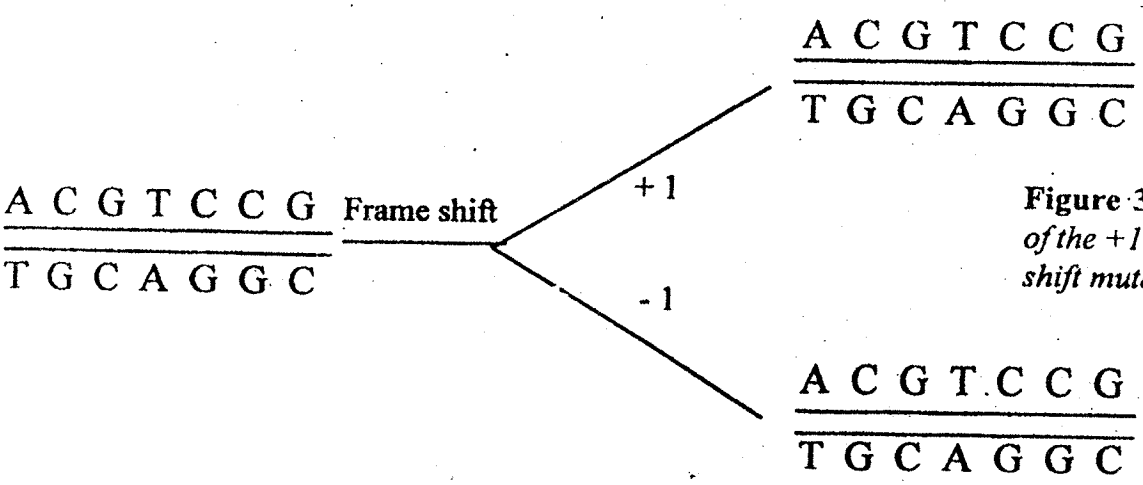


Figure 3A. *Consequences of the +1 and -1 type frame-shift mutations.*

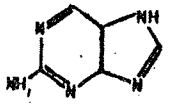
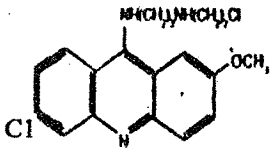
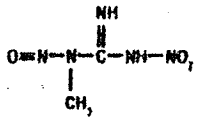
MUTAGENS

Mutagens are chemical or physical agents that increase the frequency at which mutations occur during growth of a culture. These agents act in one or the other of two quite different ways : (1) some chemical mutagens become associated with DNA (*intercalating agents*) or become incorporated into it (*base analogues*); (2) a large variety of other mutagens react chemically with DNA-usually with one of its purine or pyrimidne bases. Commonly used chemical mutagens are shown in Table 2.

The bases of DNA are most energetically stable when their oxygenaed substituents are in the keto form (=O) and their reduced nitrogen substituents are in the amino form ($-NH_2$). In these states adenine pairs with thymine and guanine with cytosine. However, at significant low frequency the bases undergo a tautomeric shift to their enol (-OH) – imino (=NH) forms. In this state their hydrogen bonding properties, and therefore the patterns of pair formation change : adenine now pairs with cytosine and guanine with thymine. If, when replication occurs, a base is in its enol-imino form, an inappropriate base will be introduced

Table 2

Examples of Types of Chemical Mutagens and their Mode of Action

Name	Structure	Action
I. MUTAGENS THAT ASSOCIATE WITH OR BECOME INCORPORATED INTO DNA		
A. Base analogue 2 aminopurine		Incoroporate into DNA causes transition mutations
B. Intercalating agents ICR – 19		Causes frame-shift mutations
II. MUTAGENS THAT REACT WITH DNA A. Alkylating agents nitroguanidine[a]		Alkylates purines; causes transitions, transversions and –1 frame shifts
B. Other DNA modifiers Hydroxylamine	$HONH_2$	Hydroxylates 6 amino groups of cytosine; cause G-C to A-T transitions.

[a] Triaval name for N-methyl-N' – nitrosoguanidine

into the newly synthesized strand, and, unless it is removed by the proofreading properties of DNA polymerase, a transition mutation will be introduced into the genome at that point (Figure 4). Base analogues are effective mutagens because they become incorporated into DNA and undergo tautomeric shifts more frequently than natural bases. The mechanism of mutagenic action of base-analogue mutagens can be illustrated by considering a commonly used mutagen of this type, 2-aino-purine. In its usual amino form it pairs with thymine; in its less frequent imino form it pairs with cytosine. Thus, it usually has pairing properties like adenine, but the probability of its being in the imino form is greater. It is a sufficiently close

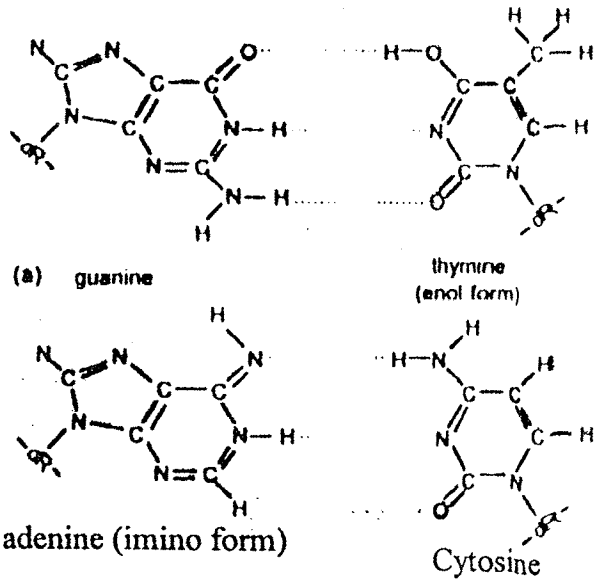


Figure 4 *Changes in base pairing as a result of tautomeric shifts. In the enol form (a) thymine forms hydrogen bonds with guanine instead of with adenine. In the imino form (b), actenine forms hydrogen bonds with cytosine instead of with thymine. Similar shifts in guanine and cytosine will also cause changes in base pairing.*

analogue of adenine to be a substrate for the enzymes of the pathway that incorporates exogenous adenine into DNA, so if 2-aminopurine is added to a growth medium it passes through this pathway and becomes incorporated in DNA. If, at the moment of incorporation it is in amino form, it enters the double helix in place of an adenine residue, and if this base analogue later undergoes a tautomeric shift during replication, the eventual consequence is an A/T to G/C transition mutation. If 2-amino-purine enters in the amino form and later undergoes a tautomeric shift, the eventual consequence is G/T to A/T transition. Thus 2-aminopurine causes both A/T to G/C and G/C to A/T transition mutations (Figure 5.).

Intercalating agents are planner molecules and can insert between the stacked pairs of bases in the core of the DNA molecule. Such inorporation distorts the backbone of the double helix in such a way that frame-shift mutations can occur when the distorted helix is replicated.

Mutagenic agents that react with DNA cause a variety of chemical changes, some of which are highly specific. For example, hydroxylamine reacts specifically with cytosine converting it to 6-hydro-xylaminouracil which pairs with adenine, a process that caused G/C to A/T transitions when this chemically altered DNA is replicated.

Nitrous acid is somewhat less specific in its action in that it reacts with all bases (A,G and C) that contain amino groups, thereby converting the amino groups to hydroxyl groups. As the altered bases have pairing properties different from those of the naturally occurring bases from which they were derived, treatment with this mutagen causes both A/T to G/C and G/C to A/T transition mutations.

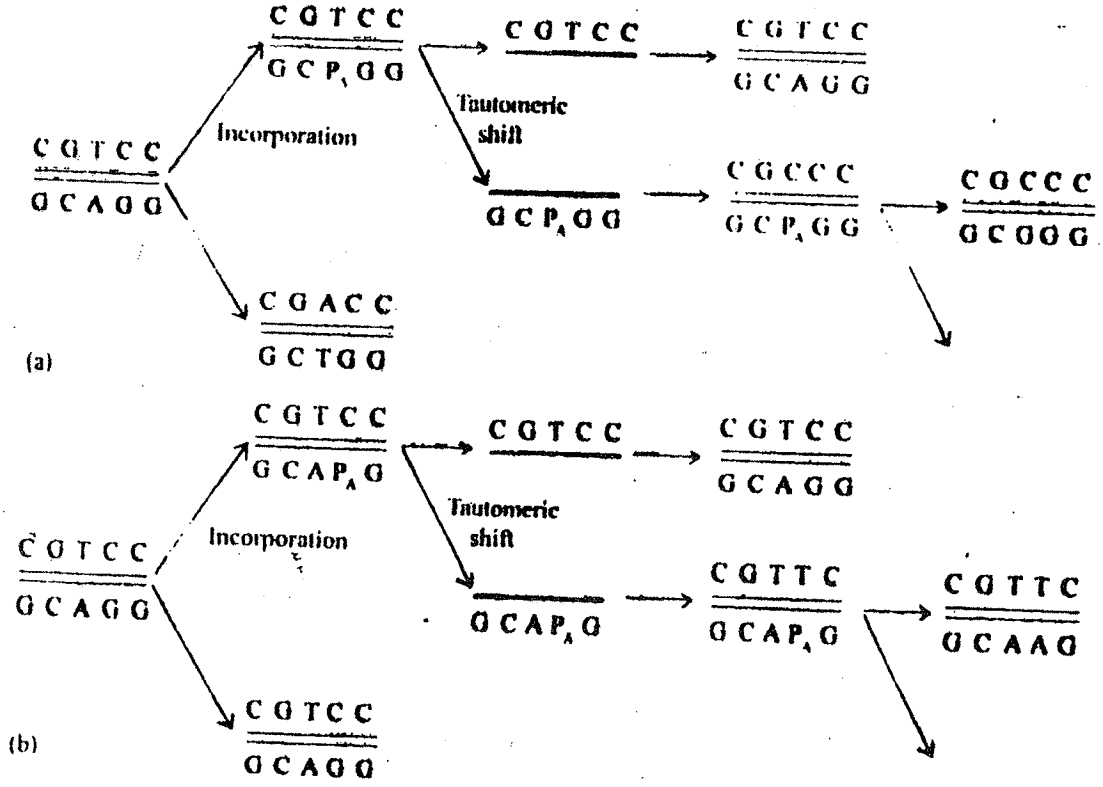


Figure 5. *Mutagenic effect of base analogues. (a) When 2-aminopuring (P) is incorporated in its amino form (P_A), a subsequent tautomeric shift causes a transition of AT to GC. (b) When it is incorporated in its imino form (P_I), a subsequent tautomeric shift causes a transition from GC to AT.*

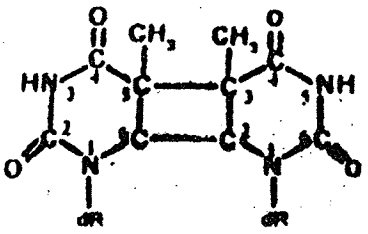


Figure 6. *Structure of a thymine dimer, the most frequent type of Pyrimidine dimers that form in DNA during ultraviolet irradiation.*
The monomers of the dimer are attached to adjacent deoxyribose (dR) residues in one strand of duplex DNA.

The most powerful known chemical mutagens include the *alkylating agents*, which add methyl or ethyl groups to the heterocyclic nitrogen atoms of the bases. Although the mechanisms by which these alkylations become mutagenic are not entirely clear, a variety of different types of mutations result. For example, alkylating agents are known to cause transition, transverstion, and -1 (but not +1) frameshift mutations.

Various types of radiation are also powerful mutagents. X-rays cause breaks in chromosomes that reform in a variety of ways, causing most types of macrolestiions. Ultraviolet (UV) light is absorbed by DNA and the energy so released causes dimerization between adjacent pyrimidine residues on the same DNA strand. The occurence of these pyrimidine dimers (Figure 6) triggers a repair mechanism that excises the pyrimidine dimers exposing a short region of single-stranded DNA on the opposite strand.

Some bacteria have enzymes, called endonucleases and exonucleases, that excise or cut out a damaged segment of DNA. Then the other enxymes, polymerases and ligasus, repair the resulting break by filling in the gap and joining the gragments together. This mechanism is illustrated in Fig. 6A and is called excision repair.

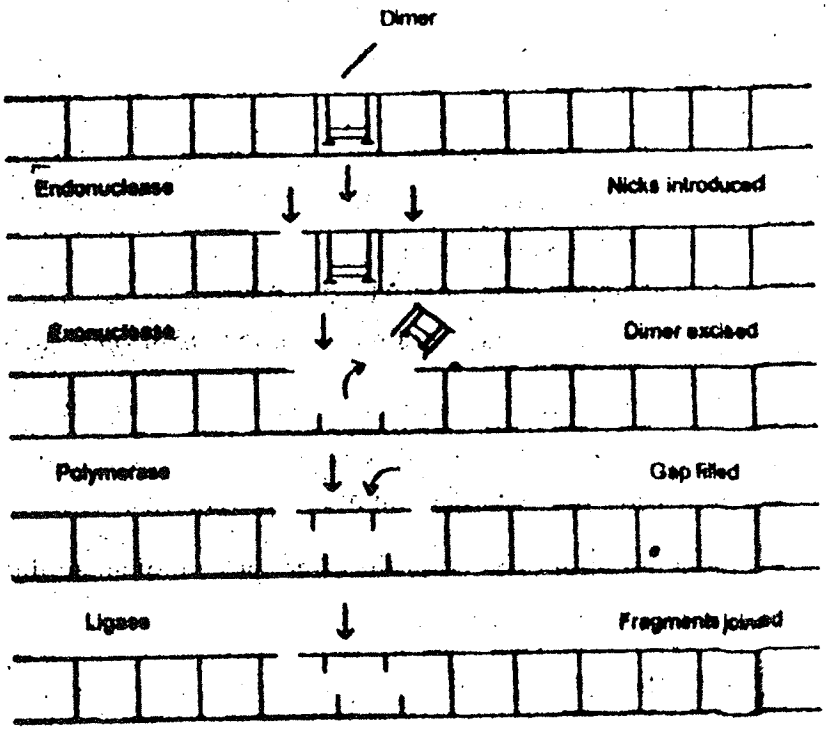


Figure 6A. *Excision repair of UVlight-damaged DNA containing a thymine-thymine dimer generated by covalent links between adjacent bases.*

The process by which E. Coli repairs large amounts of DNA damage is called inducible or SOS repair. This process is not a single discrete mechanism but includes diverse responses such as the ability to repair pyrimidine dimers, to induce various prophages, to shut off respiration, and to delay septum formation during cell division. But all the responses are coordinately regulated. The process is a very efficient one; however, it tends to insert mismatched bases and thus is error-prone and introduces additional mutation.

PHENOTYPIC CONSEQUENCES OF MUTATIONS

In the preceding sections we have discussed the various effects of mutations on a cell's genotype. Depending on the genes in which they occur and their impact on the activity of the gen's protein product, mutations can change a cell's phenotype in a variety of ways. Many mutations inactivate indispensable gene products and therefore kill the cell. But many other inactivate gene products that are not essential under all conditions of growth; i.e., loss of these products is not lethal to the cell. Clones carrying the latter type of mutations can be maintained in cultures : they differ phenotypically in a variety of ways from their unmutated parents termed *wild-type strains* (Table-3).

Table-3

Classification of Certain Mutations Based on Phenotype

Mutation	Target Gene	Mutant Phenotype
Auxotroph	In biosynthetic	Requires exogenous pathway nutrient
Carbon source	In carbon dissimilatory pathway	Cannot utilize a particular carbon source
Nitrogen source	In nitrogen dissimilatory pathway	Cannot utilize a particular nitrogen source
Antibiotic resistance	Gene encoding target protein	Can grow in presence of antibiotic
Cryptic	Gene encoding a permease	Cannot mediate a particular cellular function, although all relevant intracellular enzymes are present

A strain that carries a mutation that inactivates the product of a gene encoding an enzyme in a biosynthetic pathway loses the ability to synthesize the end product of that pathway. If the end product can enter the cell at an adequate rate (which is often the case) the mutant clone can grow in media that contain the end product. Such mutant strains are called *auxotrophs*; their parents are called *prototrophs*.

Strains that carry protein inactivating mutations in genes encoding enzymes that participate in actabolic pathways lose the ability to grow at the expense of the primary substrate of the pathway, but such mutant strains can be maintained in media provided with other primary substrates.

Other mutations that alter the target protein of an antibiotic or other toxic chemical can render the cell resistant to the antimicrobial agent.

Still other strains might have lost some non-essential capacity, such as motility, a tactic response, and so forth.

CONDITIONALLY EXPRESSED MUTATIONS

The phenotypic expression of certain mutations is conditionally dependent on the cell's environment; i.e., in certain environments the mutant clone expresses wild-type phenotype; in others, a mutant phenotype is expressed. This class of mutations is particularly valuable for studies on microbial physiology, because mutant clones can be maintained with such mutations in any gene, even one that encodes an indispensable cellular activity the loss of which would be lethal. The mutant clone can be maintained in culture in the environment in which the wild-type phenotype is expressed (*permissive condition*), and the physiological consequence of the mutation can be evaluated in the environment in which the mutant phenotype is expressed (*restrictive condition*).

The various classed of conditiionally expressed mutations along with their permissive and restrictive conditions are listed in (Table 4). The biochemical bases of conditionally expressed mutations are varied. The *temperature - sensitive* type mutations cause the gene product to become non-functional at either high or low temperature; the former subclass of mutations are termed *heat-sensitive* and the latter *cold-sensitive*. Most genes with temperature-sensitive mutations encode products that are intrinsically unable to function at the restrictive temperature, but a few, termed *temperature -sensitive-synthesis mutations*, encode products that are nonfunctional only if they are synthesized at the restrictive temperature, at which the secondary or tertiary structure of the protein product forms incorrectly.. However, if the protein is synthesized at the permissive temperature a properly folded product is synthesized that can function at the restrictive temperature as well as the permissive one.

Table 4
Types of Conditionally Expressed Mutants

Type	Defect
Temperature-sensitive	Gene product, usually a protein but sometimes a tRNA, cannot function or be synthesized (*temperature-sensitive synthesis*) at the restrictive temperature; but functions or is synthesized at permissive temperature.
Heat sensitive	High temperature is restrictive; low temperature is permissive (usually 42° and 30°C respectively in the case of enteric bacterial mutant strains).
Cold-sensitive	Low temperature is restrictive; high temperature is permissive (usually 20° and 37°C respectively in the case of enteric bacterial mutant strains).
Osmotically remedial	Permissive and restrictive conditiions are determined by the osmotic strength of the growth medium.
Streptomycin remedial[a]	Permissive condition is growth in a medium that contains streptomycin; restrictive condition is growth in a medium that lacks the antibiotic.

[a] Other aminoglycoside antibiotics, including neomycin and kanamycin can sometimes substitute for streptomycin.

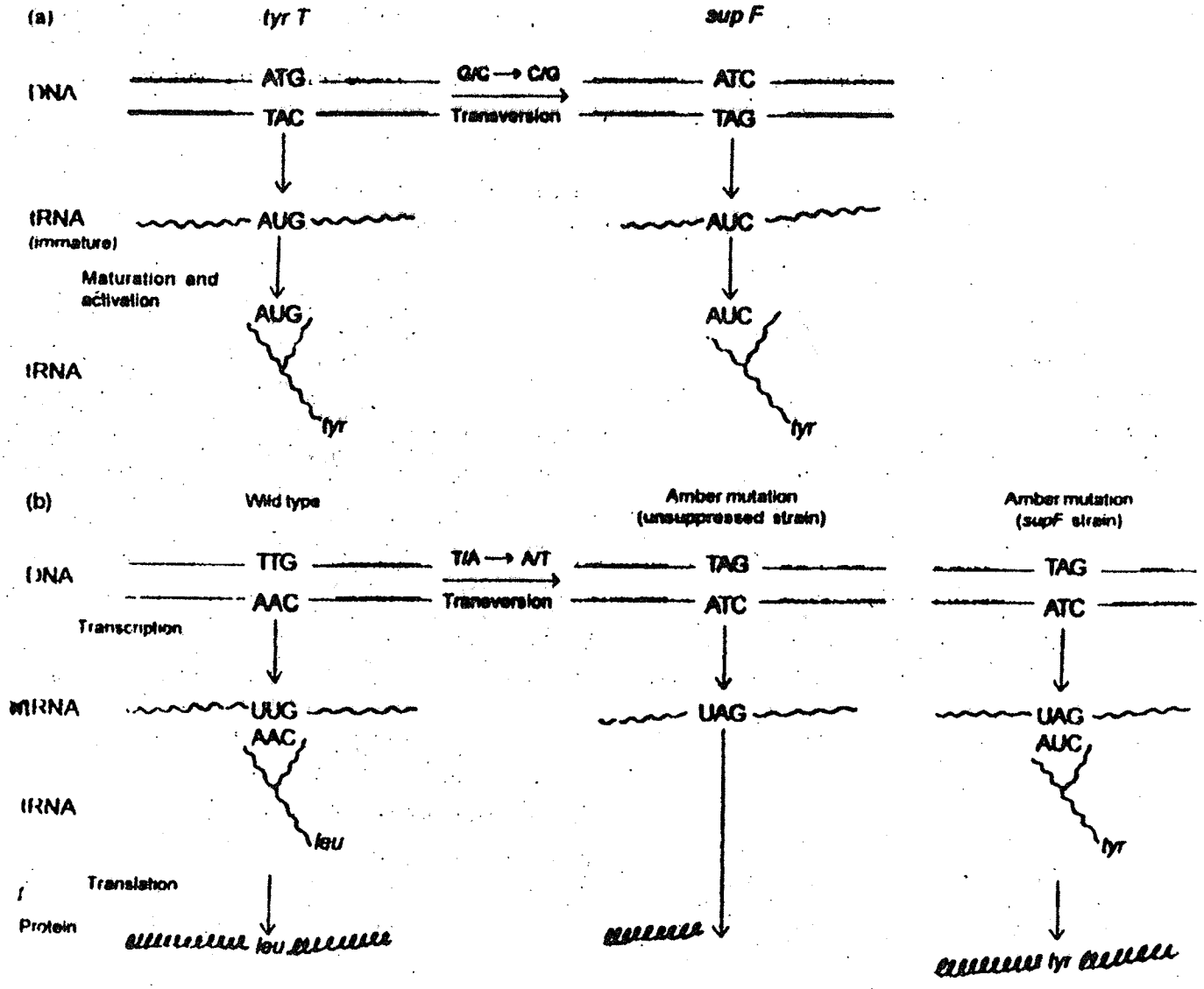
Osmotically remedial mutations cause the protein product to be particularly sensitive to osmotic strength. Usually the protein is stabel only in the presence of higher concentrations of solutes. Thus teh permissive condition for a strain with such a mutation is a medium with a higher concentration of salts. Often such strains cannot grow in commonly-employed complex media but can grow in synthetic media because the latter typically contain higher concentrations of salts.

Streptomycin - remedial mutants express a near wild-type phenotype when low levels of an aminoglycoside antibiotic (streptomycin, neomycin, or kanamycin) are added to the culture medium. These antibiotics exert their remedial effect by altering the translation mechanism rather than the gene products. These antibiotics bind to the 30S ribosomal subunit, therby increasing the error frequency of translation (the frequency with which an amino acid other than the encoded one is inserted into the growing peptide chain). Thus, in the presence of the antibiotic, incorrect forms of all the cell's proteins, including the mutual protein, are synthesized. Some of the mistranslated forms of the mutant protein are functional. By reversing the phenotypic consquences of a mutation, amino-glycoside antibiotics are said to *suppress* the defects of streptomycin - remedial mutants. Certain mutant forms of tRNA molecules also can suppress some mutations. These mutations in genes encoding tRNA molecules are called *suppressor mutations, or suppressors.* A suppressor mutation, like streptomycin, changes the translation mechanism, thereby producing some gene product that in functionally active.

The action of suppressor mutations can be illustrated by considering the action of a specific mutant allele (supF) in E. coli that suppresses the amber-type nonsense mutations (Figure 7). In the specific case considered, teh amber codon (UAG) was generated by an A/T to T/A transversion mutation, thereby changing the wild-type codon (UUG), which encodes leucine to the amber nonsense codon (UAG), which signals chain termination. The suppressor mutation is a G/C to C/G transversiion in a gene encoding one of the tyrosine tRNAs. The mutation changes the anticodon from AUG to AUG, thereby allowing it to recognize the amber codon and to insert a tyrosine residue at this site. Suppressiion occurs if the tyrosine-containing protein is functionally active. As is the case with exposure to streptomycin many faulty proteins are synthesized in cells that contain *supF* (or another suppressor mutation), because tyrosine residues are frequently inserted at the sites of other amber codons that should properly signal chain termination. But the codons designating tyrosine continue to be properly translated because the cells contain other (unmutated) species of tyro-sine-recognizing tRNA molecules.

Analogous mutant forms of tRNA suppress other mutations as well as missense or even frameshift mutations.

Figure 7
Generation (a) and function (b) of a nonsense suppressor.



MUTANT METHODOLOGY

Much of the detailed information now available about the metabolism and activities o microorganisms has come from the study of mutant strains that have lost a specific cellular function. Th rationsle of the *mutant methodology,* as this set of procedures is sometimes called, is simple, direct, an powerful : *a mutation alters or eliminates the functioning of a particular gene product; by observing th effect of genotypic change on the cell's phenotype, one can deduce the cellular function of the gene product* For example, strains with certain mutations in the *argI* gene do not synthesize a particular enzyme, ornithin carbamoyltransferase, and are able to grow only if their medium is supplemented with the amino aci

arginine or with the intermediates of the pathway leading to biosynthesis of that amino acid, provided that the intermediates enter the pathway subsequent to the genetic blockage. From such a study one learns that orni-tine carbamyltransferase catalyzes and essential reaction in the biosynthesis of arginine.

ISOLATION OF MUTANT STRAINS

The ways by which mutant strains are isolated are described in the following sections.

Phenotypic Expression

Effective mutagenic treatments are able to increase the frequency of mutant cells in a population by as much as 100,000-fold. The periods of mutagenic treatment are typically quite brief; e.g., attaining a maximal mutagenic effect of nitrosoguanidine usually involves only a 15-minute exposure. But following mutagenesis, a period of growth, sometimes quite lengthy, termed ***phenotypic lag*** must occur before the genotypically mutant cell expresses a mutant phenotype. The reasons why phenotypic expression of a mutatiion lags the genetic change that caused it can be explained by considering the case of a mutation that causes a cell to become resistant to certain bacteriophages that inflect bacteria by adsorbing to specific proteins on the cell's surface. Mutants that are resistant to these phages lack these receptor proteins or produce altered forms of them to which the phages cannot adsorb. As long as some of the wild-type form of the approximately 200 receptors are presentl on the cell surface, that cell remains are present on the cell surface, that cell remains sensitive to infectiion by the phage. Before the cell loses these receptors, it must become a homocaryotic mutant cell (i.e., contain a single type of nucleous) and pre-existing wild-type receptors must be diluted from the cell surface by growth of new surface and cell division.

Since most bacteria have several haploid nucleoi at the time of mutation, the cell is a mutant heterocaryon because it contains two different forms (alleles) of at least one gene. If a cell contains four nuclei. two cell divisions must occur before a homocaryotic mutant cell appears (Figure 8). But even the newly formed homocaryotic cell would contain many active phage receptors on its surface, and, hence, would remain phage-sensitive. Only after a number of subsequent cell divisions (about 12) would a phenotypically phage-resistant cell first appear.

Because of the requirement for homocaryon formation and gene product dilution, the phenotypic lag before recessive mutations are expressed is extended. It is typically much shorter if the mutant phenotype is dominant because there is not need either to form a homocaryon formation and gene product dilution, the phenotypic lag before recessive mutatiions are expressed is extended. It is typically much shorter if the mutant phenotype is dominant because there is no need either to form a homocaryon or dilute the wild-type gene produt; as soon as sufficient mutant gene product is produced, the phenotype of the mutant is expressed. For example, many chemicals stop the growth of bacteria by entering the cell and inhibiting the activity of specific target enzymes. Certain mutations in the gene encoding one of these target enzymes render it insensitive to the corresponding inhibitory chemical. A mutant phenotype of this class of mutations is

usually dominant and the lag between the genotypic and phenotypic change is quite brief, because as soon as some mutant (resistant) enzyme is synthesized, the cell is able to grow in the presence of the inhibitory chemical.

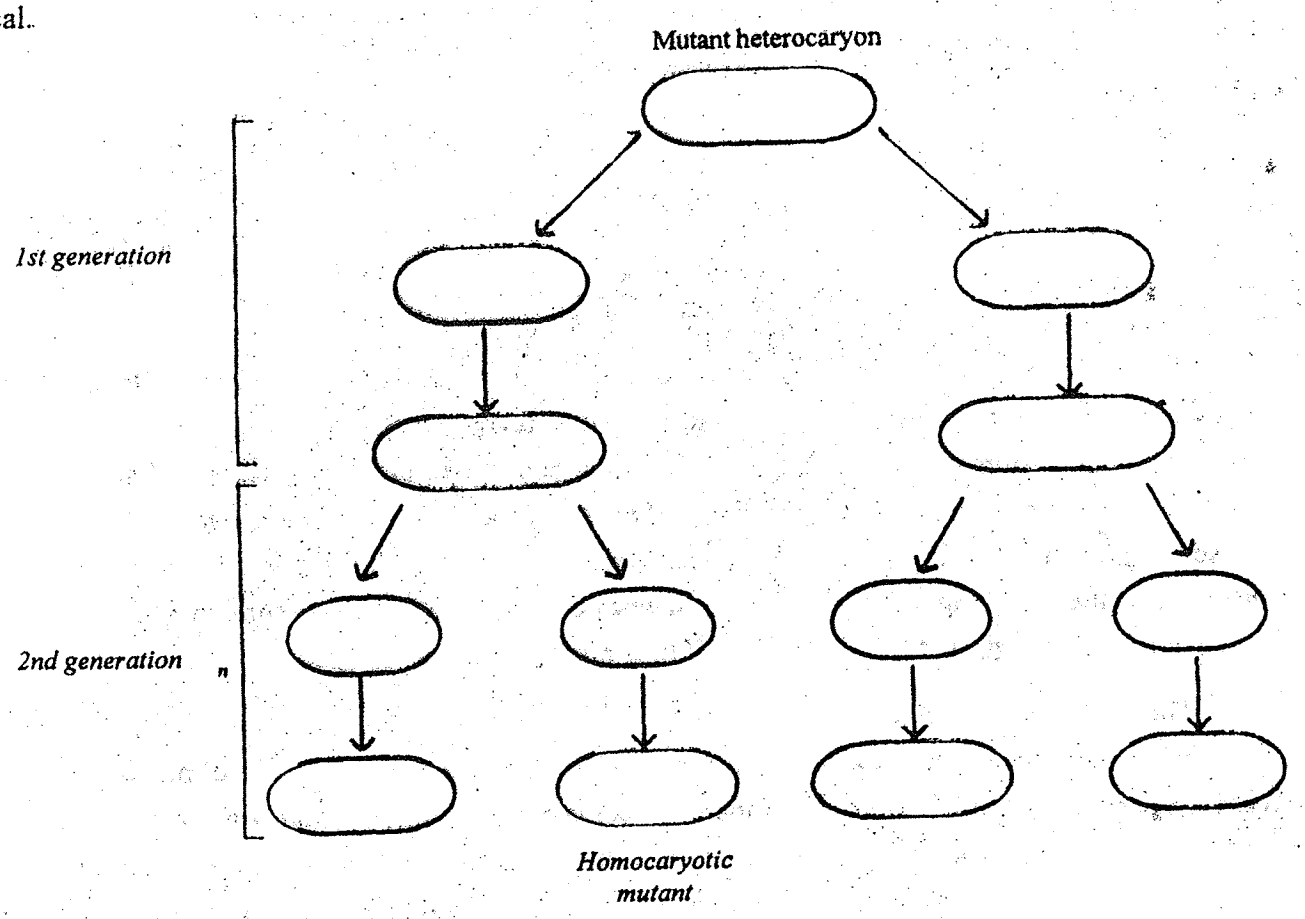


Figure 8. *Bacterial cells with either two or four nuclei if a mutation first occurs in a tetranucleate cell, two generations are required before a homocaryotic mutant cell appears. If the mutant is recessive cannot expressed until the mutant nucleus has completely segregated from unmutated nuclei.*

Enrichment of Mutant Cells in a Population

Even after effective mutagenesis, any particular mutant is relatively rare in a population and some with particular altered gene products can be quite rare. So, in order to obtain a pure culture of mutant cells, one or another of a number of schemes are employed to increase the frequency of mutant cells in the population (Table 5). Sometimes, when conditions can be designed to favor the growth of the mutant class, enrichment procedures can be quite simple. For example, phage-resistant clones can be isolated simply by plating the mutagenized, phenotypically expressed population on plates containing phage virion. Cells expressing the parental phenotype are killed; only those phage-resistant cells in the population develop into colonies. Similarly, mutant strains resistant to an antibiotic or a toxic chemical can be isolated by plating the population with the antibiotic or chemical. Such isolation or enrichment procedures are termed *direct enrichment.*

Table 5
Examples of Schemes Suitable for Enriching a Mutagenized Culture for a Particular Mutant Type

Mutant Class	Treatment
I. DIRECTENRICHMENT	
Phase resistance	Plate culuture on a medium containing phage virions
Antibiotic or chemical assistance	Plate culture on a medium containing antibiotic or chemical
II. COUNTERSELECTION	
Auxotroph	Counterselect in absence of required nutrient
Carbon	Counterselect in a medium containing only the carbon source that mutant strain cannot metabolize.
Nitrogen source	Counterselect in a medium containing only the nitrogen source that the mutant strain cannot metabolize.

However, not all types of mutants can be isolated by direct enrichment. For example, it is not normally possible to devise conditions that favor the growth of auxotrophic mutant cells in a populatiion composed principally of prototrophs, or conditions that favor mutant cells unable to utilize a particular carbon or nitrogen source in a population of cells that can utilize it. The frequency of such mutants in a populatiion can be increased by the use of one of a set of procedures, termed *counter selection*, so named because they result in killing cells that express a parental phenotype, thereby increasing the fraction of mutant cells in the surviving population. The efficiency of counterselection depends on employing a chemical or a condition of growth that kills cells only if they are growing.

The antibiotic penicillin is an effective agent for counterselection; it acts inhibiting the formation of cross-links in peptidoglycan. As a consequence, cells that grow in the presemnce of penicillin synthesize a weakened peptidoglycan layer that is incapable of containing the intracellular osmotic pressure and such a cell bursts. But nongrowing cells survive in media that contain penicillin because the antibiotic does not affect preformed peptidoglycan. Thus, in a minimal medium containing penicillin but lacking the essential nutrient of an auxotrophic mutant, the mutant cell survives because it cannot grow, but the prototrophic parental-type cell is killed as it starts to grow, synthesizes weakened peptidoglycan and bursts. Certain counterselective agents and their mechanisms of selectively killing growing cells are shown in Table 6.

Detectiion of Mutant Clones

A variety of procedures have been devised to make colonies of the desired mutant type visually distinguishable from colonies of the wild type. For example, the colorless compound tetrazolium is reduced to the brilliantly red, insoluble product, formazan, only within a narrow pH range. Thus, in a complete medium supplemented with a high concentration of a fermentable sugar, cells able to ferment that sugar lower the pH to the point where the dye is not reduced, and form white colonies. Mutant cells unable to ferment the provided sugar, however, reduce the tetrazolium intracellularly to fomazan and produce bright red colonies. By this technique it is possible to detect a single fermentation-deficient mutant colony among 10^3 wild-type colonies on a petri dish.

In some cases, the only reagents that are able to stain mutant colonies differentially are also lethal.

Table 6
Counterselective Agents and the Mechanism by which They Kill Growing Cells

Agent	Mechanism
Penicilling	Kills growing cells by inhibiting formation of cross-links in peptidoglycan.
S-Azaguanine	Growing cells incorporate 8-Azaguanine into DNA, rendering it nonfunctional.
Redioactive nutrient	Growing cells incorporate the radioactive nutrient in cellular components. These cells die slowly (during subsequent storage) as radioactive decays occur.
Thymine deprivatiion	A culture of thymine auxotrophs die when deprived of thymine. Such a culture can be used to enrich a second mutation because they will not die if they lack an essential nutrient, or are unable to grow for some other reason

For example, one may wish to select mutants that form glycogen, which can only be detected by staining (and killing) the colonies with iodine. In such cases, the technique of *sib selection by replica plating* is used : a.plate bearing thousands of colonies is replicated, as described below, and the replica plate is flooded with an iodine solutiion. If a glycogen-positive mutant colony is detected, an inoculum of *live* mutant cells can be recovered from the corresponding location on the original plate.

In replica plating, a piece of strerile velvet is stretched over a cylindrical block of wood or metal that is slightly smaller in diameter than a petri dish. The block is placed with the velvet surface facing upward; the petri dish with the lawn of bacterial colonies is inverted, and its surface is gently pressed against the velvet. The projecting fibers of the velvet, numbering thousands per square inch, act as inoculating needles, smapling every colonly in the lawn. The petri dish is removed, and a fresh plate of agar is pressed against the velvet in order to receive an inoculum from each colony. The plates are identically oriented at each application of the velvet with respect to marks placed on their rims, so that the colonies that appear on the replica plate after incubation occupy positions congruent with those of their siblings on the original plate (Figure 9).

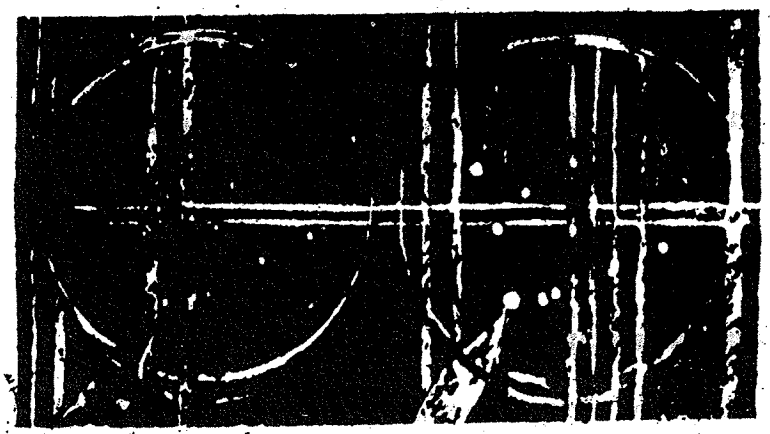


Figure 9 *Replica plating. The master plate of nutrient agar (not shown) bore twelve colonies. One replica was prepared on nutrient agar (right) and one on a synthetic medium lacking growth factors (left). The two plates are similarly oriented, and the arrow points to sister replicas of one colony. Note that although twelve colonies developed on the complex medium, only nine were formed on the synthetic medium. The three colonies that failed to give replicas on the synthetic medium were made up of mutants that required growth factors for their development.*

The inoculum on the velvet surface is usually large enough to permit a series of different agar plaes to be sequentially "printed" from it. Thus, replica plating can also be used to test inocula from a very large number of colonies on a "master plate" for their ability to grow on as many as eight or ten different selective media. This technique has made practicable the multiple analyses which are basic to microbial and molecular genetics.

The various steps involved in the isolation of a mutant are illustrated by the complete shceme suitable for isolating a heat-sensitive arginine auxotropic mutant shown in Figure 10.

Figure 10

Isolation of a heat sensitive arginine auxotrophic mutant.

Culture

↓ Treat with suitable mutagen to increase probability of desired mutant being present.

Mutagenized culture

↓ Grow for five or six doublings in a medium that permits desired mutant to replicate either at permissive temperature (30°C) or at restrictive temperatrue (40°C) with arginine present.

Phenotypically expressed culture

↓ Intiate growth of culture under conditions that preclude growth of desired mutant i.e. at 40°C in a medium lacking arginine. Add penicillin.

Culture in which the frequency of desired mutant cells is enriched

↓ Plate and incubate under conditions that support growth of mutant and parental clones e.g. at 30C on a medium lacking arginine or at 40C or a medium containing arginine. Pice colony that grows on the first set of plates but not the second.

Mutant clone

Spontaneous nature of bacterial mutation

As mentioned previously, for a long time many geneticists were very reluctant to believe that the observable changes in bacterial cultures were ever anything more than physiological adaptations or phenotypic changes. That is, they refused to believe that bacteria have stable hereditary systems and could undergo permanent changes or mutations and assumed that any changes in the characteristics of a culture were simply due to environmental influences. However, Max Delbruck and Salvador Luria believed that

bacteria have stable hereditary mechanisms, and in 1943 they performed an elegant experiment that proved the point.

Bacterial viruses called bacteriophages, or simply phages, are capable of killing bacteria. When susceptible bacteria are exposed to a phage, some of the bacterial cells survive, and they and their descendants are resistant to the phage. Some microbiologists assumed that these cells were modified by their contract with the phage so they became resistant by physiological adaptation. But Luria and Delbruck believed that phage-resistant bacteria were the result of mutations that occurred before the bacteria came into contact with the phage.

Let us suppose that resistant cells are the result of contact with phages; then if we set up a large number of identical bacterial cultures and expose them all to identical batches of phage, approximately the same number of resistant cells should appear in all cultures. However, if the resistant cells are really the result of mutations and since mutations occur entirely at random, then when we grow many identical cultures and expose them all to identical batches of phage, we should find a great fluctuation in the numbers of resistant cells, but in other cultures a mutation might have occurred very early, so that nearly all the cells are resistant (Fig. 11).

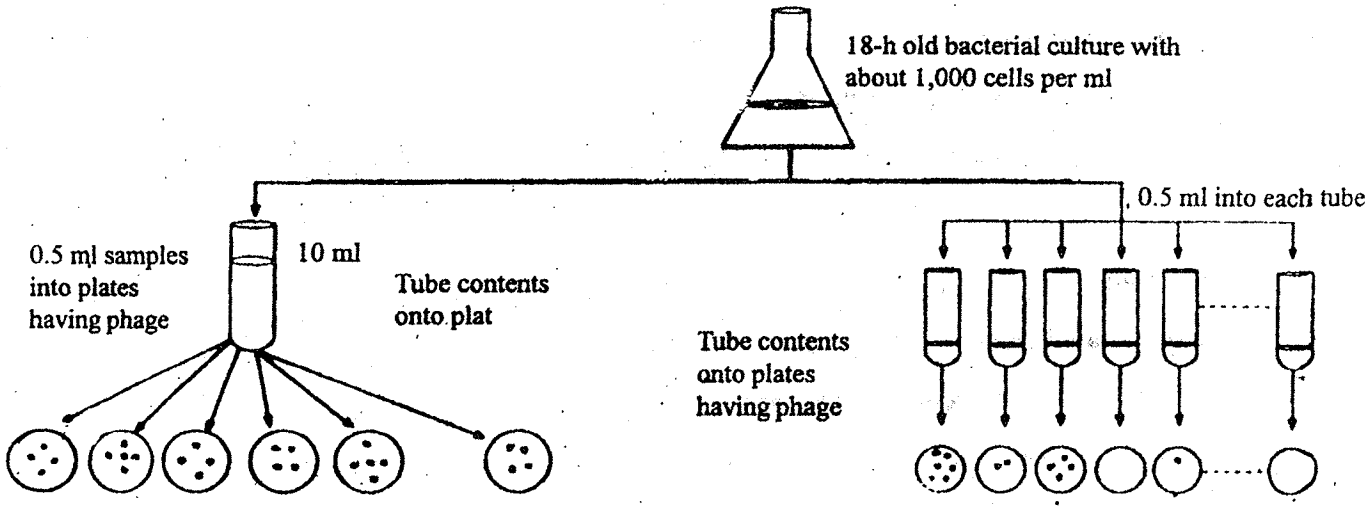


Figure 11. The fluctuatiion test was performed essentially as follows. A series of tubes containing 0.5 ml of cells was incubated without phage until a certain population size was reached. The cultures were then exposed to phage by pouring the contents of each tube into an agar plate containing phage. The number of phage-resistant mutants in each tube was thus determined. The colony counts from such a series of *similar cultures* were then compared with the results of a series of samples taken from *one culture* started with a similar density of cells per milliliter and allowed to reach a similar population number per milliliter. The results showed that resistant bacteria arise spontaneously prior to the exposure to phage since a series of similar cultures yielded results different from those obtained with a series of samples from one culture. (See text for further explanation).

Thus Luria and Delbruck predicted that physiological adaptation to the phage by the bacteria should give about the same number of resistant bacteria (within sampling error) in each culture. But if phage resistance was due to mutatiions, preexisting and merely selected by the addition of phage, the number of resistant bacteria should fluctuate widely in each culture. Luria and Delbruck found a much greater fluctuation in numbers than could be accounted for by physiological adaptation, and thus they proved statistically by their fluctuation test that phage resistance ws really the result of mutation (see Fig. 11).

Shortly after, in 1952, more direct proof of preexisting mutants was provided by Joshua and Esther Lederberg. They introduced the replica plating technique (shown in Fig. 2) and provided a direct method for demonstrating the undirected spontaneous origin of bacterial mutants; i.e., the mutants occurred independently of any selective agent or environment. The procedure made it practical to examine large numbers of clones (pupulations of cells descending from a single cell) for a particular characteristic. By using sufficiently large samples, one could, for example, demonstrate the occurrence of phage -resistant mutants in a culture which was known to be phage-sensitive. The mutant types developed and could be located on an agar-plate culture which had not encountered phage previously. Similarly, the spontaneous appearance of antibiotic-resistant strains could be demostrated without previous exposure of the culture to the antibiotic. As Fig. 12 shows, replica plating can also be used for isolating nutritional mutants. In essence the technique provides a practical means for finding the one cell in a million (more or less) which has mutated.

Thus the Lederbergs were able to isolate pure colonies of resistant E. coli to lytic phage. This experiment demonstrated that mutation against the phage had its origin in spontaneous mutation. The growth of resistant colonies on replica plates arose from cells that were already present and were already resistant on the original nonselective plate prior to exposure to the selective agent, such as a lytic phage Figure 13. shows a simplified representation of the experiment performed by the Lederbergs. Since then many other types of mutatatins have been found in bacteria, and it is now firmly established that bacteria have a hereditary system just like higher organisms.

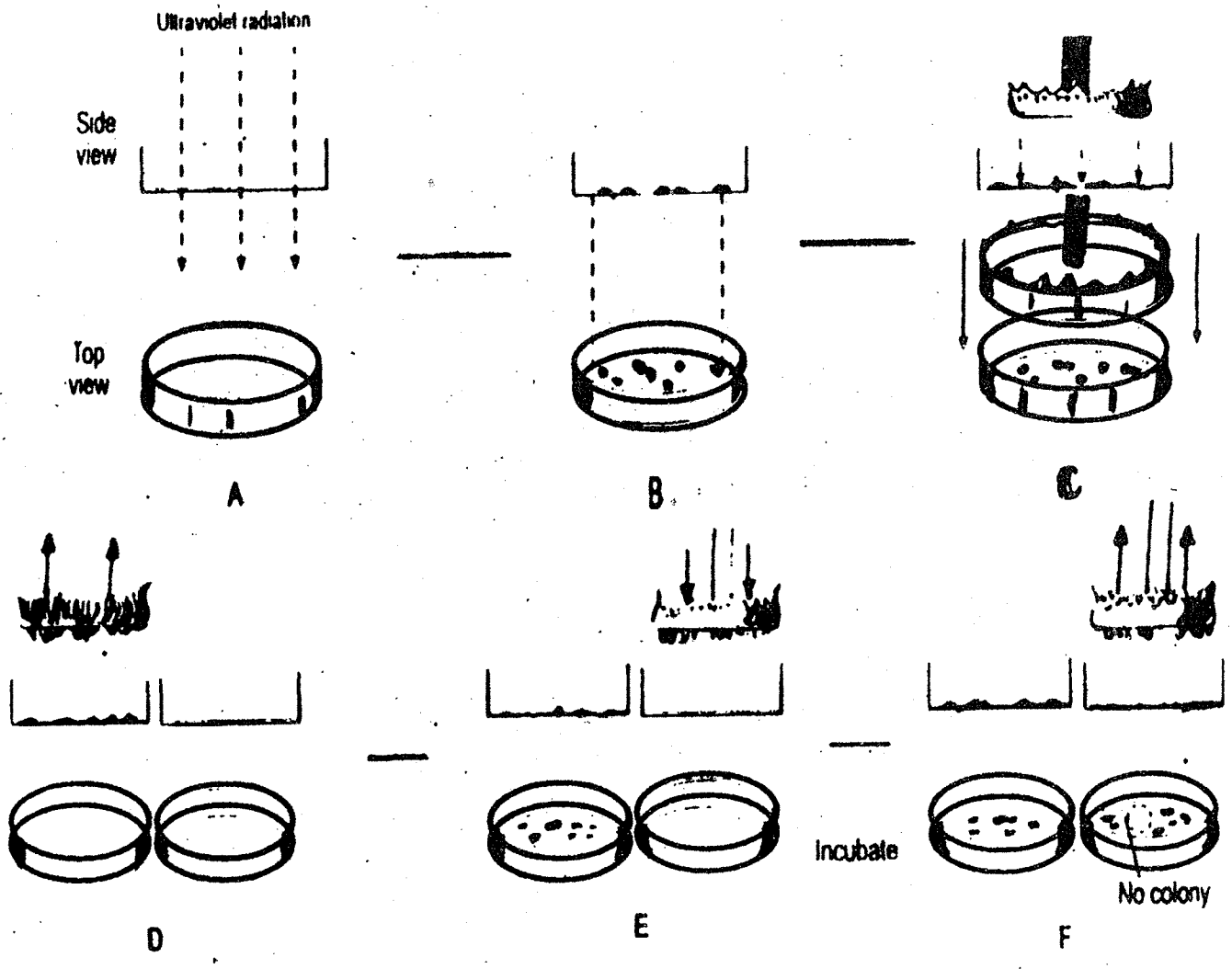


Figure 12. *Replica plating is used for isolating nutritional mutants of Escherichia coli. (A) Bacterial suspension placed in open half on Petri dish and exposed to mutatenic agent, such as ultraviolet radiation. (B) Sample from (A) plated on surface of a "complete" medium such as nutrient agar. The plate is incubated; after incubation, the exact position of colonies on the plate is noted. (C) A sterile replica plating unit is gently pressed to the surface of plate (B), then raised (D), and then pressed to the surfce of a plate of "minimal" agar medium (E). The positioning of the replica plating unit on the minimal agar must be precise, so that colony locations will be comparable on each of the two plates. The plates will be replicas of one another. The minimal agar in the plate in (E) consists of inorganic salts and glucose, nutrients which normally permit growth of E.coli. After incubation (F), colonies appear on the new plate at most, but not all, of the positions corresponding to locations of colonies on the original plate. It may be assumed that the organisms that failed to develop are nutritional mutants; that is, they are not able to grow on an inorganic salts-glucose medium, a characteristic which they originally possessed.*

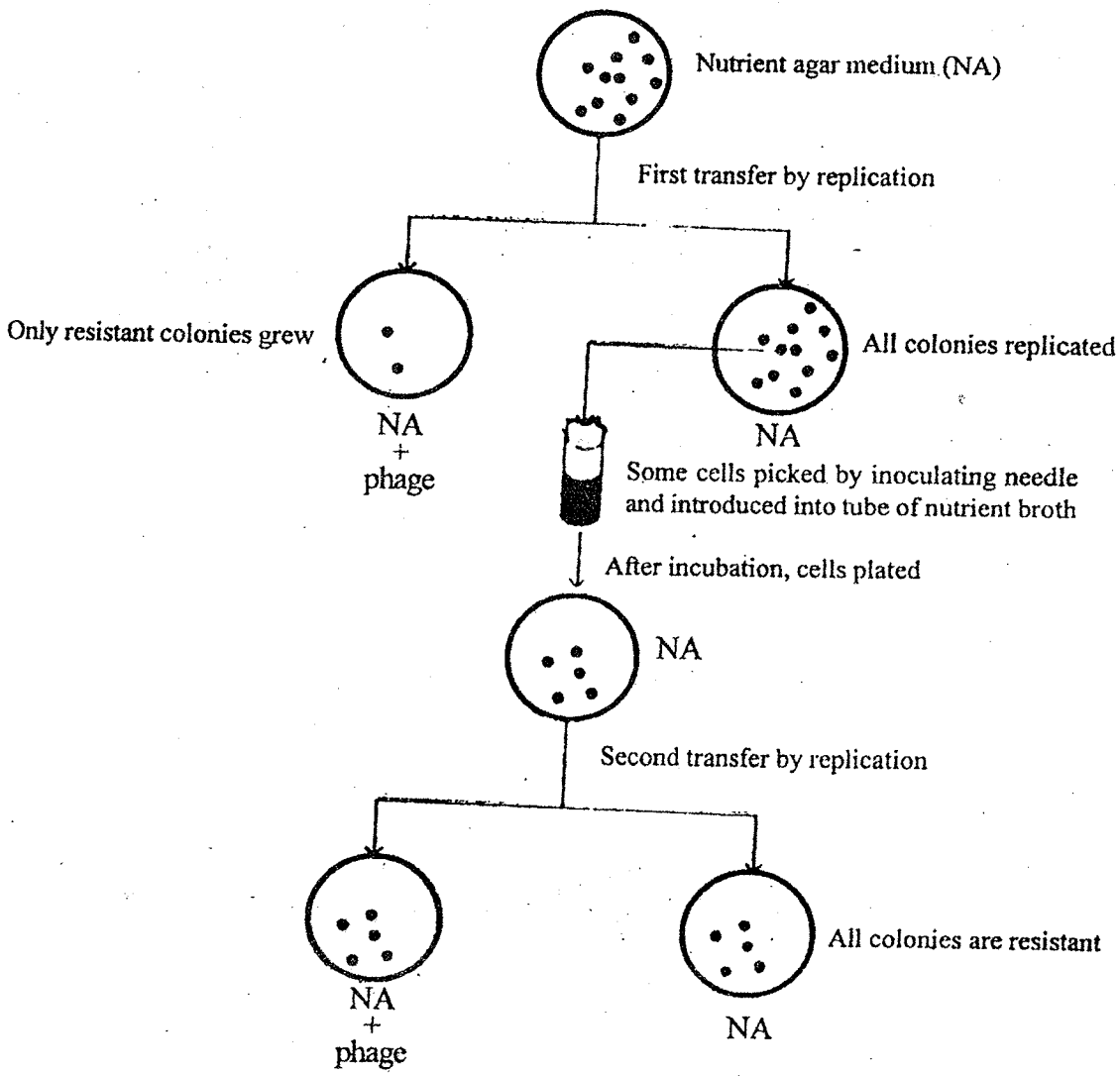


Figure 13. *Simplified representation of the experiemtn of the Lederbergs showing the spontaneous nature of mutation in bacteria. The drawing shows that isolation of a pure colony of phage-resistent bacteria from a medium is possible without prior exposure to the virus.*

Suggested Questions

1. State the mode of action of the following mutagen :-
   (a) nitrosoquanidine, (b) ICR 191, (c) 2-amino purine, (d) Hydroxylamine.
2. How will you isolate the pur culture of following mutant types ?
   (i) Phage resistance, (ii) Auxotroph.
3. How will you prove the spontaneous nature of bacterial mutation?

Ref. (i) Gen. Microbiology - Stanier (5th edition)
(ii) Mcrobiology - Pelczar (5th edition).
(iii) Microbial genetics - David Freifelder.

Botany
Chapter - 2 , Paper - I (1st Half)
Microbiology

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CHAPTER – 2

(CHEMOTHERAPY AND ANTIBIOTICS)

CHEMICAL AGENTS-CONTROLLING MICRO-ORGANISMS

A large number of chemical compounds have the ability to inhibit the growth and metabolism of microorganisms or to kill them. Commercial products which incorporate these compounds are available for controlling microbial populations in many different circumstances. For example, solutions of some chemical compounds are used to reduce the microbial flora of the oral cavity; other chemical compounds are recommended for reducing the microbial population in the dust of hospital floors. No single chemical agent is best for any and all purposes. This is not surprising in view of the variety of conditions under which they are used, their differences in mode of action, and the differences of resistance among microbial species. Experience and research have shown that certain kinds of chemicals are more appropriate and effective for certain situations. As a result, several classes of chemical substances have been identified and new compounds developed within these classes which have destructive properties in terms of their suitability for practical application.

Definition of Terms

The following terms are used to describe the processes and chemical agents employed in controlling microorganisms.

Sterilization: The process of destroying all forms of microbial life. A sterile object, in the microbiological sense, is free of living microorganisms. The terms sterile, sterilize, and sterilization therefore refer to the complete absence or destruction of all microorganisms and should not be used in a relative sense. An object or substance is sterile or nonsterile; it can never be semisterile or almost sterile.

Disinfectant : An agent, usually a chemical, that kills the growing forms but not necessarily the resistant spore forms of disease-producing microorganisms. The term is commonly applied to substances used on nanimate objects. Disinfection is the process of destroying infectious agents.

Antiseptic : A substance that opposes sepsis, i.e., prevents the growth or action of microorganisms either by destroying microorganisms or by inhibiting their growth and metabolism. Usually associated with substances applied to the body.

Sanitizer : An agent that reduces the microbial population to safe levels as judged by public health requirements. Usually it is a chemical agent that kills 99.9 percent of the growing bacteria. Sanitizers are commonly applied to inanimate objects and are generally employed in the daily care of equipment and utensils in dairies and food plants and for glasses, dishes, and utensils in restaurants. The process of

disinfection would produce sanitization; however, in the strict sense, sanitization implies a sanitary-condition which disinfection does not necessarily imply.

Germicide (Microbicide) : An agent that kills the growing forms but not necessarily the resistant spore forms of germs; in practice a germicide is almost the same thing as a disinfectant, but germicides are commonly used for all kinds of germs (microbes) for any application.

Bactericide : An agent that kills bacteria (adjective, bactericidal). Similarly, the terms fungicide, virucide, and sporicide refer to agents that kill fungi, viruses, and spores, respectively.

Bacteristasis : A condition in which the growth of bacteria is prevented (adjective, bacteriostatic). Similarly, fungistatic describes an agent that stops the growth of fungi. Agents that have in common the ability to inhibit growth of microorganisms are collectively designated microbistatic agents.

Antimicrobial Agent : One that interferes with the growth and metabolism of microbes. In common usage the term denotes inhibition of growth, and with reference to specific groups of organisms such terms as antibacterial or antifungal are frequently employed. Some antimicrobial agents are used to treat infections, and they are called **chemotherapeutic** agents.

CHEMOTHERAPEUTIC AGENTS & CHEMOTHERAPY

The treatment of a disease with a chemical substance is known as **chemotherapy;** the chemical substance is called a **chemotherapeutic agent.** Chemotherapy has been practiced for centuries, but it was only early in the present century (the mid-1930s) that this kind of therapy revolutionized the field of medicine. This turn in events is attributed to two discoveries. The first was the finding that sulfonamide compounds (sulfa drugs) could be used successfully for the treatment of certain bacterial diseases. The second was the discovery of a new and potent class of antibacterially active chemotherapeutic agents, namely, antibiotics.

Chemotherapeutic Agents and Chemotherapy

Chemotherapeutic agents are chemical substances used for the treatment of infectious diseases or diseases caused by the proliferation of malignant cells. These substances are prepared in the chemical laboratory or obtained from microorganisms and some plants and animals. In general naturally occuring substances are distinguished from synthetic compounds by the name antibiotics. Some antibiotics are prepared synthetically, but most of them are prepared commercially by microbial biosynthesis. Antitoxins and other substances produced by the bodies of infected animals are not considered to be chemotherapeutic agents. The compounds discussed in the beginning used for killing or inhibiting microbial growth in vitro are not classified as chemotherapeutic agents but usually as disinfectants, antiseptics, or germicides.

To be useful as a chemotherapeutic agent a substance must have selective toxicity for the parasite, which means a low toxicity for host cells and high toxicity for the parasite. In other words, the substance

must damage the parasite and cause little or no damage to the cells of the host. For this and other reasons antiseptics and germicides such as phenol, coal-tar derivatives, and many murcurial compounds are unsatisfactory as chemotherapeutic agents. Germicidies are not selective in their action on cells, and they interfere with such natural defense mechanisms as phagocytosis; since they do not penetrate cells and tissues well, they do not come into contact with the parasites; because they are inactivated by protein, their effectiveness is destroyed by body fluids rich in protein; and finally, the tissues killed by the germicide or antiseptic provide an excellent medium for microorganisms to grow.

Therefore a satisfactory chemotherapeutic agent must :

1) Destroy or prevent the activity of a parasite without injuring the cells the host or with only minor injury to its cells.

2) Be able to come in contact with the parasite by penetrating the cells and tissues of the host in effective concentrations.

3) Leave unaltered the host's natural defense mechanisms, such as phagocytosis and production of antibodies.

HISTORICAL HIGHLIGHTS OF CHEMOTHERAPY

Quinine and Malaria :

Europeans used natural quinone from the bark of the cinchona tree to treat malaria as early as 1630. It was used earlier by South American Indians, who relieved symptoms of malarial fever by chewing the bark of the Chinchona tree.

Ehrilich, Salvarsan and Syphilis

Syphilis is the first known disease for which a chemotherapeutic agent was used. Mercury was used to treat syphilis as early as 1495, but it was not until about 1910 when an aresenical compound known as Salvarian was synthesized by Paul Ehrlich, that a specific drug capable of curing disease without great danger to the patient was developed. Ehrilich's contributions were especially important because his was the first systematic and deliberate search for a compound that had potent microbicidal properties, low toxicity for humans and other animals, and good chemical stability. For this important discovery he was awarded a share, with Elie Metchnikoff, of the 1908 Nobel Prize in physiology and medicine, Ehrilich's compound has now been replaced in syphilis therapy by arsphenamine, neoarsphenamine, and other arsenical compounds and antibiotics.

In 1935, when Domagk showed the therapeutic value of a group of compounds known as the **sulfonamides.** These substances are not specific for a special group of organisms, as arsphenamine is for *Treponema,* but are effective against a large variety of pathogenic organisms.

The sulfonamides are especially useful in the treatment of infections caused by meningococci and Shigella, respiratory infections caused by streptococci and staphylococci, and urinary infections due to Gram-negative organisms. They are useful in the prevention of rheumatic fever, bacterial endocarditis, wound infections, and urinary-tract infections following surgery or catheterization.

Antibiotics, Fleming, and Penicillin

Antibiotics are a special kind of chemotherapeutic agent usually obtained from living organisms. The word antibiotic has come to refer to a metabolic product of one microorganism that in very small amounts is detrimental or inhibitory to other microorganisms. It has been known for many years that antagonisms can exists between microorganisms growing in a common environment. The term antibiosis was first defined by Vuillemin in 1889 as a condition in which "one creature destroys the life of another in order to sustain his own the first buning entirely active and the second entirely passive; one is in unrestricted imposition to the life of the other". However, it can be seen that this definition is not entirely compatible with the present day use of the term antibiotics proposed by Waksman in 1945 as applying to those chemical substances of microbial origin which in small amounts exert antimicrobial activity.

The first systematic search for, and study of antibiotics, made by csratia and Dath about 1924, resulted in the discovery of actinomycetin in strans of actinomycetes, soil organisms that are representative of the group that has given us a number of antibiotics since 1940. Actinomycetin was never used for the treatment of patients but was used to lyse cultures of bacteria for the production of vaccines.

In 1929 Alexander Fleming noticed that an agar plate inoculiated with **Staphylococcus aureus** had become contaminated with a mold and that the mold colony was surrounded by a clear zone, indicating inhibition of bacterial growth, or lysis of the bacteria. He was inspired to solate and identify the mold and study its activities, but not until there was an urgent need for a better means of preventing death from infection of war wounds was the importance of Fleming's observation realized. With the aid of many investigators in England and the United States, and at a great deal of expense, the inhibitory substance from Fleming's "contaminant mold" became a "miracle drug". Because the mold was identfied as a **Penicillium sp.**, Fleming called the antibiotic penicillin.

In 1939, Rene Dubos isolated from New Jersey soil a culture of **Bacillus brevis** which produced a substance that killed many Gram-positive bacteria. The cell-free extract produced from **B.bravis** by Dubos was found to contain two active principles now known as gramicidin and tyrocidine. These successes were followed closely by the discovery of streptomycin by Selman Waksman and associates.

Several thousand antibiotic substances have been isolated and identified since 1940. Many of them are of no practical importance as yet, but a few have changed the entire concept of chemotherpy. The popularity of antibiotics is due to their ability to desory many kinds of pathogens and to their relatively nontoxic properties to the host when given systemically. Few developments in the field of medicine have

had as dramatic an effect as have antibiotis in the treatment of microbial infections.

Characteristics of Antibiotics that Quality them as Chemotherapeutic Agents

To be useful as chemotherapeutic agents antibiotics must have the following qualties :

1. They should have the ability to destroy or inhibit many different species of pathogenic microorganisms. This is what is meant by a "broad-spectrum" antibiotic.
2. They should prevent the ready development of resistant forms of the parasites.
3. They should not produce undesirable side effects in the host, such as sensitivity or allergic reactions, nerve damage, or irritation of the kidneys and gastrintestinal tract.
4. They should not eliminate the normal microbial flora of the host, because dining so may upset the "balance of nature" and permit normally nonpathogenic microbes, or particularly pathogenic forms normally restrained by the usual flora to establish a new infection. The broad-spectrum antibiotics, for example, may aliminate the normal bacterial flora but not Monilia from the intestinal tract. Under these conditions the Monilia may establish an infection that is not controlled by antibiotic therapy.

Consult : [Ref:- Pelzar's Microbiology & The pharmacological basis of therapeutics – Goodman and Gillman].

Antibiotics and their mode of Action:

The major points of attack of antibiotics on microorganisms include :

1. Inhibition of cell wall synthesis.
2. Damage to the cytoplasmic membrane.
3. Inhibition of nucleic acid and Protein synthesis.
4. Inhibition of specific enzyme system.

The most common classification has been based on chemical structure and proposed mechanism of action as follows :-

1. Agents that inhibit synthesis of or activate enzymes that disrupt bacterial cell walls to cause loss of viability and often, cell lysis; these includes Penicillins and Cephalosporins which are structurally similar and dissimilar agents such as cycloserine Vancomycin bacitracin.
2. Agents that act directly on the cell membrane of the micro organisms, affecting permeability and leding to leakage of intracellualr compounds these includes detergents, polymyxin and colistimethate and the polyene antifungal agents mystatin and amphotericin B that bind to cell wall sterols.
3. Agents that affect the function of bacterial ribosome to cause a reversible inhibition of protein synthesis, these bacterio static drugs include chloramphenicol, the tetracyclines, erythromycin and clindamycin.

4. Agents that bind to the 30s ribosomal submit and alter protein synthesis which eventually leads to cell death, these includes aminoglycosides-Streptomycin.
5. Agents that affect nucleic Acid metabolism such as rifamycins (e.g. rifampin) which inhibit DNA dependent RNA polymerase and the quinolones which inhibit DNA supercoiling and DNA syntehsis.
6. The antimetabolites including trimethoprim and the sulfonamides which block specific metablolic steps that are essential to micro-organisms.
7. Nucleic Acid analogs such as Zidovudine, ganciclovir, Vidarabine and acyclovir, which bind to Viral enzyme that are essential for DNA synthesis, thus halting viral replication.

At the present time the precise mechanism of action of some antimicrobial agents is unknown.

The Penicillins

The first of the modern antibiotics, and still one of the most useful, penicillin is produced by Penicillium notatum, Penicillium chrysogenum and by other species of molds. As previously noted, the first of those was isolated by Fleming in 1929, when he found it as a contaminant on a culture pladte. Florey and his associates at Oxford University for Gram-positive bacteria, some spirochetes, and the Gram-negative diplococci (Neisseria). Although is rarely toxic in human patients, it may give rise tosensitivity reactions which vary from a mild skin reaction to severe anaphylaxis.

Penicillins are a class of β-lactam antibiotics of related structure with slightly different properties and activities. All penicillin have a common basic nucleus, a fused b-lactam-thiazolidine ring with different side chains which give each its unique properties (Fig.1). Several chemically different penicillins are produced by biosynthesis in a single fermentation.

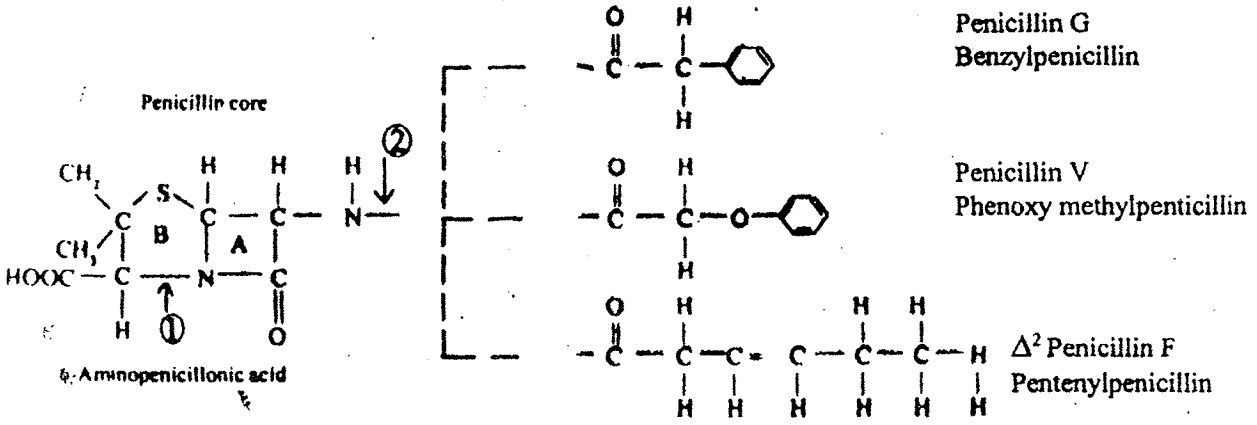


Figure 1 : Some "natural penicillin showing the basic core of 6-aminopencillonic acid with side cahins which differ, thereby conferring special properties. A → Thirzolidine ring B.→ β-lactam ring 1. → Site of action of Penicillinase 2. → Site of action of amidase.

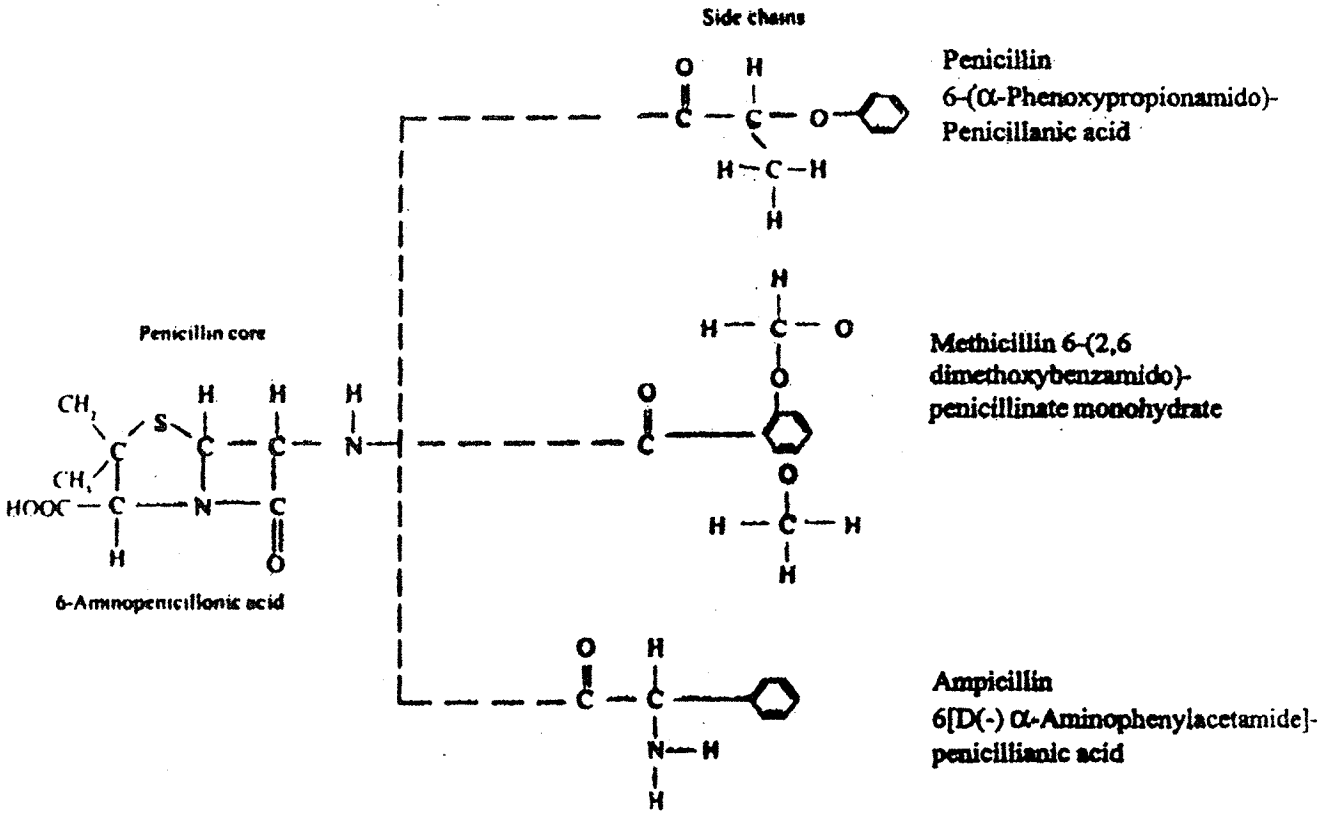


Figure 2- Structural formulas of some semisynthetic penicillins.

Natural penicillins can be prepared as salts of sodium, potassium, procaime and other bases. The crystalline sodium of potassium salts are freely soluble in water, ethyl alcohol, ether, esters, and dioxane but only slightly soluble in chloroform and benzene. In pure crystalline form penicillins are colorless. The natural penicillins are inactivated by heat, cysteine, sodium hydroxide, panicillinase and hydrochloric acid. They are not affected by the action of saliva or bile. Penicillin V exhibits greater stability than others in acids. Some of the new semisynthetic penicillins may be much more stable than those produced by biosynthesis, the "natural penicollins.

The first break in the production of the new semisynthetic penicillins was the discovery that the basic nucleus of the molecule, common to all penicillins, is 6-aminopenicillanic acid. The next step was to obtain 6-aminopericillanic acid in quantity so that suitable side chains could be attached to it. This was a very difficult task, but is was then discovered that under suitable conditions P. chrysogenum would produce the basic nucleus in abundance by "interupted biosynthesis", and that the side chains could be removed from penicillin G. produced by biosynthesis, by amidase enzymes, leaving the 6-aminopenicillamic acid free for attaching new side chains as desired.

One of the first semisynthetic penicillins to be produced for clinical use was phenethicillin. It is

more readily absorbed than penicillin V and just as effective as penicillin G. Another of themsemisynthetic penicillins methicillin is more resistant to penicillinase and therefore is less likely to be inactivated.

Ampicillin

Ampicillin, another semisynthetic penicillin, acts against a broad spectrum of bacteria. It is strongly bactericidal and lacks toxicity, but it is not resistant to penicillinases. It is relatively stable to gastric acid and hence can be administered orally. The chemical structures of these three penicillins are shown in Fig.2. Several additional semisynthetic penicillins have been developed for theche motherapeutic use.

Mode of Action

Penicillins interfere with the final stages of peptidoglycan biosynthesis. The penicillins inhibit the transpeptidase reaction, namely, the cross-linking of the two linear polymers. The penicillins are bactericidal to growing cells.

Actually Penicillin inhibits the cross linking reaction by acting as a structural analogue of the terminal D-alanine residue of the peptidoglycan strand. The similarity between the conformation of the penicillin molecule with dipeptide D-alanyl-D-alanine is responsible for such reaction.

Transpeptidse first reacts with the substrate to form the acyl enzyme intermediate with the elimination of D-alanine and that this active intermediate then reacts with another strand to form the crosslink and regenerate the enzyme. Because Penicillin is an alanogue of alanyl alanine; it should fit the substrate binding site, with the highly reactive CO-N-Bond in the β-lactum ring in the same position as the bond involved in the transpeptidation. It thus has the potential to acylate the enzyme forming penicilloyl enzyme and thereby inactivate it. In support of this view is the fact that penicilloyl is the piece of the antibiotic found in inhibited enzymes.

Further Reading:-

1. Pharmatological Basis of Therapentics by Godman and Gillman.
2. Biochemistry by Zube.

Antibiotics: These are chemical substances synthesized by micro-organisms and are effective against other micro-organanisms than the producing one when it used in very low concentration.

Unit of Penicillin:

One unit of penicillin is that amount of penicillin which when added to 50 ml of nutrient broth will not allow the growth of *staphylococcus aurens* for 24 hrs. only.

* The weight of this amount is varied on the extent of purification. So weight is not used.

* Penicillin breaks up after prolong storage.

Highly purified 1 mg. Wt. Of benzyl pencillin=1667 units.

International unit of Penicillin is the amount of activity produced under defined conditions 0.5988 mg of the international standard.

Cephalosporins:

Cephalosporins are a group of antibiotics produced by a species of marine fungus, *Cephqlosporium acremonium*, which bears considerable resemblance to *penicillium spp.* They are effective against Gram-positive and Gram-negative bacteria. The cephalosporins have antibacterialproperties similar to those of the semisynthetic penicillins. They are effective therapeutically and have a low toxicity. The nucleus of the cephalosporins (Fig. 3) resembles that of penicillin. As with penicillin, several semisynthetic cephalosporins have been manufactured commercially for therapeutic use.

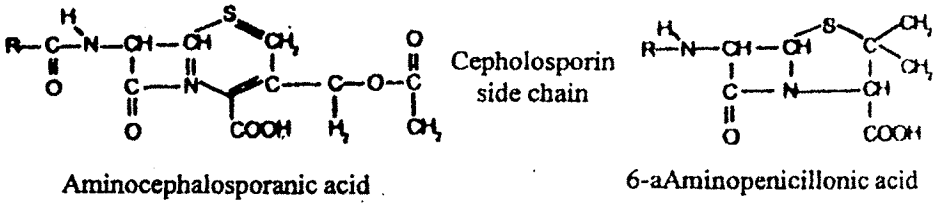


Figure 3 *A comparison of the nucleus of cephalosporin (aminocephalosporanic acid) with the nucleus of penicillin (6-aminopenicillanic acid).*

As would be anticipated from the similarity in chemical structure of penicillin and cephalosporin, the mode of action of the cephalosporins is that of inhibitiion of the cross- linking transpeptidase. They are bactericidal to growing cells.

Cycloserine:

Cycloserine, a relative simple compound, is related in structure to almine (see Fig. 4). It was originally discovered as an antibiotic produced by streptomyces and is now manufactured through chemical synthesis. The main use of this antibiotic is in tuberculosis therapy. However, because of potential undesirable side effects, its utilization is limited.

Cycloserine manifests its inhibitory effect on peptidoglycan synthesis by interference with synthesis of the peptide moiety of the peptidoglycan. Specifically, it inhibits both alanine racemase and D-alany-D-alanine synthetase, this enzymes involved in the synthesis of the pentapaptide side chains.

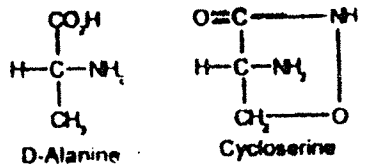


Figure 4. *The chemical structure of D-alanine and the antibiotic cycloserine.*

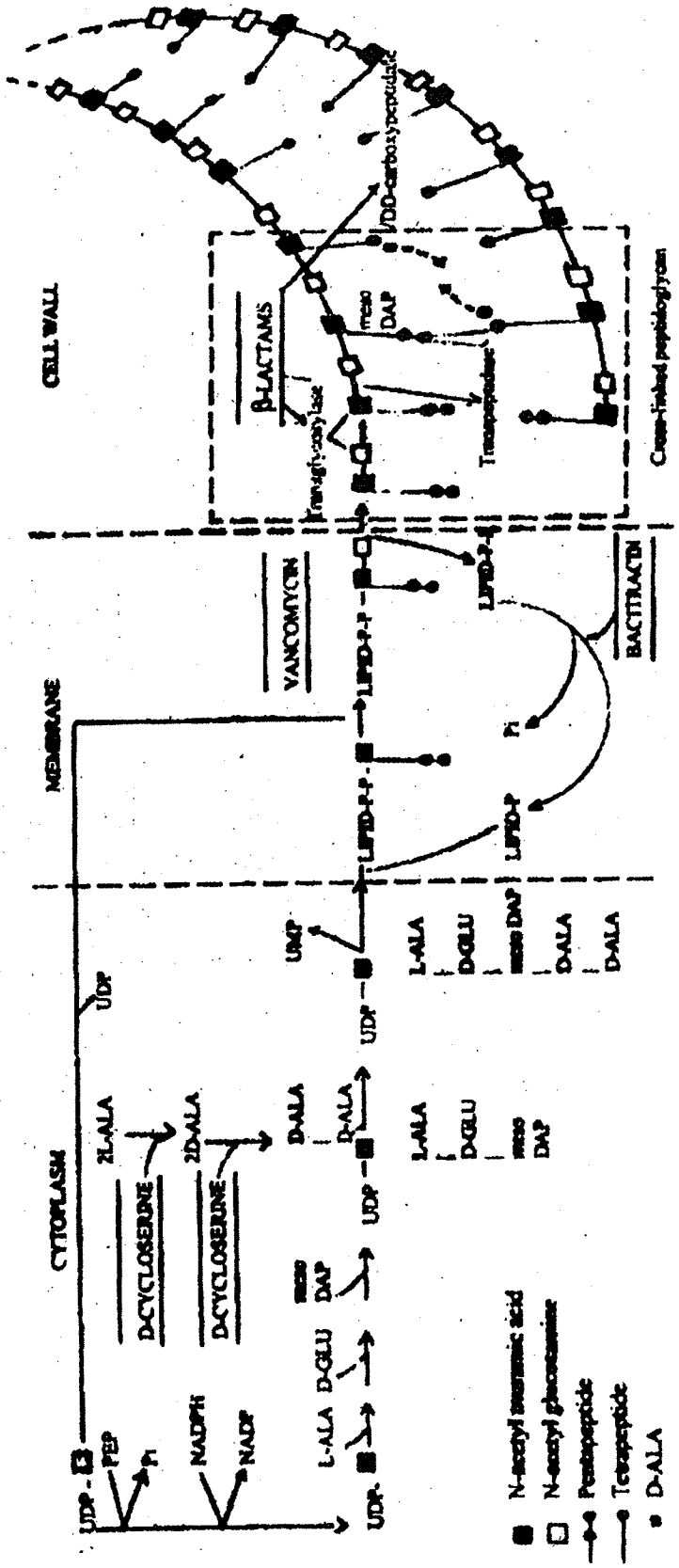


Figure 5: *Schematic illustration of sites of attack of antibiotics on cell-wall synthesis (formation). (Erwin F.Lessel, illustrator).*

Bacitracin:

Bacitracin is a product of *Bacillus subtilis* and chemically is a polypeptide. Because of its toxicity to animal and human cells it cannot be used for systemic chemotherapy. It does have application for topical treatment of infection's caused by Gram-positive bacteria.

Bacitracin interferes with regeneration of the monophosphate form of bacto-prenol from the pyrophosphate form (lipid-P-P in Fig.-5).

Vancomycin :

Vancomycin is an antibiotic produced by *Streptomyces orientalis.* It is a complex chemical entity consisting of amino acids and sugars.

Vancomycin inhibits peptidoglycan synthesis by binding the D-alanyl-Dalanine group on the peptide side chain of one of the membrane-bound intermediates.

A schematic summary of the modes of action of some antibiotics that exert their antibacterial effect through interference with cell-wall synthesis is shown in Fig. 5.

Damage to Cytoplasmic Membrane :

Several polypeptide antibiotics produced by *Bacillus spp.* have the ability to damage cell-membrane structure. They adversely affect the normal permeability characteristics of the cell membrane. Included in this category are the polymysins, gramicidins and tyrocidines (see Fig-6).

The polymyxins are particularly effective against Gram-negative organisms, while the tyrocidines and gramicidins are more effective against Gram-positive organisms.

Mode of Action:

These agents are bactericidal; they cause a leakage from the cynplasmic content of the cell. Because of their toxicity to tissue they have limited application in chemotherapy.

The positively change peptide ring is thought to bind electro statically to the anionic phosphate head groups of the *membrane phospholipid,* displacing magnesium ions which normally contribute to membrane stability. At the same time the fatty acid side chain is inserted into the hydrophobic inner region of the membrane. The effect is disturb the normal organization of the membrane and to alter its permeability characteristics.

In Gram-bacteria there is a further binding to the outer membrane affecting mainly the lipopolysaccharide but with similar disorganizing effects.

The tyrocidins are also bactericidal & promote leakage of Cytoplamic solutes. Their action on the bacterial membrane permits passage it into the cell of ions that are normally excluded and under same

conditions this causes uncoupling of oxidative phosphoylation as a secondary effect. Gramicidins a closely related compound, acts similarly.

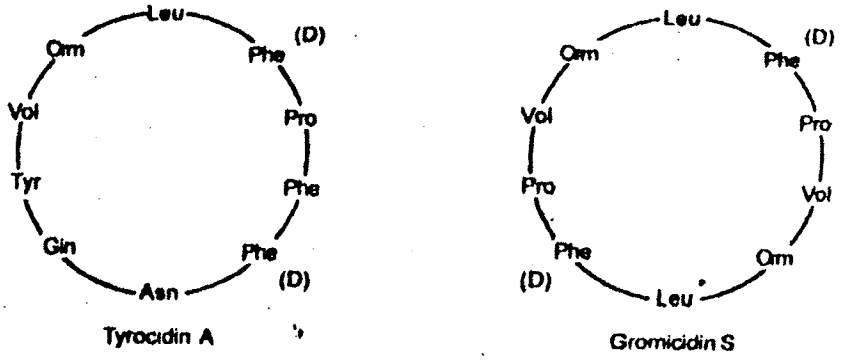


Figure 6: *The structural formulas of tyrocidine A and gramicidin S. polypeptide antibiotics which exert their antibacterial action through binding with the cytoplasmic membrane. (Amino acid configuration is L except for those marked D.)*

Another category referred to as polyene antibiotics are large ring structures with many double bonds. Examples are *nystatin,* produced by *Streptomyces nodosus.* Polyne antibiotics act upon cells which have sterols in their cytoplasmic membrane. They act upon fungi (including yeasts) and animal cells but do not affect bacteria. Their antimicrobial action is attributed to their ability to increase cell permeability.

AMPHOTERICIN-B

Mode of Action : [Polyene antifungal antibiotics]

The antifungal activity of it is partly dependent on its binding to a sterol moiety. Primarily ergosterol present in the membrane of sensitive fungi. By virtue of their interaction with the sterols of cell membranes, polyenes appear to form pores or channels. The result is an increase in the permeability of the membrane allowing leakage of a variety of small molecules. Additional damage to fungal cells at least in vitro (Sokol – Anderson et al 1986) and some capability to enhance cell mediated immunity in the host (Medoff et. Al 1983).

Used in the treatment of *Mucormycosis in vasive aspergoillosis and cryptococcosis.*

Nystatin:

1. Produced by *streptomyus noursei.*
2. Str. Similar to Amphotericin-B.
3. Same mode of Action same as amphotericin – B.
4. More toxic – not used systemically.

Tropical Antifungal Agent.

IMIDAZOLE:

Antifungal compounds (Ketoconazole Miconazole)

1. Active against broad spectrum fungi.

2. Mode of Action:

At concentrations achieved during systemic use, the major effect of imidazoles on fungi is inhibition of sterol 14-a-demethylase, a microsomal cytochrome P-450 dependent enzyme system. Imidazoles thus impair the biosynthesis of ergosterol for the cytoplasmic membrane and lead to the accumulation of 14-a-methyl sterols (Vanden Bossche et. Al 1986). These methyl sterols may disrupt the close packing of acyl chains of phospholipids, impairing the functions of certain membrane bound enzyme systems and inhibiting growth.

Streptomycin:

Streptomycin is produced by *Streptomyces griseus,* a soil organism isolated by Shatz, Bugle, and Waksman, who reported on its antibiotic activities in 1944. It is particularly important because it inhibits many organisms resistant to sulfonamides and penicillin. Its antibacterial spectrum includes many Gram-negative bacteria, including *Francisella tularensis* and some organisms in the salmonella group. It is inhibitory for several species of *Mycobacteium tuberculosis.* Highly purified streptomycin is untoxic to humans and other animals when given in small doses, but it appears to have a cumulative detrimental effect on a specific region of the nervous system when given as a medication over long periods of time.

Streptomycin is characterized chemically as an aminoglycoside antibiotic; its structure is shown in Fig. 7. Other aminoglycoside antibiotics are kanamycin, produced by *Streptomyces kanamyceticus and neomycin* produced by *Streptomyces fradiae* and other species of streptomyces.

Mode of Action

Streptomycin and other aminoglycoside antibiotics inhibit protein synthesis by combining irreversibly with the 30S submit mRNA. Thus the normal synthetic sequence is disrupted.

The mechanism of bacterial killing by Streptomycin and other aminoglycosides has reexamined by Davis (1980). His hypothesis is that misread proteins can became incorporated into the bacterial membrane making it leaky to small molecules including the antibiotic itself. The sequence of events is as follows.

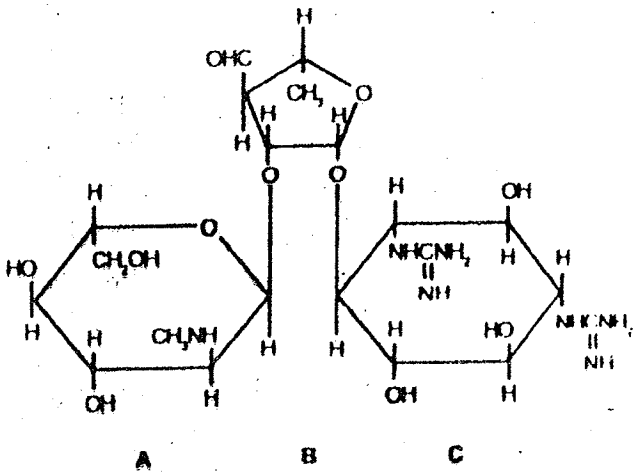


Figure 7: *Streptomycin. This antibiotic consists of three components, linked glycosidically : (A) N-methyl-L-glycosamine, (B) streptose and (C) streptidine.*

1. A *small amount of antibiotic enters the cell and causes ribosome misreading.* (mechanism unclear)
2. Misread protein is incorporated in the Cytoplasmic membrane, creating channels which allow more antibiotic into enhance the effect.
3. The antibiotic concentration reaches a level which blocks further protein synthesis.
4. The irreversibility of the ribosome blockage results in cell death.

Tetracyclines:

Chlortetracycline, oxytetracycline, tetracyclie, doxycycline and minocycline are generic names for five antibiotics having similar biological and chemical properties. As a group they are commonly called tetracyclines. Their structural formulas are shown in Fig. 8. Note that the antibiotic produced by *Streptomyces auteofaciens* is chlortetracycline, while *Streptmyces rimosus* produces oxytetracycline. They are broad-spectrum antibiotics with similar antimicrobial spectra and cross resistance of bacteria to them is common.

Tetracycline, oxytetracycline, chlortetracycline, minocycline and dioxycycline are chemically very similar. It is not surprising, therefore, that there are no great differences in their activity. The antimicrobial spectra are similar and all are bacteriostatic in their action.

Mode of Action:

The tetracyclines inhibit protein synthesis through interference with the binding of aminoacyl-tRNA to the 30S subunit ribosome. Tetracyclines inhibit bacterial protein synthesis. Their site of action is the bac. ribosme, but at least two processes appear to be required for these antibiotics to gain access to the ribosome of gram-negative bacteria (Chopra & Howe 1978). The first step is passive diffusion through the hydrophilic channels formed by the porin proteins in the outer cell membrane. Minocycline & Deoxycycline are more lipophilic than the other congeners and pass directly through the lipid bilayer. The second process

involves an energy-dependent active transport system that pumps all tetracyclines through the inner cytoplasmic membrane. Such transport may require a periplasmic protein carrier. Although permeation of these drugs into gram-positive bacteria is less well understood, it too requires an energy dependent system. Once the tetracyclines gain access to the bacteria cell, they bind principally to the 30S submits of bacterial ribosomes. They appear to prevent access of amino acyl tRNA to the aceptor site on the mRNA-robosome complex these prevents the amino acids to the growing peptide chain. Only a small portion of the drug is irreversibly bound, and the inhibition affects of the tetracyclines are reversible (when the drug is removed).

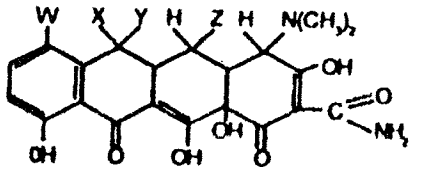


Figure 8 *Tetracyclines, broad-spectrum antibiotics produced from Streptomyces, differ slightly in chemical structure as shown in the positions labeled "W", "X", "Y" and "Z" in the above molecule.*

Antibiotic	Position on molecule			
	W	X	Y	Z
Tetracycline	-H	$-CH_3$	-OH	-OH
Oxytetracycline	-H	$-CH_3$	-OH	-OH
Chlorotetracycline	-Cl	$-CH_3$	-OH	-H
Minocycline	$-N(CH_3)_2$	-H	-H	-H
Deoxycycline	-H	$-CH_3$	-H	-OH

Chloramphenicol:

Chloramphenicol is a broad-spectrum antiobotic active against many Gram-positive and Gram-negative bacteria. Its antimicrobial spectrum is similar to that of tetracycline. It is also bacteriostatic. Chemically, it is a nitrobenzene ring with nonionic chlorine (Fig. 9). The possibility of serious side affects such as blood dyscrasias have limited the used of this antibiotic as a general antibacterial agent.

Mode of Action:

Chloramphenicol inhibits protein synthesis by combining with the 50S sub-unit ribosome. The transpeptidation and translocation functions associated with this site are blocked.

It inhibits protein synthesis in bacteria and to a lsser extent, in eukaryotic cell. The drug readily penetrates into bacterial cells, probably y a process of facilitated diffusion. It acts primarily by binding reversibly to the 50S ribosomal sub-unit. Although binding of tRNA at the codon recognization site on the 30S ribosomal sub-units is thus undisturbed, the drug appear to prevent the binding of the amino acid containing end of aminoacyl tRNA to the acceptor site on the 50's ribosomal sub-unit. The interaction between peptidyl transferase and amino acid substrate can not occur, and peptide bond formation is inhibited. (Pratt & Fekety, 1986).

Chloramphenicol can also inhibit mitochondrial protein synthesis in mamalian cells perhaps mitochondrial ribosomes resemble ribsome (both are 70S).

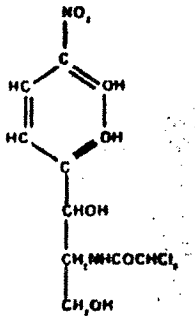


Figure 9 *Structure of Chloramphenicol, a broad-spectrum antibiotic from Streptomyces venexuelae.*

Erythromcycin:

Erythromycin is produced by a strain of Streptomyces erythraeus isolated from soil collected in the Philippines. Erythromycin is active against the Gram-positive bacteria, some Gram-negative bacteria and pthogenic spirochetes. With regard to antimicrobial spectrum and clinical usefulness, it resembles penicillin, but it is also active against organisms that become resistant to penicillin and streptomycin. It is therefore, often prescribed to those patients with cillin and streptomycin. It is, therefore, often prescribed to those patients with allergies when penicillin is indicated.

Erythromycin belongs to the chemical class of antibiotics known as macrolides. Structurally it contains a large lactone ring linked with amino sugars through glycosidic bonds. Erythromycin inhibits protein synthesis as a result of binding on the 50S subunit ribosome; the steps of transpeptidation and translocation in protein synthesis are blocked.

Mode of Action

Erythromycin can interfere with the binding of Chloramphenicol, which also acts at this site. Erythromycin does not inhibit peptide bond formation directly but rather inhibits the transloction step wherein a newly synthesized peptidyl tRNA molecule moves from the acceptor site on the ribosome to the peptidyl (or donor) site.

RIFAMPIN [after Nucleic Acid Metabolism]

1. PRODUCED BY *streptomyces mediterranei.*
2. Semisynthetic derivative of rifamycin B.
1. Inhibit the growth of Gram +ve bacteria as well as Gram –ve bacteria.

Mode of Action:

In inhibits DNA dependent RNA polymerase of mycobacteria and other micro organism leading to supression of intiation of chain formation (but not chain elongation) in RNA synthesis. More specificatty

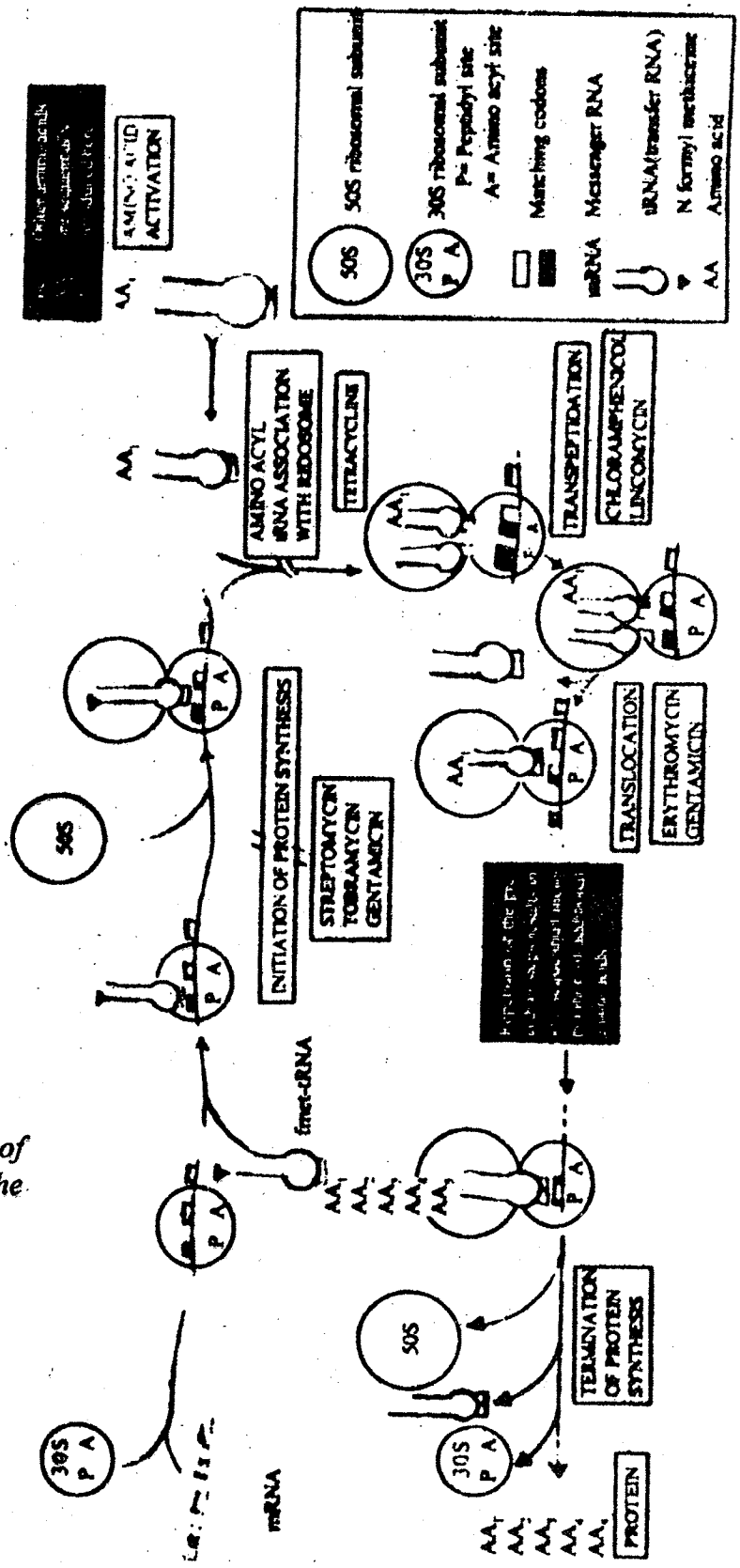


Figure 10. *Schematic illustration of sites of action of antibiotics on the sequence of protein synthesis.*

the B subunit of this complex enzyme is the site of action of the drug, although rifampin binds only to the holoenzyme. Nuclear RNA polymerase from a variety of eukaryotic does not bind rifampin, and RNA synthesis is correspondingly unaffected : while rifampin can inhibit RNA synthesis in mamalian mitochondira, considerably higher concentration of the drug are required them for the inhibition of the bacterial enzyme. Rifampin is bactericidal for both extracellular & intracellular micro organism.

The specific steps in protein synthesis which are interrupted by various antibiotics are summarized in Fig. 10.

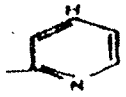
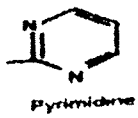
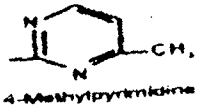
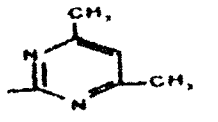
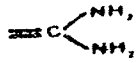
Inhibition of Specific Enzyme Systems:

The sulfonamides, represent a category of compounds whose antibacterial attack is directed toward a specific essential enzyme. There are numerous sulfonamides as shown in Table-1. All of them have the same basic structure. This structure is related to the compound p-aminobenzoic acid (PABA) as a precursor

Table-1. Some examples of Sulfonamides.

The basic structure for the sulfonamides is 

which is a p-aminobenzenesulfonamide. The sulfonamides differ primarily by virtue of the different substituents in the R, position, as indicated.

Name*	R'
Sulfanilamide	
Sulfapyridine (N'-2-Thirzolysulfanilamide)	
Sulfadiazine (N' –2- Sulfanilamidopyrimidine)	
Sulfamerazine [N'- (4-methyl-2-pyrimidyl) – sulfanilamide]	
Sulfamethazine {n' – (4,6-Dimethyl-2-pyrimidyl) – sulfanilamide]	
Sulfaguanidine (N'-Guanylsulfanilamide)	

*The common name is followed by the systematic name.

to their synthesis of the essential coenxyme tetrahydrofolic acid (THFA). PABA is a structural part of the THFA acid molecule. The selective action of sulfonamides is explained by the fact that the PABA molecule and a sulfonamide molecule are so very similar that the sulfonamide may enter the reaction in place of the PABA and block the synthesis of an essential cellular constituent, which in this case is THFA, as shown in Fig. 11. The cellular functions of the THFA co-enzyme include amino acid synthesis, thymidine synthesis, etc. Lack of this coenzyme will quite obviously disrupt normal cellular activity. Sulfonamides will inhibit growth of those cells which synthesize their THFA from PABA and will not interfere with the growth of those cells (including mammalian host cells) which require the vitamin folic acid and reduce it directly to THFA. This accounts for the selective antibacterial action of sulfonamides and makes them useful in the treatment of many infectious diseases.

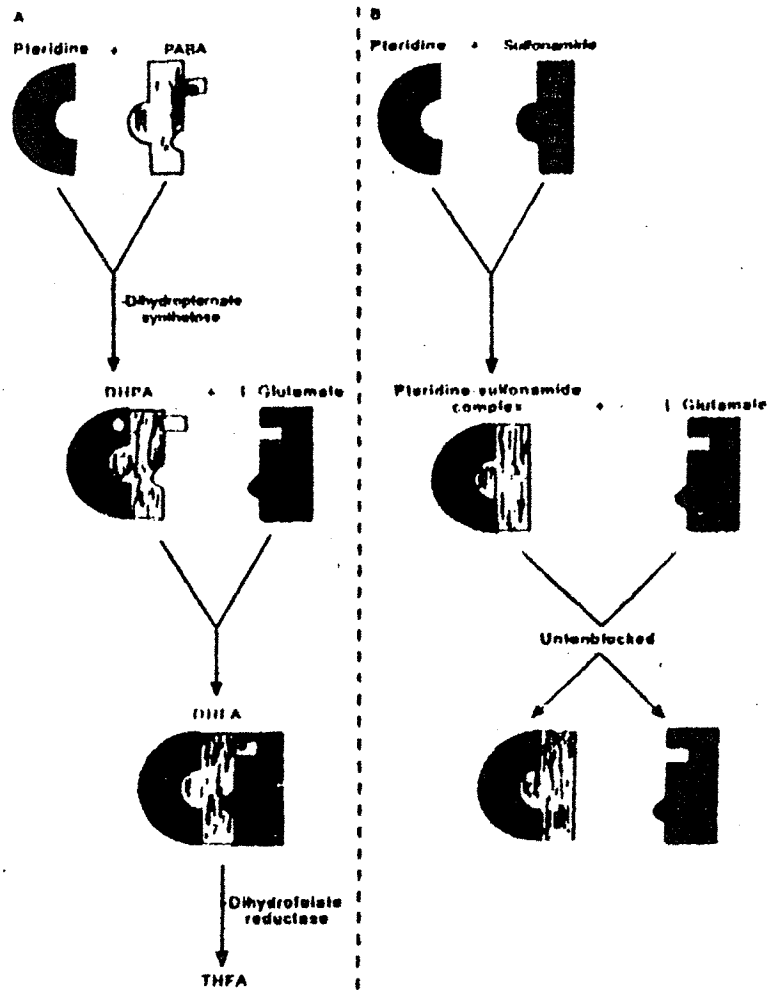


Figure 11. *The mode of action of sulfonamides in inhibition of tetrahydrofolic acid synthesis (Erwin F. Lessel, illustrator).*

This mode of action is an example of competitive inhibition between an essential metabolite (PABA) and a metabolic analog (a sulfonamide).

ANTIFUNGAL ANTIBIOTICS:

Nystatin is an antifungal agent useful in the therapy of nonsystemic fungal infections.

It is produced during fermentation by a strain of Streptomyces maursei. This antibiotic was discovered in 1950 by Elizabeth Hazen and Rachel Brown.

Mod of Action : [Discussed earlier.]

Griseofulvin:

Griseofulvin is obtained from *Penicillium griseogulvin*. It is used in the treatment of many superficial fungus infections of the skin and body surfaces and is also effective in the treatment of some systemic (deep-seated) mycoes. The drug is administered orally.

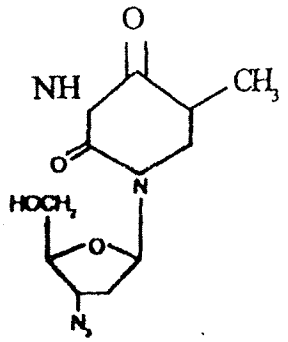
Mode of Action:

A prominent morphological manifestation of the action of griseofulvin is the production of multinucleate cells as the drug inhibits its fungal mitosis. It causes disruption of the mitotic spindle by interacting with polymerised microtubules (Similar to Colchicine).

Zidovudine :

It is a thymidine analog in which 3 hydroxyl of the deoxyribose moiety has been replaced by azodo group.

Its structure :



Active against human imunodeficiency virus (HIV-1) the cansative agents of AIDS and other mammalias netroviruses.

Mode of Action:

Zidovudine is phosphorylated in vivo by cellular enzymes to the corresponding deoxynucleoside diphosphate derivative. In this form the drug inhibits viral RNA dependent DNA polymerase (reverse tranecriptase). Its antiviral selectivity is due to its great in affinity for reverse transcriptase than for human DNA polymerase.

Antiviral Chemotherapeutic Agents

Viruses are intracellular, and hence the chemotherapeutic agent, in order to attack the virus, must

enter the host cells. aLso the agent must not be toxic to the host cell while exerting an inhibiting action on the virus. This demands a high level of selective toxicity. In cases of infection by acteria, fungi, or protozoa the infectious agent is acted upon outside the host cells. Additionally, there are many metabolic processes that can be interrupted with these microorganisms.

Among the more promising of the chemotherapeutic agents for treating viral diseases is interferon. Interferons are small glycoprotein substances of which two types are leukocytic interferon and fibroblast interferon. Cells exposed to interferon develop antiviral properties. The antiviral actiion of interferon is attributed to interference of protein synthesis.

Natural interferons are in very short supply and are expensive. Recent advances in recombinant DNA techniques (genetically engineered bacteria like *Esherichia coli* to produce interferon on a large scale commercially) have increased the availability of interferon for both chemotherapeutic and experimental use.

Acycloguanosine is a nucleoside analog which is active against the herpes virus in animals. Its mode of action appears to be that of inhibition of nucleotide utilization. A synthetic nucleotide analog, 5'-iododeoxyuridine (see Fig. 12) has been shown to have antiviral activity and promise as an antiviral chemotherapeutic agent. Its made of action is most likely that of inhibition of nucleic acid synthesis – preventing the incorporation of thymidine into DNA.

Amantadine is a low – molecular – weight compound which is very effective against influenza A virus. Infections is greatly reduced by used of this drug. The mode action of amantadine is that of interfering with the uncoating of virus particles and the subsequent release of their nucleic acids.

It also causes chain termination during DNA synthesis. Thus if azidothymidine tri phosphate is incorporated into a growing stand of DNA, additional nucleotides can not be added because of the modification in the 3-position of the drug.

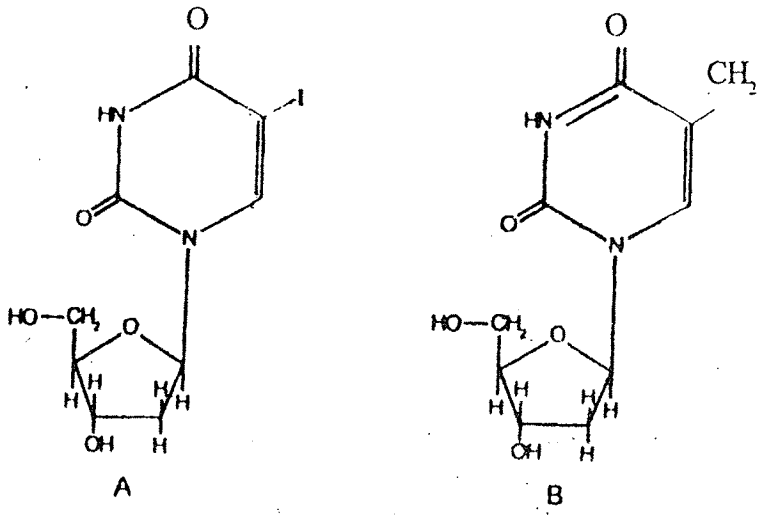


Figure 12 (A) *The pyrimidine analogue 5'-iodideoxyuridine. (B) Thymidine, the pyrimidine which the analog resembles. This pyrimidine analog exhibits antiviral activity.*

ACYCLOVIR

Acyclovir is a synthetic purine nucleoside analog in which a linear side chain [9-2-hydroxy ethoxy) methyl] guanine, has been substituted for the cyclic sugar of the naturally occuring *guanosine* molecule.

Activity confined to *herpes viruses* particularly active against herpes simplex-I.

Mode of action

It inhibits viral replication by inhibiting DNA synthesis. Selectivity in this action comes from two distinct interactionn of the drug with viral proteins. In order to inhibit DNA synthesis, acyclovir must be phosphorylated, first by viral thymidine kinase. After synthesis of acyclovir monophosphate in virally infected cells normal cellular enzyme catalyze the sequential synthesis of acyclovir GDP & acyclo GTP. Acyclo GTP then selectively inhibits the viral DNA polymerase by competing with *deoxyguanosine triphosphate* and, to a much lesser extent the cellular polymerases. In addition *acycle-GTP* is incorporated into the elongating viral DNA, where it causes termination of bio synthesis of the viral DNA strand (Elion 1986).

ANTITUMOR ANTIBIOTICS

Some antibiotics have been found to posses antthramycin group (anthramycin, tomaymycin and neothramycin) us an example of potent antitumor agents. One of the complicating factors associated with the potential use of these anticancer agents is that they are also cardiotoxic, a fact that illustrates the need for a high level of specificity in a chemotherapeutic agent. The antitumor action of these antibiotics is directed toward DNA structure and function. One of the problems is that of determining whether, through the manipulation of the structure of an antibiotic, e.g. anthromycin, one can cut out the cardiotoxic property without destrowing the antitumor property.

SYNTHETIC CHEMOTHERAPEUTIC AGENTS

Nitrofurans

The nitrofurans are antimicrobial drugs which differ from the antibiotics in that they do not occur naturally. The prototype of the nitrofuran derivatives is furfural, which can be prepared from corncobs and constalks, oat tulla, beet pulp, peanut hulls, and other vegetable by-products.

The chemical structures of some chemotherapeutic nitrofurans are shown in Fig. 13. As a class, the nitrofurans generally are effective against a broad spectrum of both Gram-positive bacteria, several pathogenic protozoa, and some fungi which cause superficial infections in both humans and other animals.

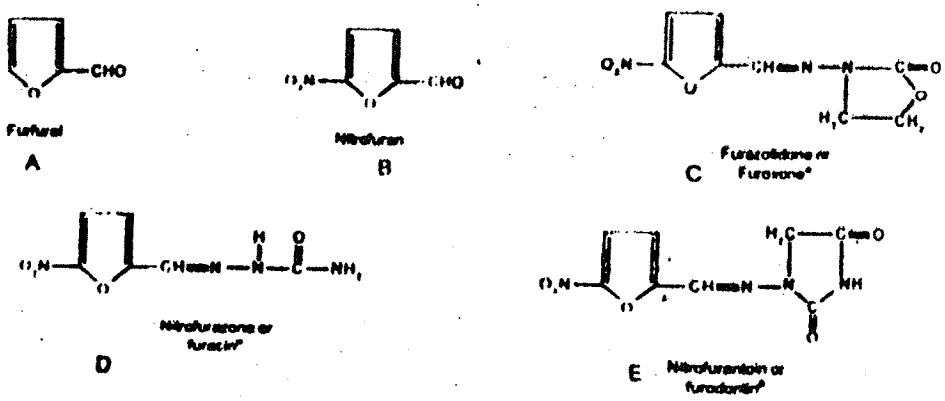


Figure 13 *Furfural (A) is the prototype of nitroguran compounds, and (B), (C), (D) and (E) are chemotherapeutic derivatives of furfural.*

Isonicotinic Acid Hydrazide (Isoniazid)

Isoniazid has an important, though restricted, application in the therapy of disease. It is an example of competitive inhibition affecting a restricted group of microorganisms, the mycobacteria. It has proved to be very useful in the control of tuberculosis in humans and is more effective when given alternately with streptomycin. Because it is a structural analog of pyridoxine, or vitamin B_6 (see Fig. 14) and nicotinamide, isoniazid can block pyridoxine and nicotinamide-catalyzed reactions. This may account for its antimicrobial activity.

Nalidixic Acid

Nalidixic acid is a synthetic chemical with a structural formula as shown in Fig. 15. It is a useful chemotherapeutic agent for urinary-tract infections caused by Gram-negative. Its antimicrobial activity is attributed, at least in part, to inhibition on DNA synthesis.

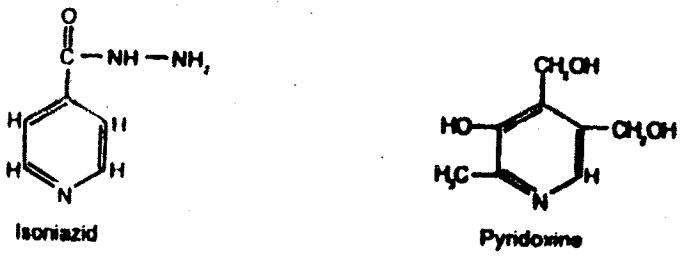


Figure 14 *Isoniazid, a structural along of pyridoxine (vitamin B_6), may prevent the growth of microorganisms by blocking pyridoxine-catalyzed reactions in the microbial cell.*

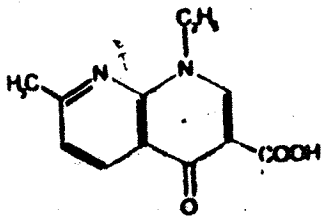


Figure 15 *Nalidixic acid is a synthetic antibacterial drug with a selective action against bacterial DNA synthesis.*

MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency of antibiotic content in samples can be determined by chemical, physical and biological means. An assay is made to determine the ability of an antibiotic to kill or inhibit the growth of living micro-organisms. Biological tests offer the most convenient means of making an assay.

Chemical Assay

Where antibiotics exist in pure chemical form, their concentration can be expressed in micrograms of the pure chemical per miligram of the specimen. To be of value, such tests must give results that correlate well with those obtained in biological assays. Chemical-assay methods are generally more accurate and require less time than biological methods, but they are less sensitive, and caution must be used lest biologically inactive degradation products give misleading results.

Biological Assay

Biological potency is expressed in terms of either micrograms or units determined by comparing the amount of killing or bacteriostasis of a test organism caused by the substance under test with that caused by a standard preparation under rigidly controlled conditions (see Fig.16)

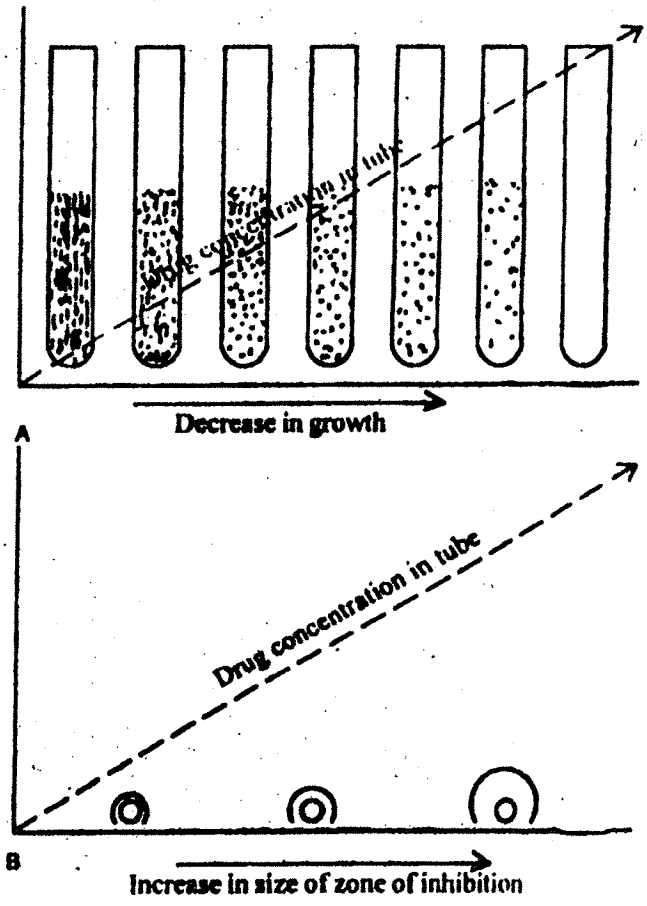


Figure 16 *Microbiological assay of antibiotics and some other chemotherapeutic agents is accomplished by either the tube-dilution or the cylinder-plate method (a variation of the paper-disk-plate technique). (A) In the tube-dilution technique, the inhibition of growth (decrease in turbidity) produced by the unknown sample is compared with that produced by the known or standard sample. The amount of antibiotic present in the unknown sample can then be calculated. (B) The cylinder plate technique follows much the same procedure for determining antibiotic potency, except that inhibition of growth is measured in terms of the size of the zones of inhibition.*

Microbial susceptibiltiy to Chemotherapeutic agents

Species and strains of species of microorganisms have varying degrees of susceptibility to different antibiotics. Furthermore, the susceptibility of an organism to a given antibiotic may change, especially during treatment. It is therefore important for the clinician to know the identity of the microbe and the specific antibiotic which may be expected to give the most satisfactory results in treatment. For this information the microbiologist will be called upon to make an accurate microbiological diagonosis and to determine the susceptibility of the organism to various antibiotics. From time to time during the course of therapy the microbiologist may be required to make estimates of any change in the susceptibility of the pathogen to the drug and possibly even to assay the antibiotic concentration in the body fluids.

Tube-Dilution Technique

The susceptibility of a microorganism to antibiotics and other chemotherpeutic agents can be determined by either the tube-dilution technique, one can determine the smallest amount of chemotherapeutic agent required to inhibit the growth of the organism in vitro (see Fig. 17). This amount is referred to as the MIC (minimal inhibitory concentration).

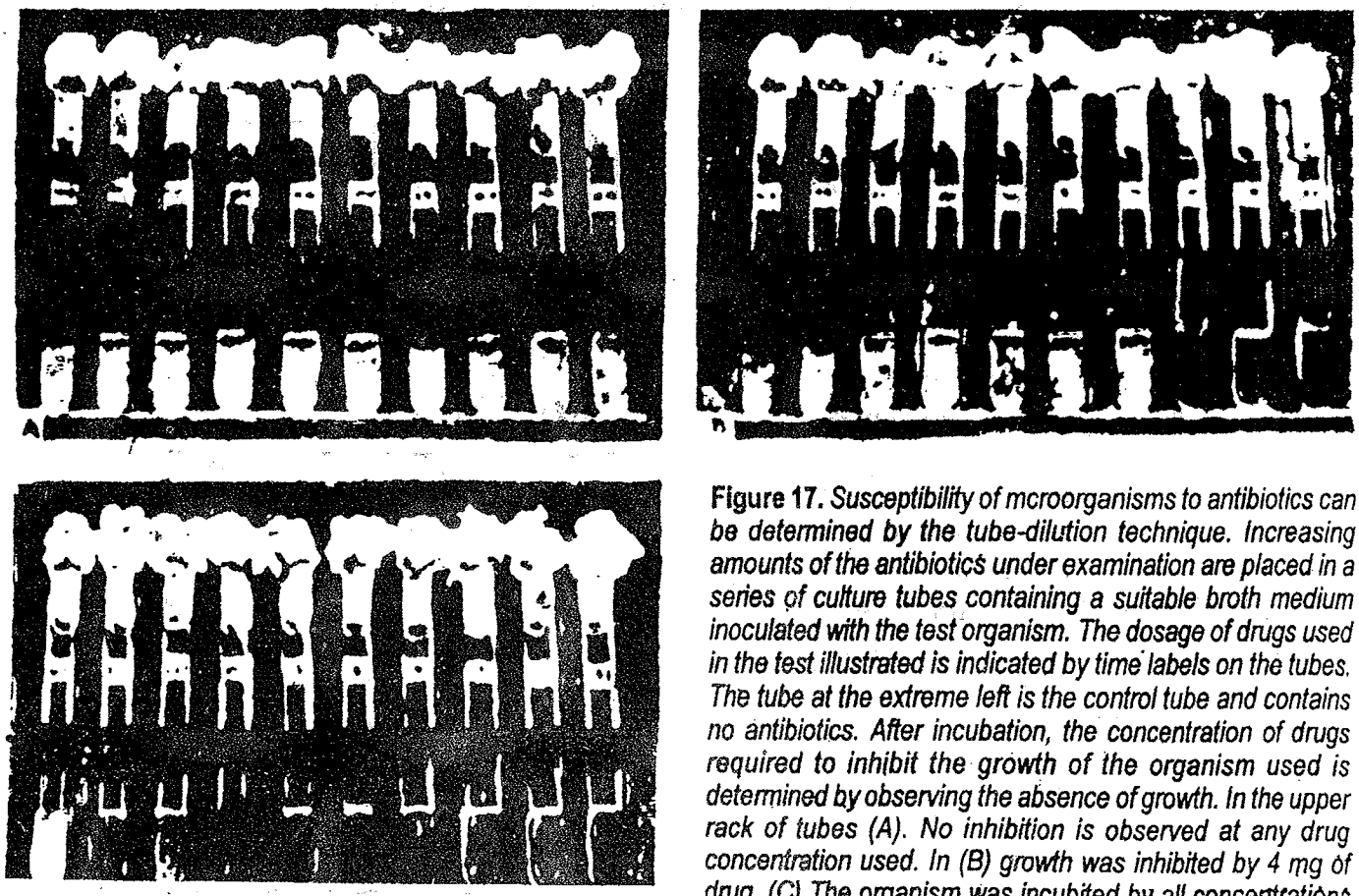


Figure 17. *Susceptibility of mcroorganisms to antibiotics can be determined by the tube-dilution technique. Increasing amounts of the antibiotics under examination are placed in a series of culture tubes containing a suitable broth medium inoculated with the test organism. The dosage of drugs used in the test illustrated is indicated by time labels on the tubes. The tube at the extreme left is the control tube and contains no antibiotics. After incubation, the concentration of drugs required to inhibit the growth of the organism used is determined by observing the absence of growth. In the upper rack of tubes (A). No inhibition is observed at any drug concentration used. In (B) growth was inhibited by 4 mg of drug. (C) The organism was incubited by all concentrations of the drug. (Courtesy of Abdon Laboratories).*

Disk-Plate Technique

The paper-disk-plate method is the most commonly used technique for determining susceptibility of microorganisms to chemotherapeutic agents. Small paper disks impregnated with known amounts of chemotherapeutic agents are placed upon the surface of an anoculated plate. After incubation, the plates are observed for any zones of inhibition surrounding the disks (see Fig. 18). A zone of inhibition (a clear area) around the disk indicates that the organism was inhibited by the drug, which diffused into the agar from the disk.

The single-disk method for susceptibility testing currently recommended by the FDA is a slight modification of the procedure developed by Bauer, Kirby, Sherris and Truck in 1966. This is a highly standaridized technique; the amount of antimicrobial agent contained in the disk is specified as well as the test medium, size of the inoculum, conditions of incubation, and other details, when the susceptibility test is performed in conformity with the FDA procedure, one can correlate the sizes of the zones of inhibition with the MIC of the drug for the microorganism in question; it is possible to determine whether the microorganism is resistant or susceptible to the antimicrobial agent. The relationship of MIC's to zone-of-inhibition diameters for the antibiotic cephalothin against several bacteria is shown in Fig. 19.

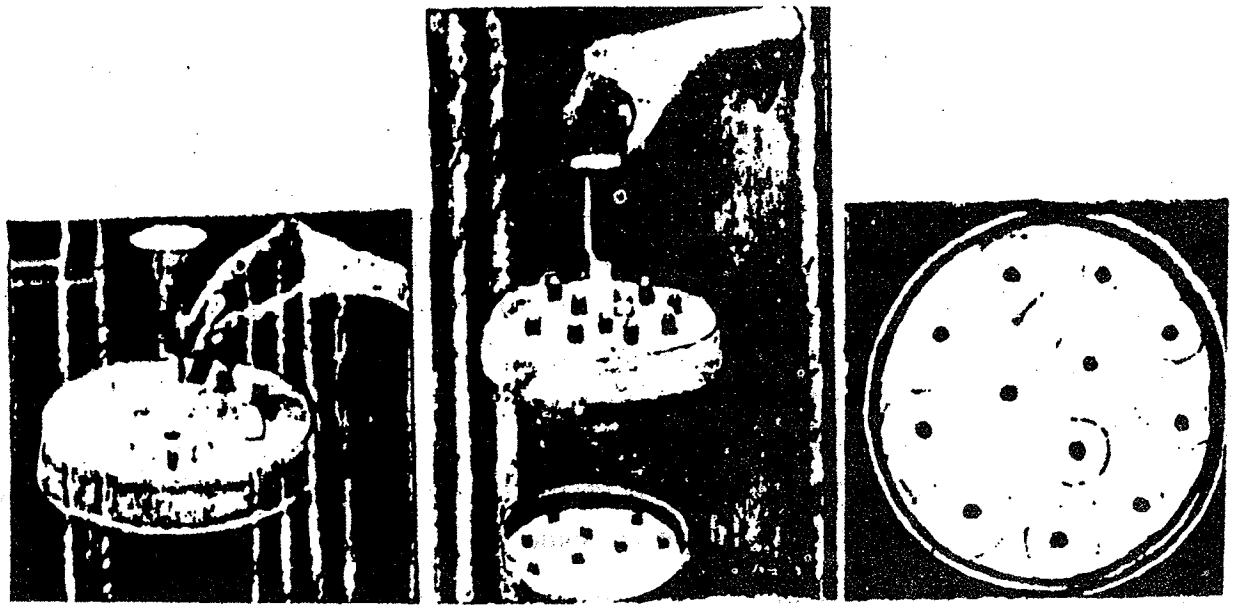


Figure 18. *The paper-disk-plate method for determining the susceptibility of microorganisms to antibiotics. (A) Automatic dispenser of paper disks impregnated with antibiotics. (B) Disks positioned on inoculated Petri dish before incubation. (C) Zones of inhibition develop after incubation around each disk that contains an antibiotic that inhibits growth of microorganisms. (Courtesy of Becton-Dickinson, BBL Microbiology Systems).*

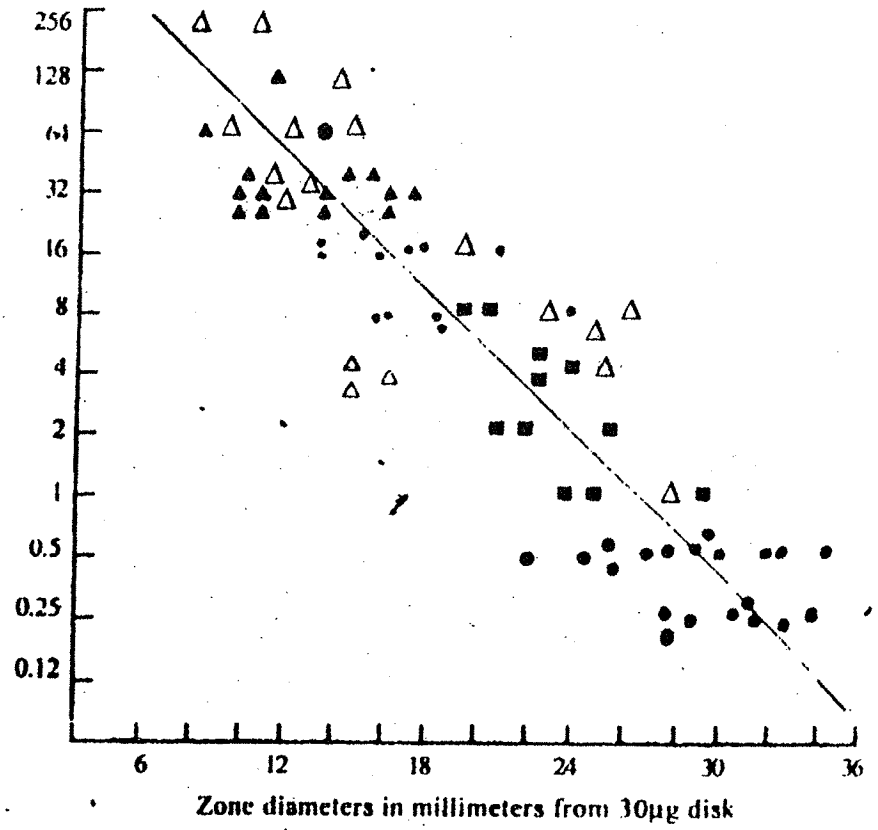


Figure 19. *The relationship between the dilution and diffusion methods of testing the ability of an antibiotic to inhibit bacterial growth is demonstrated here for cephalothin. The size of the inhibition zone produced by an antibiotic disk goes up as the MIC goes down. All test conditions must be held constant. ▲ Enterococci, • Staphylococcus aureus, • Escherichia coli, Δ Enterobacter-Klebsiellsa, ■ Haemophilus (Courtesy of K.J. Ryan. F.D. Schoenknecht and W.M. M. Kirby, Hosp Pract, p 99, 1970).*

NON MEDICAL USED OF ANTIBIOTICS

Antibiotics are used as growth stimulants in poultry and livestock feeds. After the discovery that many domestic food-producing animals require Vitamin B_{12} for optimum growth when fed a diet consisting of plant protein, it developed that by adding wastes from fermentation by-products to feeds, growth was stimulated more than could be accounted for B_{12} alone. Even when adequate amounts of B_{12} were present in the diet, more rapid growth of young animals was noted when they were fed mash from the antibiotic fermenters. Use of pure antibiotics has given similar results. Commercially, the addition of auromycin, terramycin, or penicillin to swine or poultry feeds at the rate of 5 to 20 g per ton of feed increases the rate of growth of young animals by at least 11 percent and sometimes by as much as 50 percent.

The stimulating effect of antibiotics on growth of domestic animals may be explained in several ways :

1. The antibiotics may destroy bacteria and other intestinal parasites that cause subclinical disease and retard growth and development. For example, it has been suggested that pigs respond dramatically to the addition of tetracycline to their diet because the antibiotic inhibits the growth of **Clostridium perfiringens** in their intestines and prevents or reduces a chronic but subclinical toxemia.

2. Removal of the saprophytic bacteria from the intestinal tract may have a beneficial effect on the nutrition of the animals.

3. Streptomycin may have a "sparing effect" on the B_{12} in the diet, making it available in greater quantities for utilization by the anumals.

The Practice of supplementing animal feed with antibiotics has raised the issue of widespread development of bacterial resistance. The broad exposure of microorganisms to antibiotics provided by antibiotic supplemental feeds has led to restrictions on this practice.

Because they inhibit the growth of some bacteria and do not affected others, antibiotics have been widely used to prevent bacterial growth in tissue and culture fluids and in chick embroys used for the cultivation of viruses. Fleming's first used of penicillin was to add the crude mold-culture filtrate to media used for the isolation of *Haemophilus influenzae* from nose-and-throat washings. The antibiotic inhibited the Gram-positive cocci present but permitted *H. influenzae* to grow.

Some antibiotics are effective against plant pathogens and are attractive for treatment of plant diseases. The extent of this practice is limited mainly by economic factors, i.e., the cost of the antibiotic.

Suggested Questions

1. What are the characteristics of an ideal chemotherapeutic agent?

2. What are the major modes of antibacterial action of chemotherapeutic agents?

3. Describe the mode of antimicrobial action of the following chemotherapeutic agents : penicillins, cephalosporins, streptomycin, chlortetracycline, bacitracin and sulfonamides.

4. Why are some antibiotics used to supplement animal foods?

5. Which genera of microorganisms produce the most antibiotics?

6. What is objection to using antibiotics as a food preservative?

BIOCHEMICAL MECHANISMS OF DRUG RESISTANCE

Some years ago the American microbiologist Bernard Davis listed a number of possible mechanisms for drug resistance. While this list continues to be useful, experience has shown that mechanisms 1-4 (below) are the most common, at least in bacteria

Summary of possible mechanisms

1. Conversion of action drug to an inactive derivative by enzyme(s) produced by the resistant cells.

2. Modification of the drug – sensitive site.

3. Loss of cell permeability to a drug.

4. Synthesis of an additional drug-resistant enzyme or overproduction of a drug-sensitive enzyme.

5. Increased concentration of a metabolite that antagonizes the inhibitor.

6. Conversion of an inactive derivative.

β -LACTAMS

The destruction of penicillin by bacteria that produce penicillinase (β–lactamase) involves the opening of the β–lactamase bond of penicillin to give the inactive penicilloic acid (Fig. 20). As we shall see later, the nature of the side chain, R, can be of critical importance in determining the susceptibility of the β–lactam bond to penicillinase. The related antibiotics, the cephalosporins, are also degraded by β–lactamase. The immedite end product of cephalosporin degradtion is cephalosporanoic acid, although this compound is so unstable that it spontaneously undergoes a complex series of degradative reactions. Penicillin and cephalosporin β–lactamases are produced by many Gram-positive and Gram-negative bacteria. Individual enzymes exhibit preferences for either penicillins or cephalosporins as substrates, so that cross-resistance between the two classes of β–lactam antibiotics is frequently incomplete.

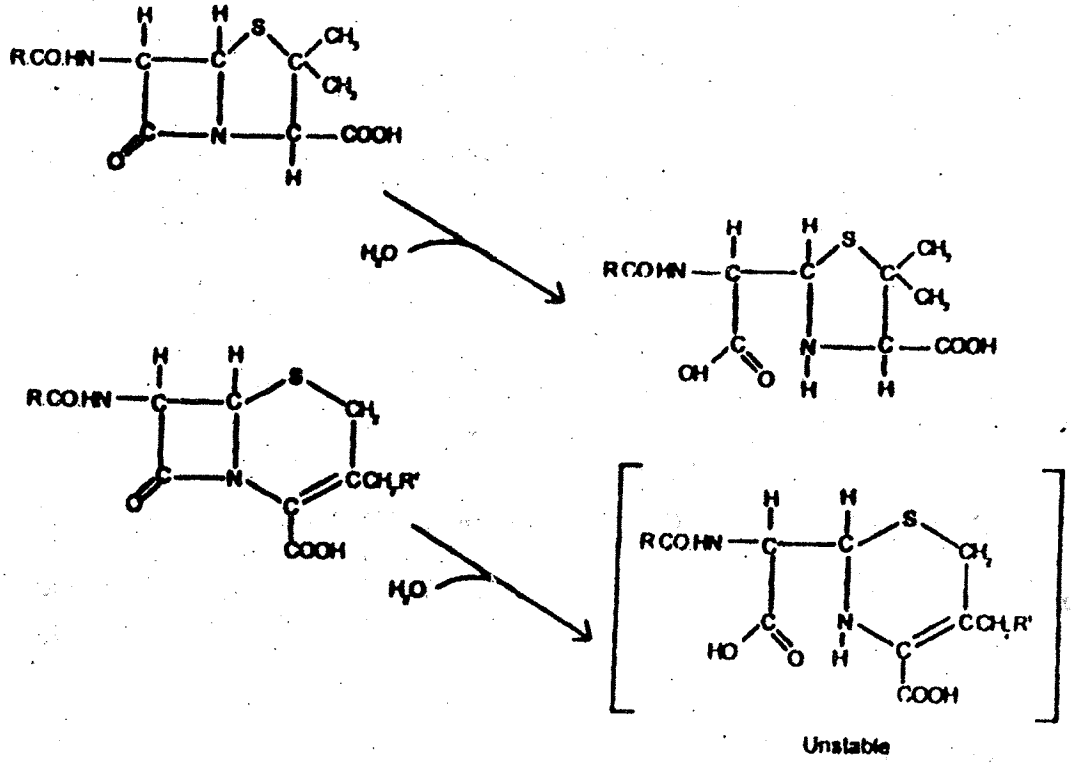


Figure 20. *Inactivation of (a) penicillins and (b) cephalosporins by β–lactamase. In both cases the β–lactam bond is broken by a hydrolytic mechanism, but whereas penicilloic acid is relatively stable, the corresponding cephalosporin product is highly unstable and decomposes spontaneously to a complex mixture. R and R' indicate variable side chains.*

CHLORAMPHENICAL

Another important example of bacterial drug resistance due to inactivation of the antibiotic is the enzymic acetylation of chloramphenicol by resistant strains of both Gram-positive and Gram-negative bacteria. The enzyme responsible, chloramphenicol acetylransferase (CAT), is usually plasmid encoded and the gene for a major subtype is found on a transposon, Tn9 in Gram-negative bacteria. A chromosomally determined form occurs in *Pseudomonas mirabilis*. CATs are widely distributed in bacteria and despite differences in amino acid sequences, all CATs appear to be tetramers with identical subunits with molecular weights in the range of 23,000 to 25,000. In Gram-positive bacteria the enzymes are inducible whereas in Gram-negative organisms enzyme synthesis is constitutive. As shown in Fig. 21 the enzyme first converts

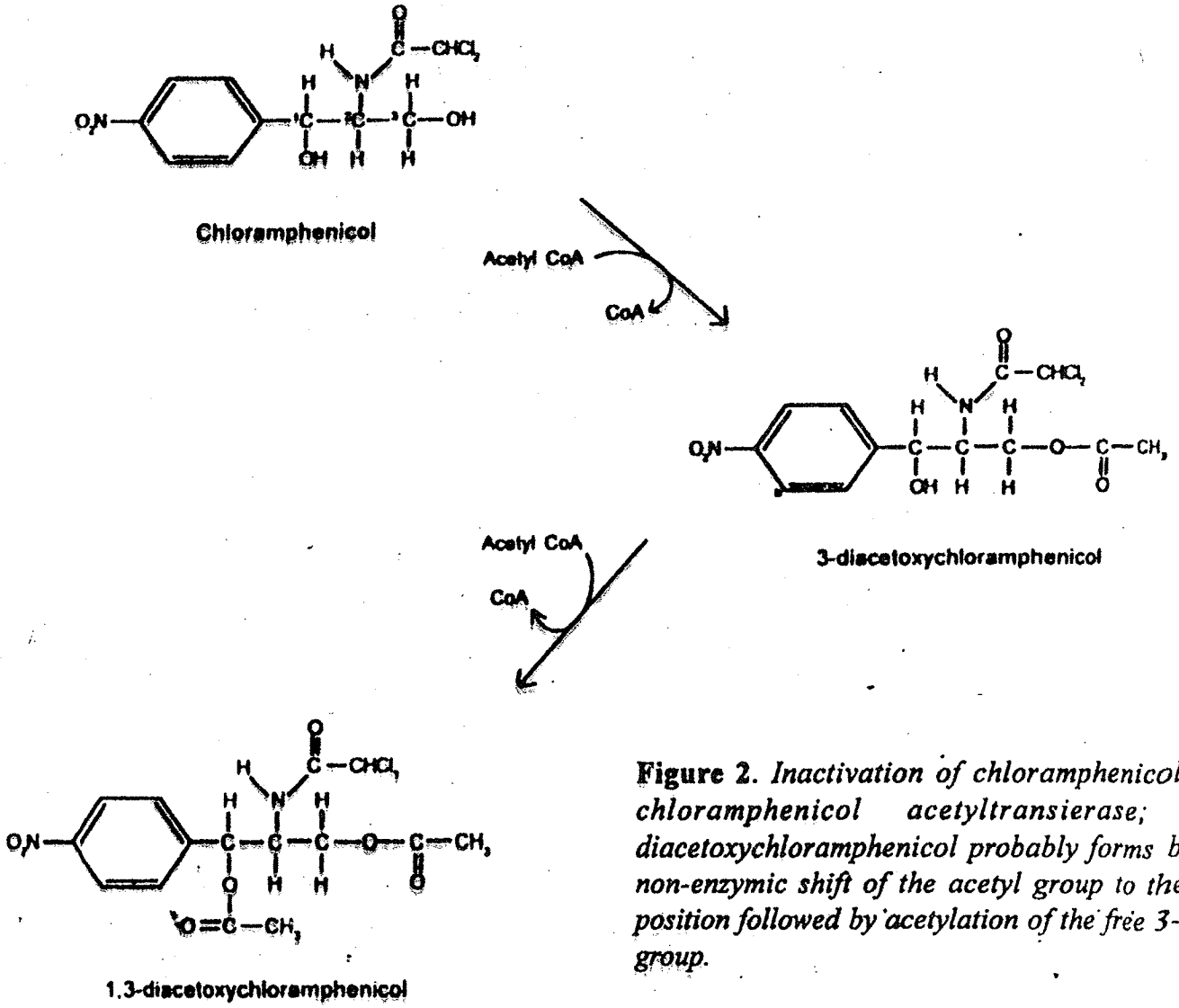


Figure 2. *Inactivation of chloramphenicol by chloramphenicol acetyltransierase; 3-diacetoxychloramphenicol probably forms by a non-enzymic shift of the acetyl group to the 1-position followed by acetylation of the free 3-OH group.*

chloramphenicol to the 3-acetoxy derivative using acetyl-Co A as an essential cofactor. A non-enzymic intramolecular rearrangement then follows which transfers the acetoxy group to the 1-position. Further enzymic acetylation at the 3-position generates the final product. 1.3-diacetoxychloramphenicol. Both the mono- and di-acetoxy derivatives are inactive as antibiotics so that the two-step acetylation sequence is somewhat metabolically ineficient. Recently, several new derivatives of chloramphenicol have been described which are not subject to acetylation and yet retain antibacterial activity. The key substitution appears to be the replacement of the *terminal* OH group of the side chain with flurine. None of these compounds has yet established a role in clinical medicine.

(2) Modification of the drug –sensitive site

STREPTOMYCIN

A stiking example of the modification of a drug-sensitive site resulting in a high level of resistance is the loss of ribosomal sensitively to streptomycin. The change in ribosomal structure has been traced to a single amino acid replacement in either of two positions in the S12 protein of the 30S ribosomal subunit. The changes in the S12 protein are caused by chromosomal mutations. The modified 30S subunit no longer binds streptomycin and the drug cannot therefore exert its characteristic effects on protein biosynthesis. The S12 protein may also be modified in mutants showing streptomycin dependence. Streptomycin resistance due to modified ribosomes is much less significant clinically than the plasmid-mediated resistance, although ribosomal resistance to streptomycin has been found in clinical isolates of *Neisseria gonorrhoeae*. Staphylococcus aureus, *Pseudomonas aeruginosa and Streptococcus faecalis*.

ERYTHROMYCIN

In natural isolates of *Staphylococcus aureus* and *Streptococcus* spp. Resistance to erythromycin is associated with a change in the 23S RNA of the 50S ribosomal subunit. A plasmid-mediated ribosomal RNA methylase induced by erythromycin is responsible for the alteration in the 23S RNA which involves N-dimethylation of a specific moiety using S-adenosylmethionine as the methyl donor. The chemical modification of the adenine residue is believed to induce a conformational change in the ribosome that diminishes its affnity for erythromycin. The modified ribosomes are crops-resistant to lincomycin. This mechanism of resistance provides an intriguing contrast with that of kasugamycin where a spcific *loss* of RNA methylation is involved.

The inductive mechanism does not involve the interaction of erythromycin with a repressor protein and a subsequent increase in transcription but rather an increase in the rate of translation of the message is hindered by specific secondary structure in the mRNA which is indirectly relieved by the action of erythromycin on the ribosomes. Paradoxically this permits limited biosynthesis of the N-dimethylase. As the methylated ribosomes escape the inhibitor effects of erythromycin, translation of the message for N-dimethylase slows once again.

RIFAMYCINS

Another important example of a mutation causing a change in the drug-sensitive site is seen in the altered DNA-dependent RNA polymerase of mutants resistant to the rifamycins. Resistance to thre rifamycins depends on a modification of the B-subunit of the 'core' enzyme of the polymerase loss of sensitivity of the polymerase to rifampicin is accompanied by a failure of the 'core' enzyme to bind the antibiotic. The change in the enzyme is determined by a chromosomal mutation.

(3) Loss of cell permeability to a drug

Several mechanisms of resistance minic a fall in cell permeability to a drug. A familiar example occurs where resistant cells convert a drug to a derivative that does not bind to its intracellular target. The cells no longer sequester the drug and a false impression of diminished permeability is created. Similarly, a change in the target site leading to a loss of affinity for a drug results in a reduced cellular uptake of drug.

Despite these traps for the unwary investigator resistance to certain antimicrobial drugs clearly depends on cellular changes that hinder drug access. This loss of permeability barrier may be due to one of several mechanisms.

(i) An existing permeability barrier may be modified. The greater intrinsic antibiotic insensitivity of Gram-negative bacteria compared with Gram-positive organisms depends on the non-specific permeability barrier provided by the outer membrane of Gram-negative cells. Mutations affecting the lipopolysaccharides, porins and nutrient receptor proteins of the outer membrane may all adversely affect the inward movement of antibiotics. These changes may not in themselves always give rise to significant resistance, but combined with other factors, such as drug-inactivating enzymes or a reduction in target sensitivity, they may be sufficient to afford the cells adequate protection. The reduced uptake of streptomycin and erythromycin by some resistant pneumococci may be due to the development of an altered capsular permeability barrier. Since these strains are less susceptible to DNA transformation because of a diminished uptake of DNA, the permeability barrier is probably non-specific.

(ii) When the drug gains intracellular access by a specific transport mechanism, diminished uptake by resistant cells can be due to a mutation that results in a partial or complete loss of transport function. As we saw in Chapter 7, drugs may subvert permeation mechanisms designed for nutrients. A mutatation affecting such a permeation mechanism may give rise of resistance if the cells can withstand or circumvent the loss of the nutrient. A good example is found in a series of mutants of *Escherichia coli* resistant to D-cycloserine. In sensitive cells this drug is accumulated by the amino acid-transport system that normally carried D-alanine or glycine. Mutants exhibiting graded loss of function of this transport system are resistant ty cycloserine. Resistance to the clinically valuable antifungal drug 5-fluorocytosine (Chapter 6) can arise from mutations affecting the activity of acytosine permease. This enzyme is exploited by 5-fluorocytosine in order to penetrate the fungal cell membrane.

(iii) Specific antagonism of antibiotic transport. The most commonly encountered mode of resistance to tetracyclines in Gram-positive and Gram-negative bacteria depends on a dininished cellular accumulation of these compounds. Many tetracycline-sensitive bacteria accumulate tetracyclines by an energy –dependent process which involves the cytoplasmic membrane. Tetracycline accumulation in resistant cells is blocked by a pritially inducible system that is determined by plasmid genes in Gram-positive and Gram-negative bacteria. When resistant cells are exposed to a subinhibitory concentration of tetracycline there is a rapid increase in the level of resistance which can be prevented by blocking protein or RNA synthesis in the cell. The increase in resistance is associated with a fall in the uptake of tetracycline by the cells. In enteric bacteria two transposons, Tn 1921 and Tn 10, commonly carry the genes for tetracycline resistance. Tn 10 encodes two proteins: a membrane-located protein of molecular weight 36,000 that appears to mediate resistance and a repressor protein associated with the inducibility of tetracycline resistance. In Gram-positive bacteria the induction mechanism does not appear to involve a repressor protein but depends upon a transnational attenuation mechanism of the type previously described for erythromycin resistance.

The actual basis for tetracycline resistance has proved to be an efflux process that specifically pumps tetracycline out of the cells against a concentration gradient. The pump, presumably associated with the inducible membrane protein, is therefore energy-dependent and appears to harness the proton motive force across the cell membrane. Although the mechanism of tetracycline resistance is essentially the same in Gram-positive and Gram-negative bacteria, there is some evidence that the proteins concerned may have evolved separately in the two genera.

(4) Synthesis of an additional drug-resistant enzyme or overproduction of drug-sensitive enzyme.

SULPHON AMIDES

Resistance to the sulphonamides has been recognized for nearly forty years and the resistance determinant was found on R-plasmids when they were discovered in Japan in the 1950s. The plasmids carry the genetic information for two forms of digydropteroate synthase which are highly resistant to inhibition by sulphonamides. R-plasmid-determined dihydropteroate synthase binds sulphonamides 10,000 times less tightly that the chromosomal enzyme, while the K_m for the substrate, *p*- aminobenzoic acid, is the same for both enzymes. Cells with the additional resistant enzyme utilize *p*-aminobenzoic acid normally, despite high concentrations of sulphonamides. Pneumococci resistant to sulphonamides also synthesize a dihydropteroate synthase that is not inhibited by sulphonamides.

METHICILLIN

Another example of drug-resistance dependent upon the production of a modified form of the target enzyme is found in the potentially dangerous methicillin-resistant strains of *Staphylococcus aureus* that plague many hospitals. Most of these strains have the ability to make a unique penicillin binding protein, PBP2a, which has a lower affinity for methicillin and B-lactams in general than the usual form of this

protein. The production of PBP2a appears to be stimulated by the presence of methicillin although the regulatory mechanism involved is unknown. The gene encoding PBP2a may be located on a transposon.

(5) Increased production of a metabolite that antagonizes the inhibitor

When a drug inhibits growth by the competitive antagonism of a normal metabolite, resistance to such an inhibitor may be due to increased production of the metabolite: the inhibitor is competitively displaced from its enzyme binding site. This type of resistance is exemplified by certain mutants resistant to sulphonamides. In these cells the concentration of *p*-aminobenzoic acid is said to be substantially higher than in sulphonamide-sensitive cells although the mechanism underlying the increased production of *p*-aminobenzoic acid us uncertain.

BACTERIAL TOLERANCE

A special type of resistance to β - lactam antibiotics has been encourntered amongst clinical isolates in recent years. The β - lactams are characterized by their very useful ability to kill growing bacteria. However, below the concentrations required for bactericidal activity β - lactams also arrest cell growth. β - lactam-tolerant mutants show no change in their sensitivity to the growth-inhibiting action of β - lactams but are much more resistant to their bactericidal activity than the parent organisms. The consequence for the patient is that the cause of their infection and treatment period is unduly prolonged since the successful application of β - lactam therapy depends to a considerable extent upon the expected bactericidal component. The basis for β - lactam tolerance does not appear to rest upon β - lactamase activity or changes in the sensitivity of the molecular targets of β - lactams but stems changes in the sensitivity of the molecular targets or β - lactams but stems from a decline in the normal drug-induced disruption of the structure of the expanding peptidoglycan envelope of growing bacteria. This disruption leads to the inappropriate activation of autolysins, or peptidoglycan hydrolases which cause disintegration of the bacterial cell wall and rapid cell death. β - lactam-tolerant mutants have a defect in their autolysin activity which, though causing some abnormality in cell morphology, affords protection against the lethal effects of β - lactams. Such tolerant organisms have been reported amongst clinical isolates of *Pneumococci* and other Gram-positive bacteria. There is little evidence so far to suggest that the β - lactam tolerance poses a major threat to the successful treatment of Gram-negative infections.

PRACTICAL APPROACHES TO THE CONTROL OF DRUG RESISTANCE

We have reviewed the genetic and biochemical ingenuity of micro-organisms in combating the toxic effects of growth-inhibiting substances. Highly successful mechanisms of resistance against antibiotics were evolved by micro-organisms long before the introduction of these substances into medical and veterinary

practice. The development of resistance against novel chemically synthesized agents unrelated to antibiotics, natural metabolites or growth factors is more difficult to explain, although the high rate of cell division in most microbial populations combined with their extraordinary genetic flexibility facilitates 'high speed' evolution. Nevertheless, the capacity of bacteria to develop resistance is not unlimited. Clinical experience with β - lactamase-stable derivatives gives some support to this view, since resistance to these agents has appeared much more slowly than to the original penicillin.

Apart from the preparation of new chemical agents there are a number of other measures which can limit problem of drug resistance.

(1) Treatment of an infection usually begins with the administration of a high 'loading' dose of antimicrobial drug in the hope of eliminating resistant organisms in the body. Therapy is continued with doses large enough and frequent enough to maintain an effective antibacterial level of drug in the blood until the infection is overcome.

(2) Micro-organisms are often cross-resistant to chemically related drugs. Simultaneous therapy with chemically unrelated inhibitors, however, presents a more formidable challenge to a microorganism especially when the inhibitors attack essential biochemistry at two different points. Although R-plasmids conferring resistance to the sulphamethoxazole-trimethoprim combination are no longer uncommon, several years of 'grace' were obtained before this occurred. In the treatment of tuberculosis, where the need for resistance, the use of a combination of isoniazid, streptomycin and *p*-aminosalicylic acid successfully avoids the frequent emergence of resistant strains. The clinical use of combinations of inhibitors of β - lactamase and β - lactams is proving to be useful in countering the threat of β - lactamase-producing pathogens.

(3) Undoubtedly, the careless use of antimicrobial agents in human medicine and in farming for non-therapeutic has assisted the spread of resistant organisms. An encouraging development, however, is towards much sticter control over the use of these drugs. The proper and careful deployment of antimicrobial agents can produce dramatic results in reducing the number of resistant organisms in the environment.

FURTHER READING

Bryan, L.E. (ed.) (1984) *Antimicrobial Drug Resistance,* Academic Press, London.

Antimicrobial drug action –*Franklin & Snow.*

BOTANY

Module No. - 04

Part - I, Paper - I (1st Half)

Title : Antigens and Antibodies : Nature of immunological reactions. diagnostic applications, vaccinations and their manufacture.

The human body is like an island which is continuously being invaded by strangers with different needs, different food requirements and different localities in which to raise their progeny. The immune system faces the task of providing a defence mechanism to establish a state that is known as immunity to infection.

What is immunity?

The contemporary defination of immunity is therefore : "All those physiological mechanisms that endow the animal with the capacity to recognize materials as foreign and to neutralize, eliminate or metabolize them with or without injury to its own tissues."

It serves these broad functions -

(a) Defence against microorganisms

(b) **Haematostasis;** removal of damaged cells.

(c) **Surveillance;** recognition and destruction of mutant cells.

Types of immune response :-

Immune responses have been broadly classified as **non-specific and specific**

By non-specific immune response we mean the general ability of certain cells to resist most pathogenic bacteria, virus and fungi. The engulfment by macrophages which is otherwise known as phagocytosis is one example of such immunity. Non-specific immunity is the first line of defence after pathogens break through the anatomical barriers of the body.

A specific immune response is elicited by a wide variety of individual molecules that are foreign to the vertebrate host. Such foreign molecules, known collectively as *immunogens*, trigger a response that has three major characteristics : **Specificity, memory** and **tolerence**. Antigen antibody reaction is a specific immune response which provides the body with a second line of defence directed solely towards a particular microorganism or part of it.

Antigen and Antibody :-

The terms immunogen and antigen are often used synonymously. However these two terms imply

two closely related, but different entities. A molecule that provokes an immune response is called an *immunogen*. They are usually macromolecular components of the pathogen, such as surface proteins. Foreign molecules are known as *antigens* when they are recognized by the immune system of the host.

Antigens are passed along to antigen specific cells, the T-lymphocytes or T-cells by a process called antigen penetration. The T-cells can interact directly with the antigen (cell mediated immunity) or they can pass the information to a second group of antigen specific cells, the B-lymphocytes or B-cells, which make specific proteins known as **immunoglobulins or antibodies.**

Therefore, *antigen can be defined* as a molecule which reacts with the components of cell mediated immunity or with the antibody produced as a result of its own invasion.

Antibody can be defined as a cellular protein produced by B cells that interact with antigens. They are chemically known as immunoglobulins.

Properties of Antigen:

There are some requirements for any molecule to qualify as an antigen -

(a) The substance must be genetically foreign to the host. When body constituents are recognized as foreign by the host, then it leads to the autoimmune disease.

(b) Particles with a molecular weight less than 10,000 are non-antigenic or very weakly antigenic in nature.

(c) Chemical complexity is another important characteristic. For example, monomeric proteins may actually induce tolerence but in polymeric state they are highly antigenic.

(d) Conformation of a protein is important. For example if disulphide bridges of a protein molecule are broken by any treatment then this protein loses its antigenicity.

(e) Amino acid sequence of a protein when disturbed then it leads to loss of antigenicity.

(f) That portion of the antigen which reacts with the antibody is known as antigenic determinant. This determinant should be accessible to the antibody to evoke any response.

Antibodies:

Antibodies are protein molecules that are able to combine with the antigenic determinants. They are found the serum and in other body *fluids* such as gastric juices and milk. They are classified into a group of proteins known as *globulins* and referred to as immunoglobulins for their immunogenic properties. The serum containing antigen specific antibody is often called antiserum. Immunoglobulins (Ig) can be separated into five major classes on the basis of their physical, chemical and immunological properties : IgG, IgA, IgM, IgD and IgE. Immunoglobulin class IgG is further subdivided into four classes namely IgG, IgG_2, IgG_3 and IgG_4.

In response to antigen the first immunoglobulin to be produced is IgM. Later IgG appears

In most individuals about 80% of serum immunoglobulins are IgG proteins and therefore those molecules have been studied extensively.

Basic Structure of Immunoglobulin G or IgG : (Fig-1)

Immunoglobulins are glycoproteins - The basic unit is a monomer consisting of four polypeptide chains. The chains are held together by both intrachain and interchain disulphide (s-s) *bridges*. The two short chains are identical in amino acid sequence and referred to as *light chains* or *L-chains*- Similarly two larger chains are identical to one another and referred to as *Heavy Chains* or *H-chains*. Each polypeptide chain contains an amino terminal and a corboxy terminal. The amino terminal portion of the L-chain is variable in respect of amino acid sequence from one Ig molecule to other, unless they are produced by from same mother cell. The constant (C) region of the light chain, by contrast, is identical in all the molecules of a class of immunoglobulin. Thus light chains have a variable (V_L) and a constant (C_L) region. Similarly the heavy chains have a variable (V_H) region and a constant (C_H) region. However, in the heavy chain the C-region is subdivided into 3 distinct areas (domains) each of which is about 110 amino acids in length. These areas are designated as CH_1, CH_2 and CH_3 regions.

The antigen binding region is formed only by small number of amino acids in the 'V' region of H and L chains. There is a *hinge region* between CH_1 and CH_2, which is flexible and more exposed to enzymes. Enzyme papain acts on hinge region to fragment into two portions :

(a) Antigen binding Fab fragment

(b) Crustallizable Fe fragment, which lacks the ability to bind antigen.

Disulphide bridges are present between H and H, H and L, L and L regions. It is interesting to note that in many cases the removal of disulphide bridges does not cause the loss of structural integrity of the antigen. Depending upon minor differences in their C_H region the IgG have been classified into IgG_1, IgG_2, IgG_3 and IgG_4 sub-classes.

Other classes of Immunoglobulins :- (Figure - 2)

How do immunoglobulins of other classes differ from IgG? The C_H of any given immunoglobulin determines its class. Five amino acid sequences exist among the Ig molecules. These sequences are identified as, α, μ, δ and ε sequences. These are characteristic for IgG, IgA, IgM, IgD and IgE respectively.

IgM : An aggregate of 5 immunoglobulin molecules attached as shown in figure - 2, by short peptides called *J-chains*. IgM accounts for 5-10% of serum immunoglobulins and it is the first class of antibodies which are produced in response to a bacterial infection.

IgA : It is present in the body secretions such as saliva, tear, breast milk etc. They have a different structure

compared to IgG as they are present in the form of a dimer attached to a protein body called *secretory piece* or J-chain. It performs the role of defending the exposed internal surface of the body.

IgE : Found in extremely small quantities in the serum. Despite its low conc. it is important because the allergic reactions in response to specific type of antigens called *allergens* are mediated by IgE. Its mol. wt. is higher than other immunoglobulins.

IgD : It is also present in low concentration. Its structure is like IgG but has an extended hinge region. Its function is not very clear. However it is found abundantly on the surface of B-cells alongwith IgM and it is suggested that they serve as signals for B-cells to start antibody production in case of infection.

Antigen-Antibody Reactions :

The principle of antigen antibody reaction lies in the specific combination of determinants on the antigen with the variable region (V_H) of the antibody molecule. All antigen-antibody reactions therefore rely on antigen binding as the first step. Some typical antigen-antibody reactions which are important in clinical medicine are described below :-

i) **Neutralization** - Nutralization of microbial toxins by specific antibody can occur when toxin molecules and antibody molecules directed against toxin combine in such a way that the active portion of the toxin is blocked. An antiserum containing an antibody that neutralises a toxin is known as *antitoxin*.

ii) **Precipitation** - Since antibody molecules generally have two combining sites (because they are bivalent as in IgG) it is possible for each site to combine specifically with different antigen molecules. If the antigen also has more than one determinants, then all antigen binding sites of an antibody molecule may be occupied by different antigenic determinants of the same molecule acting as antigen. Such a case will lead to the formation of a precipitate which is actually an aggregate of antigen and antibody. As they are observed easily, the precipitation reactions are serologically very important. Precipitation only occurs when antigen and antibody are present in optimal quantities. Excess of any one of them will not give an ideal result.

iii) **Agglutination** - If the antigen is not in the solution but on the surface of a cell or other particles, an antigen antibody reaction can lead to clumping of particles or cells-called agglutination.

For example, red blood cells (RBC) contain a variety of antigens on their surface. They will form clumps when subjected to specifric antibodies causing agglutination. The clumping of RBC is referred to as *haemagglutination*. People differ in the antigens present on their RBC. The classification of blood antigens which is also known as *blood typing* is a good example of agglutination reaction, because antibodies, specific for a particular blood surface antigen, cause

RBC to visibly clump. The classification of blood types on the basis of agglutination reaction is as follows.

Blood type	Haemagglutination with	
	Anti-A	Anti-B
Type - O	No	No
Type - A	Agglutinate	No
Type - B	No	Agglutinate
Type - AB	Agglutinate	Agglutinate

iv) **Complement fixation** : Complement is a system of 11 proteins, designated as C_1, C_2, C_3 and so on, which bind specific antigen-antibody complexes to bring about reactions which would not occur otherwise. Therefore, a major function of the antibody is to recognize the antigen and to activate the complement system to attack the infectious agent.

In a system, where there are antigen, antibody and the complement proteins present, the consumption of complement can be used as a test to detect and measure the antibody, the antigen or the both. This test is known as *complement fixation test.*

v) **Hypersensitivity** : Hypersensitivity is over-reaction of antibodies causing host damage. This antibody mediated hypersensitivity is often known as *allergy*. Antigens which induce such a reaction are known as *allerglens*. When an allergen (such as pollen grain) enters the body. it causes an *anaphylactic reaction* causing rapid production of IgE. These are not circulating antibodies like IgG or IgM. They tend to adhere to the *Mast cells* or *Basophils* of the blood. When they are exposed to allergens, they produce a number of chemical mediators like *histamine* and *seratonin*. The release of these substances cause dilation of blood vessles and contraction of smooth muscles which are clinically known as *allergy* or *anaphylaxis*.

Immunity:

The major role of the immune system of the body is to protect the individual from the consequences of infection. The importance of antibodies in disease resistance is shown dramatically in individuals with genetic disorders where antibodies are not produced. Such individuals are unusually sensitive to infection and rarely survive. The general lack of antibody response is also observed in those suffering from AIDS.

An animal or human may exhibit two types of Immune response to a disease :

I) **Natural Immunity** : When an individual is exposed to infection, he or she develops immune response which is a natural outcome of the infections. The first exposure to an antigen evokes

a *primary response*. After introduction of antigen there is a latent period during which the antigen is recognized. Thereafter the antibody production takes place.

The *secondary response* occurs upon the next exposure to same immunogen weeks, months, years later.

II) **Acquired Immunity** :

a) **Passively acquired Immunity** - It is acquired (i) by the newborn from the mother (ii) by administering preformed immunoglobulins to an individual from another individual who has already suffered the disease.

b) **Actively acquired immunity** - Other than suffering the disease and surviving it, *vaccination* is the only other way of acquiring active immunity against an infection. The material used in inducing active immunity is known as *vaccine*.

Vaccines are of two types-

i) Some are *killed infectious agents* which do not lose their antigenic determinants and therefore able to elicit antibody formation.

e.g. **Bacterial vaccines for :**

Typhoid (*Salmonella typhi*)

Cholera (*Vibrio cholerae*)

Plague

Viral vaccines for :

rabies

poliomylitis

hepatitis B etc.

ii) **Attenuated vaccines** : Often it is possible to isolate a mutant strain of a pathogen that has lost its virulence but still retains the immunizing antigens; these antigens are called *attenuated antigens* which can evoke a normal immune response system in the body, so that the antibodies are produced against them.

e.g. (a) Bacterial BCG - a live *attenuated Mycobacterum bovis* for tuberculosis.

(b) Viral vaccines, like live *vaccinia* virus for small pox, rubella, measles mumps etc.

Apart from vaccines, active immunity to toxin caused diseases, can be obtained by injecting the

toxin, modified in such a way so that, they retain their antigenicity but lose their toxicity. Such a modified exotoxin is called *toxoid*. Some important *toxoids* are :

i) diptheria and tetanus

ii) cholera toxin B.

Key to preparation of vaccines :-

1) **Killed micro-organisms** :- Bacteria killed by some laboratory procedure e.g. exposure to 75% alcohol (TAB vaccine against typhoid fever caused by *Salmonella typhi*).

2) **Attenuated organisms** :- Virulence of *Mycobacterium tuberculosis* is greatly reduced by growing it in a high temperature. In many cases it may be a mutant variety with same antigen but lacking the virulence. Sometimes, there is a chance of attenuated strain reverting back to its virulent form. To minimize this chance attenuation incorporates several gene mutations instead of one. Preserving the vaccine in a cold temperature from laboratory to the field is absolutely essential and a typical problem faced in the tropical countries.

3) **Toxoid** :- Inactivation of the toxin is done by laboratory treatments like treatment with formaldehyde (eg toxoid against *corynebacterium diptheriae* causing diptheria).

4) **Mild strain of virus** - Using closely related but non-pathogenic strain e.g. Small pox/cow pox (variola virus grown in cow used to confer immunity in man).

Further study :-

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2) Scientific American Medicine : Scientific American Inc., New York, 1991.

3) Textbook of Immunology : Nandini Shetty. Wiely Eastern Ltd.; India 1993.

4) Brock's Microbiology, (New edition).

Questions :-

1) Characterize antigens and illustrate in brief the principles of different antigen and antibody reactions.

2) What is antibody? Describe the basic structure of Immunoglobutia G. What are the other classes of Immunoglobulins and what are their biological roles.

3) What are the principal forms of immune response. Discuss the methods by which the immunity can be acquired? Cite suitable examples in each case.

4) What is immunity? What are the different types of immune response shown by the human body? What are the principal antigen-antibody reactions and how these reactions are applied in clinical biology?

5) **Write short notes on:-**

a) IgG

b) Complement fixation.

c) hypersensitivity

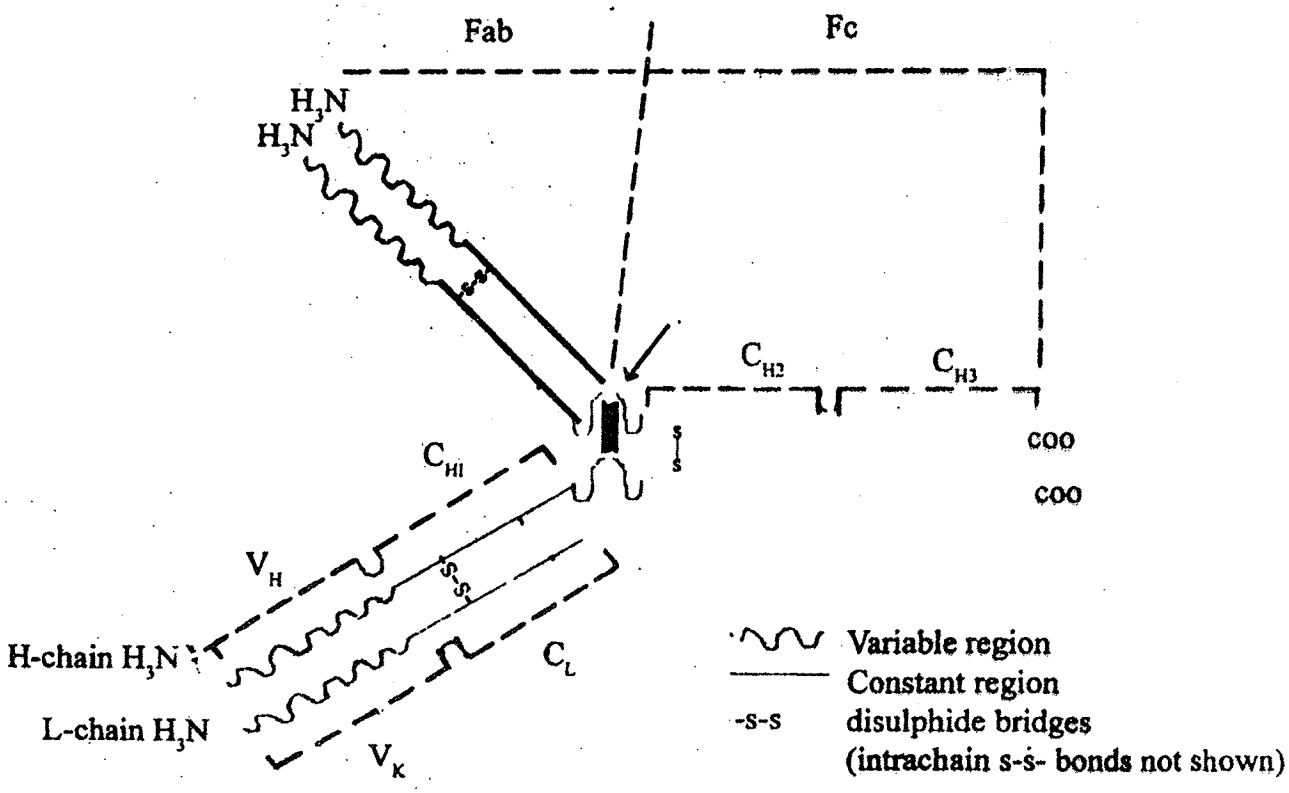
d) agglutination

e) blood typing

f) vaccines and vaccination

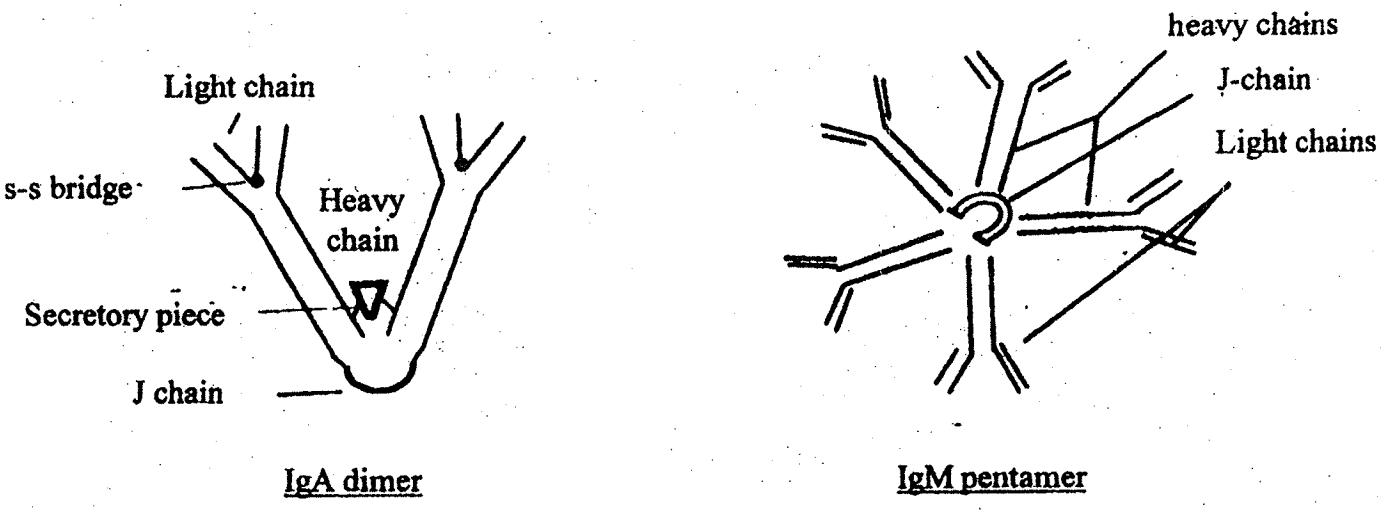
Figure - 1

Papain cleavage



IgG molecule

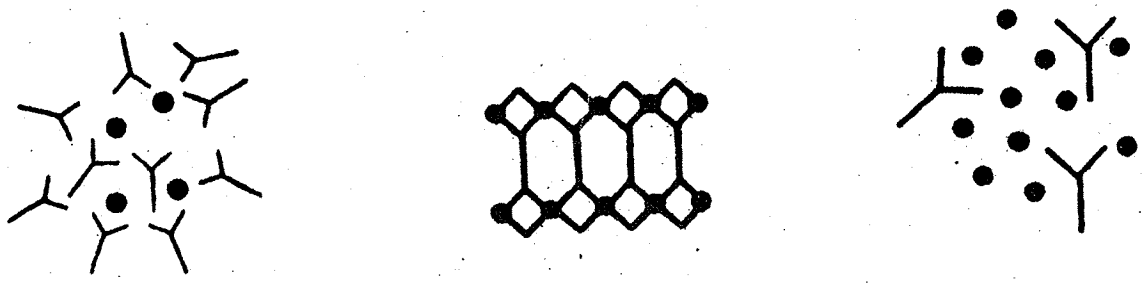
Figure - 2



IgA dimer

IgM pentamer

Figure - 3



Antibody Excess [No ppt.]

Antigen-antibody optimum [ppt.]

Antigen excess [No ppt.]

Antigen-antibody Reaction : Precipitation.

BOTANY

Module - 5
Part - I, Paper - I (1st Half)

Microbiology

Contents

CHAPTER – 1

VIRUSES

CONTENTS

- Discovery
- Nomenclature of the virus particles and its parts
- Morphological categories of viruses.
- Chemical composition of viruses.
- Classification of viruses.
- Identification of viruses.
- Cultivation of animal virus.
- Isolation and purification of plant viruses.
- Cultivation of plant viruses.

The Discovery of viruses:

Viruses were discovered in the 19th Century, some 50 years before the development of the electron microscope. The first demonstrations that viral diseases could be transmitted from one host another under controlled laboratory conditions were published by L. Pasteur in 1884 in studies on rabies and by A. Mayer in 1886 in studies on mosaic disease of tobacco plants. In 1892 D. Iwanowsky by a simple experiment that the causative agent of tobacco mosaic disease is smaller than any bacterium. From his experiment he established that infectious agents that could pass through fine filters became known as filterable viruses. In 1898 M. Beijerinck established that viruses posses the property of replication that is common to all living things by demonstrating that Tobacco mosaic virus (TMV), the filterable virus that causes tobacco mosaic disease. In the same year F. Loeffler and P. Frosch showed that the filterable agent of foot and mouth disease proliferates in cattle. Beijerinck further established that TMV proliferates only in growing plant tissues and made a conclusion that virus proliferation occurs intracellularly and independent on the active metabolism of host cells. F. Twort and F.D. Hevelle (1915, 1917) independently discovered bacterio phages.

For several decades viruses were distinguished by three properties.

i) they are infections agent of disease
ii) invisible in the light microscope
iii) do not proliferate in culture media

In 1935 W. Stanley Crystallised the Virus and showed that it is composed largely of protein.

Nomenclature of the virus particles and its parts:

A virus particle capable of infecting a specific host organism is termed a **Virion**. The two major components of a virus particle, namely the **nucleic acid** and the **protein sheath** are known as the core or

the **Nucleoid** and **Capsid** respectively. The Capsid is formed by a member of structural subunits called the Capsomeres. These are made up of polypeptide chains, numbering two to four coiled in a specific manner. The number of Capsomeres and their proper alignment are specific and are characteristics of individual capsids. Some animal and plant viruses have a **lipoprotein** membrane surrounding them. The membrane structure is called the **Envelope**. There are often projection on these envelope known as spike. Non enveloped Viruses are referred as **naked** virion.

Morphological Categories in Viruses:

Viruses could be categorised into the following principal morphological groups. (Fig.1).

(1) Naked elongated (helical) viruses. (TMV)
(2) Naked spheroidal (icosahedral) Viruses (Polio virus)
(3) Enveloped elongated (helical) viruses (Sendai virus)
(4) Enveloped spheroidal (icosahedral) viruses (Herpes viruses)
(5) Tailed (binal) viruses (Bacteriophage)
(6) Uncertain type (Pox viruses)

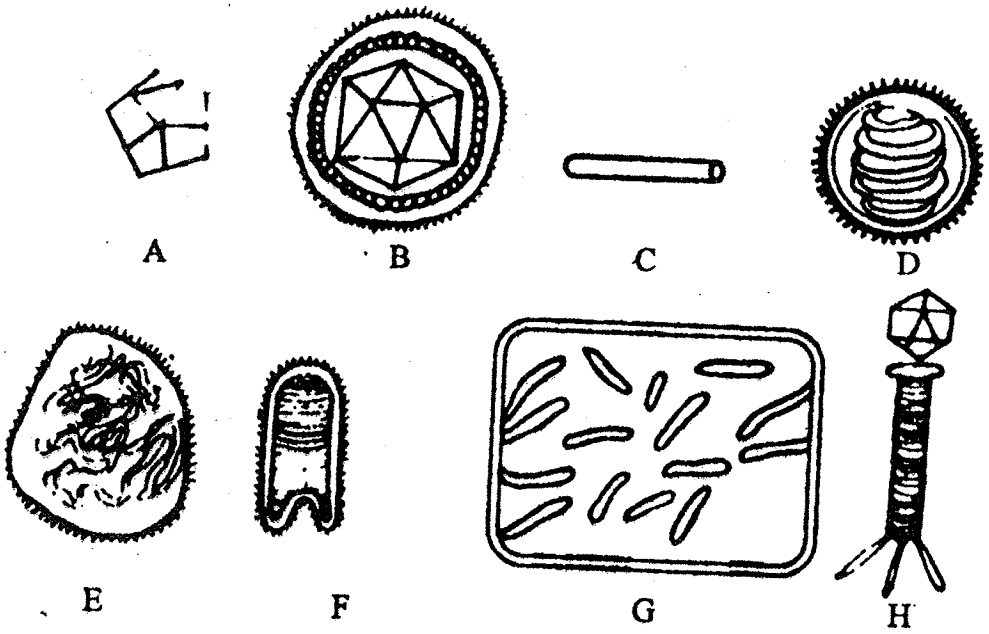


Figure-1. *Morphology of some well-known viruses. Icosahedral symmentry : (A) polio, wart, adeno, rota; (B) herpes. Helical symmetry : (C) tobacco mosaic (D) influenza; (E) measles, mumps, parainfluenza; (F) rabies. Complex or uncertain symmetry : (G) pox viruses; (H) T-even bacteriophages. (Redrawn by Erwin F. Lessel after a drawing by Frances Doane, University of Toronto).*

[For details consult **Microbiology** by Pelczar, Chan & Krieg, Chapter-4, Page-440]

[& General Microbiology by Stanier et al. Chapter – 9, Page – 213] (5th eds.)

Chemical Composition of Viruses :

Chemically, viruses are nucleoproteins. The majority of viruses consist of two components, one is a single usually linear molecule of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The other is an outer proteinaceous covering or sheath surrounding the inner nucleic acid molecule. However, some of the larger animal and bacterial viruses are reported to contain lipids. Lipids are also associated with even a few plant viruses. The general chemical composition of some plant, animal and bacterial viruses are listed in

Table 1, 2 and 3.

TABLE 1. CHEMICAL COMPOSITION OF SOME PLANT VIRUSES

(As percentages)

Virus	RNA	DNA	Proteins	Lipids	Carbohydrates
Alfalfa mosaic	19	-	81	-	-
Broad bean mottle	22	-	78	-	-
Carnation latent	6	-	94	-	-
Carnation each ring	-	-	-		-
Cauliflower mosaic	-	16	84	-	-
Cyanophage-LPP I	-	40	60	-	-
Dahlia mosaic	-			-	-
Golden yellow mosaic	-	23	77	-	-
Pea enation mosaic	29	-	71	-	-
Potato spindle tuber (Virioid)	100	-	-	-	-
Potato X	6	-	94	-	-
Potato leaf roll	-			-	-
Tobacco necrosis	19	-	81	-	-
Tobacco mosaic	5	-	95	-	-
Tomato bushy stunt	17	-	83	-	-
Tomato spotted wilt	5	-	71	19	5
Turnip yellow mosaic	34	-	67	-	-
Wound tumour	23	-	77	-	-

Adapted after Shepherd (1968), Fujisawa et al (1968, 1972), Best (1968), Knight (1974), Sarkar (1976), Goodman (1977).

TABLE 2. CHEMICAL COMPOSITION OF SOME ANIMAL VIRUSES

(As percentages)

Virus	RNA	DNA	Proteins	Lipids	Carbohydrates
Adenovirus	-	13	87	-	-
Equine encephalo mvelitis	4	-	42	54	-
Fowl plaque	2	-	69	26	2
Herpes simplex	-	9	67	22	2
Influenza	1	-	74	19	6
Poliomyelitis	26	-	74	-	-
Polyoma	-	16	84	-	-
Reovirus	21	-	79	-	-
Rous sarcoma	2	-	62	35	1
Shope papilloma	-	18	82	-	-
Simion virus 5 (SV 5)	1	-	73	20	6
Tipula iridescent	-	13	82	5	-
Vaccinia	-	5	88	5	2

Adapted after Knight (1974); Wyatt & Cohen 1953.

TABLE 3. PERCENTAGE COMPOSITION OF SOME BACTERIAL VIRUSES

Virus	RNA	DNA	Proteins	Lipids	Carbohydrates
Coliphage f_2	30	-	70	-	-
Coliphage M_{13}	-	12	88	-	-
Coliphage Ø X 174	-	26	74	-	-
Coliphage T_4	-	55	40	-	5
Coliphage T_2	-	50	50	-	-
Coliphage lambda (λ)	-	56	44	-	-

After Butler (1970); Knight (1974); Wyatt and Cohen (1953).

Classification of Viruses:

Properties used for classification of virus in Table – 4.

Table – 4. Properties used for Classification of Viruses

Primary Characteristics

Chemical nature of nucleic acid :

Secondary Characteristics

Host range :

RNA or DNA; single or double stranded;
Single or segmented genome; (+) or (-) strand;
Molecular weight

Host species; specific host tissues or cell types

Mode of transmission :
e.g. feces.

Structure of virion:
Helical, icosahedral, or complex; naked or Enveloped; complexity; number of capsomeres For icosahedral virions; diameter of nucleo-capside for helical viruses

Specific surface structures :
e.g. antigenic properties

Site of replication :

Nucleus or cytoplasm

In all viral taxonomies, individual viruses are grouped by the nucleic acid, they contain (DNA or RNA), their size and the architecture of their capsid. Presence of an envelope and chromosome structure (circular versus linear, and single stranded versus double stranded molecules) are also important properties in viral taxonomies. In some group of RNA virus, a virion contains several chromosomes each encoding one or two viral proteins. Virions that exhibit this highly unusual genetic organisation are said to possess a segmented genome.

In some of the single-stranded RNA viruses, the chromosome also serve as viral mRNA in others transcribed RNA, complementary to the RNA chromosome serve as in RNA. Virion RNA that can function as mRNA is fermed **plus-strand RNA** while RNA that is complementory to virion in RNA is termed **minus-strand RNA.**

Animal plant and bacterial Viruses are usually classified separately. Animal Viruses are often grouped into families, genera and species which are given Latin names, but English names are also used. Selected families of animal Viruses together with their properties are given in **Table-5.** A taxonomy employing Latin family names also has been proposed for bacterial Viruses (Table – 6). Plant viruses are grouped on the barsis of (1) the structure of the virion (2) whether it contains DNA or RNA and (3) its mode of transmission. In most cases the groups of plant viruses are named after a prominent representative. For example a group of plant viruses closely related to TMV is termed the tobacco group. Selected groups of plant viruses together with their defining properties are listed in **Table – 7.**

TABLE – 5

Some Families of Animal Viruses

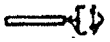
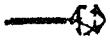
Family Name	Morphology	Enveloped (E) Or Naked (N)	Approximate Size (nm)	Nucleic Acid[b]
Proxviridae (Poxviruses)		E	350 x 250	Linear ds DNA
Herpesviridae (herpesviruses)		E	200	Linear ds DNA
Adenoviridae (adenoviruses)		N	75	Linear ds DNA
Parvoviridae (parvoviruses)		N	20	Linear ss DNA
Papovaviridae (papovaviruses)		E	50	Circular ds DNA
Baculoviridae (baculoviruses)		E	300 x 40	Circular ds DNA
Picornaviridae (picornaviruses)		N	27	Plus-strand RNA
Togaviridae (togaviruses)		E	50	Plus-strand RNA
Retroviridae (retroviruses)		E	50	Plus-strand RNA
Orthomyxoviridae (orthomyxoviruse		E	110	Segmented : 8 minus-strand RNA molecules
Paramyxoviridae (paramyxoviruses		E	200	Minus-strand RNA
Rhabdoviridae (rhabdoviruses)		E	170x70	Minus-strand RNA
Reoviridae (reoviruses)		N	65	Segmented : 10-13 ds RNA molecules

[a] Protein and nucleoprotein components are diagramed in black, nucleic acid in grey.

[b] Single-stranded (ss) or double-stranded (ds) nucleic acid.

TABLE – 6.

Some Families of Bacteriophages

Family Name	Morphology	Nucleic Acid[a]	Example
Myoviridae		Linear ds DNA	T2, T4, T6, P2
Styloviridae		Linear ds DNA	T5, λ
Pedoviridae		Linear ds DNA	T3, T7
Microviridae		Circular ss DNA	Ø x 174
Inoviridae		Circular ss DNA	m13, fd
Leviviridae		linear ss RNA	Qβ, R17, MS2
Cystoviridae		Linear ds RNA	Ø 6.

[a] Single-stranded (ss) or double-stranded (ds) nucleic acid.

TABLE 7.

Some Groups of Plant Viruses

Group (Representative)	**Morphology and Approximate Size**	**Nucleic Acid[a]**
Tobamoviruses		
(Tobacco mosaic virus)	Rigid helices 18 x 300 nm	Linear ss RNA
Potyviruses		
(Potato Y virus)	Flexible Helices 11 x 700 nm	Linear ss RNA
Comoviruses		
(Cowpea mosaic)	Icosahedral with 30 nm diameter	Linear ss RNA
Caulimoviruses		
(Cauliflower mosaic)	Icosahedral with 50 nm diameter	Circular ds DNA

Single-strand (ss) or double-strand (ds) nucleic acid.

The families of viruses infecting vertebrates and plants are given in Fig. 2, 3.

THE FAMILIES OF VIRUSES INFECTING VERTEBRATES

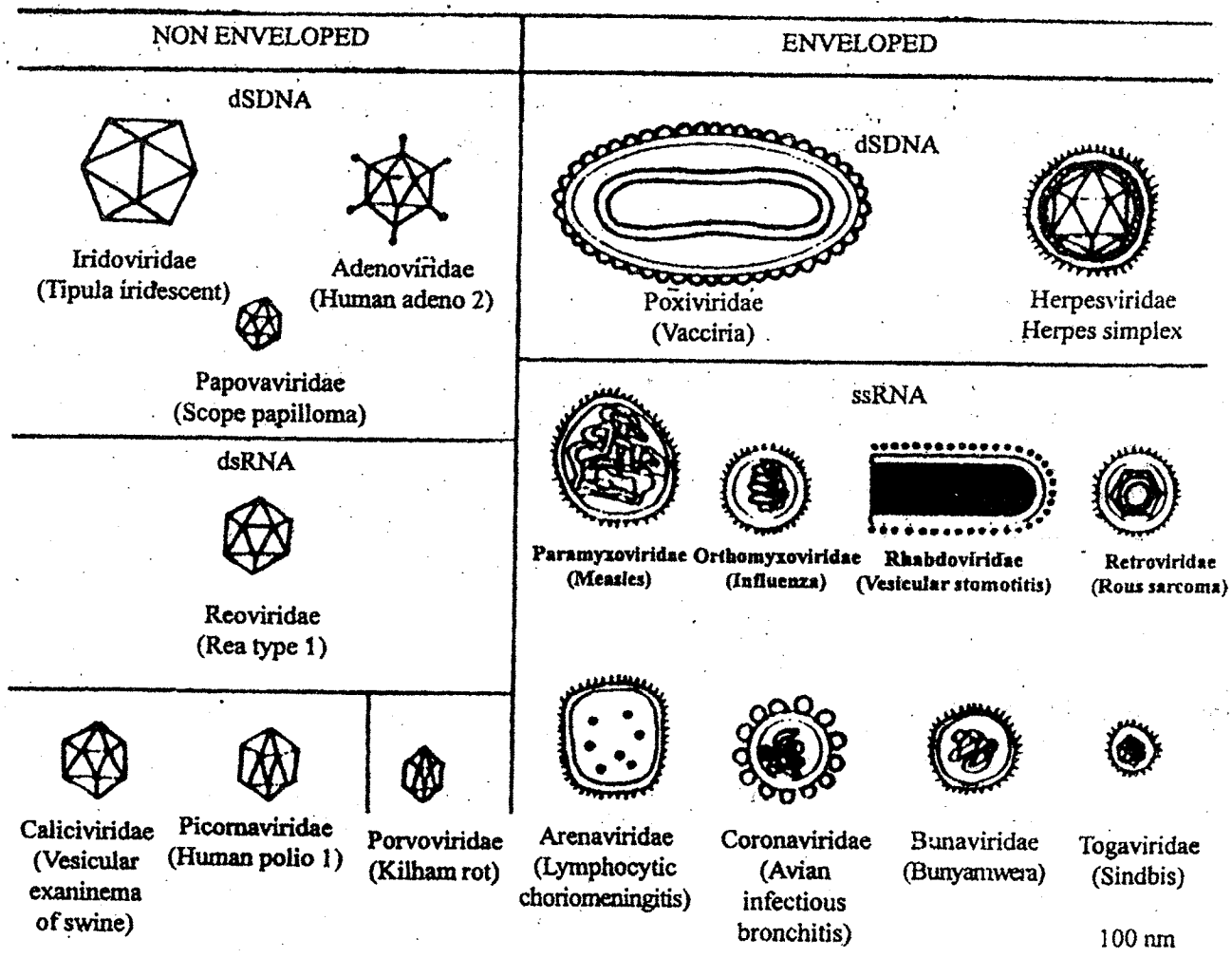


Figure 2.

Line drawings of the families of viruses infecting vertebrates. All diagrams have been drawn to the same scale. For each drawing the family name is given together with a well-known member of it (but the dimensions and shape used for the drawing may not be exactly those of the virus named). (Reproduction from drawings by Mrs. J. Keeling in R.E.F. Matthews, "Classification and Nomenclature of Viruses," Intervirology, 17 : 1-99, 1982. By permission from S. Karger AG, Basel, Switzerland).

THE FAMILIES AND GROUPS OF VIRUSES INFECTING PLANTS

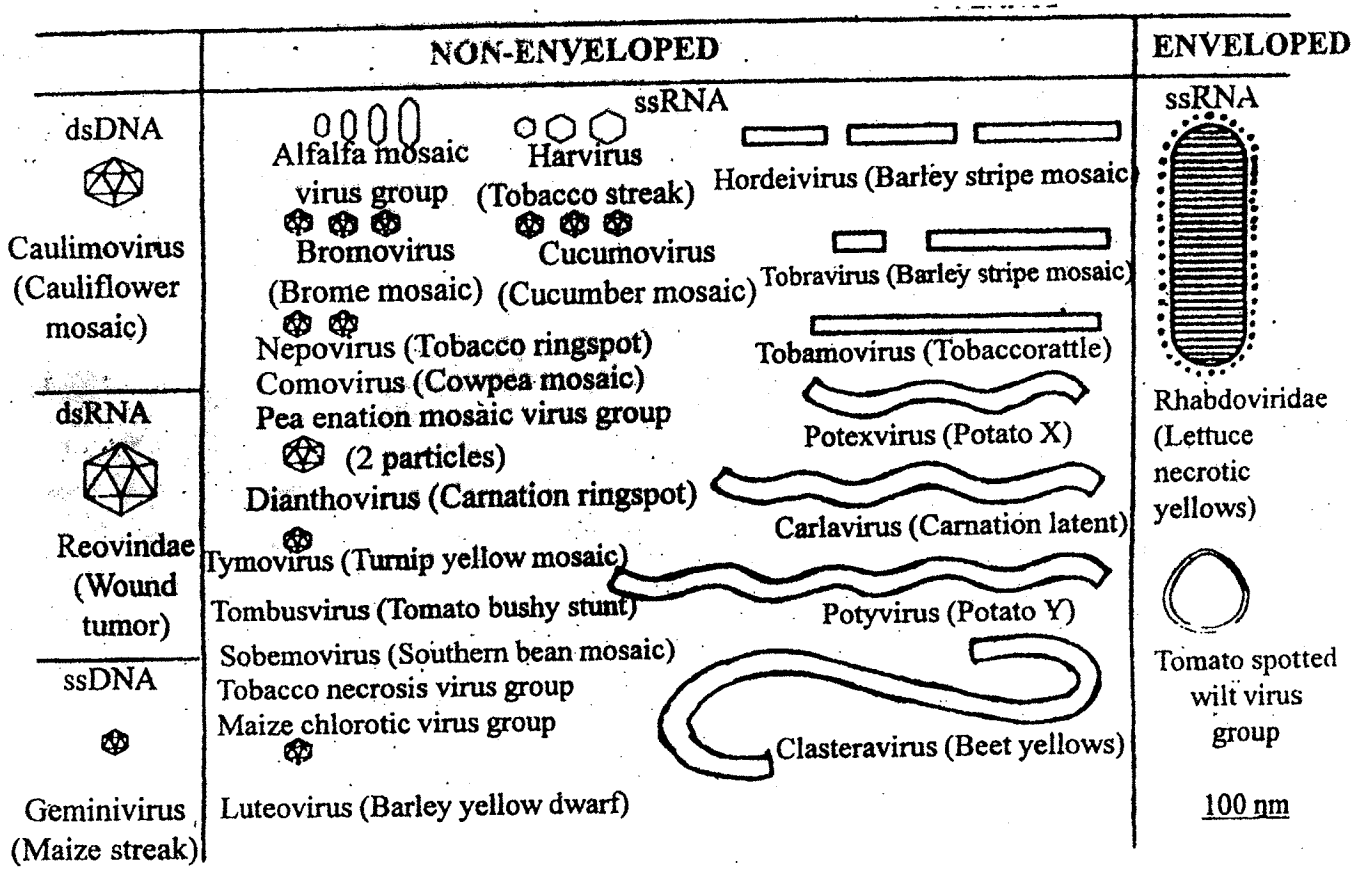


Figure 3.

Line drawings of the families and groups of viruses infecting plants. All diagrams have been drawn to the same scale. For each drawing the group name is given together with a well-known member of it (but the dimensions and shape used for the drawing may not be exactly those of the virus named). (Reproduced from drawings by Mrs. J. Keeling in R.E.F. Matthews, "Classifications and Nomenclature of Viruses, "Intervirology, 17 : 1-99, 1982. By permission from S. Karger AG, Basel, Switzerland.

Identification of Viruses:

Identification of Viruses from clinical specimens or materials for research purposes can be accomplished by a number of different methods, but no single technique is satisfactory for all viruses or every kind of specimen. The first step in laboratory identification of a virus is the proper collection and care

of specimens until susceptible animals tissue cultures, embryonated eggs or other appropriate media are inoculated. This includes making the specimen bacteria-free by filtration, differential centrifugation or treatment with bactericidal agent.

If a virus is present, characteristic antibodies i.e. hemaglutination inhibiting, Complement-fixing, or neutralizing antibodies may be produced. Next to that serological test are performed to identify these antibodies.

Cultivation of Animal Viruses:

Chicken embryo technique : It is one of the most economical and convinient methods for cultivating a wide variety of animal viruses. **(Fig.4).**

Figure – 4. *Embryonated hen's egg is used for the cultivation of many mammalian viruses.*

Fig. 4

Chick embryos contain several different types of cells in which various viruses will replicate. By using embroys of various eggs and different methods of inoculation. **(Fig. 5).**

Figure – 5. *Diagrammatic representation in sagittal section of the embryonated hen's egg 10 to 12 days old. The hypodermic needles show the routes of inoculation of the yolk sac, allantoic cavity, and embryo (head). The chorioallantoic membrane is inoculated after it has been dropped by removing the air from the air sac.*

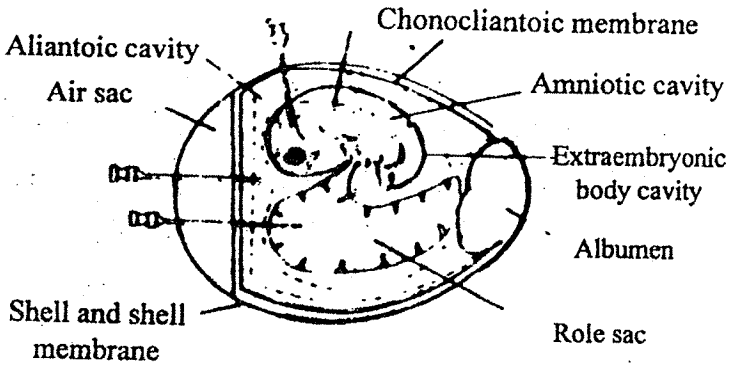


Fig. 5

It is possible to grow the type of virus desired. For example Vaccinia virus can be grown on the chorioallantoic membrane and produce lession or pocks. The yolk sac and the embryo sac can be used to grow viruses. The chick embryo technique has been used in the production of vaccines against small pox, yellow fever, influenza, and other diseases.

Tissue Cultures.

Cell cultures are to day the method of choice for the propagation of viruses for many reasons (convenience, relative economy of maintenance compared to animals, observable cytopathic effects). On the basis of their origin and characteristics cell culture are of three types **Primary Cell Culture, double cell strain and continuous cell lines.** Primary cell cultures are obtained from normal tissue of an animal (Chicken or monkey tissue). When cells from these tissues are prepared and cultured, the first monolayer is referred to as a primary culture. (A monolayer is a confluent layer of cells covering the surface of a culture Vessel). The cell from subcultures are called **secondary cultures**. Cell cultures prepared from fresh tissue resemble more closely the cells in the whole animal than do the cells in continuous cell line unfortunately, cells derived in this manner can be subcultured on a limited number of times before dying.

Diploid cell strains are derived from primary cell cultures established from particular type of tissue such as lung of kidney, which is of embryonic origin. They are of a single cell type and can undergo 50 to 100 divisions before dying, they possess the normal diploid karyotype. Such diploid cell spains are the host of choice for many viral studies, especially in the production of human vaccine virus. Vaccines prepared from tissue cultures have an advantage over those prepared from embryonated chicken eggs in minimizing the possibility of patient developing hypersensitivity or allergy to egg albumen. (The salk polio myelitis Vaccine is prepared through such tissue culture method.)

Continuous cell line appear to be capable of an infinite number of doublings such cell lines may arise with the mutation of cell strain, or more commonly from the establishment of cell cultures from malignant tissue. The karyotype of these cells is aneuploid (a variable multiple of haploid chromosome number) and not diploid. Such cells obviously are not used in virus production for human vaccine.

It has been seen that the tissue structure deteriorates as the virus multiplies. This deterioration is called the cytopathic effect (CPE).

Isolation and Purification of

PLANT VIRUSES

(Modified after Markham and Smith 1949)

1st Step : Infected plant materials or tissue and cell cultures harbouring virus particles are collected.

2nd Step : The infected tissue are then cut into small pieces and then homogenized by any available means. A suitable buffer is used as the extracting medium.

3rd Step : Extract is filtered through a fine piece of cloth to separate cell debris.

4th Step : The filtrate is then centrifuged at low speed (2500 x g) to remove remaining cell debris.

5th Step : Soluble impurities like plant protein and gummy substances are being removed from the crude preparation by precipitation [by heating at 55°C] [or by treating the suspension with a specified amount of ethanol].

OR

by treating with chloroform and centrifuging the mixture at low speed 92500 x g) for 20 mins. Proteins mostly go into chloroform layer or are aggregate at the interface and the aqueous layers contains viruses.

6th Step : The semi purified preparation thus obtained can be further purified by precipitation of the virus particle with 30% NH_3SO_4 or with cold ethanol. Saturated ammonium sulphate solution is added to the suspension till the final concentration reaches 30% level. Proteinaceous virus particles precipitated along with other protein impurities. These are then collected by centrifugation if desired process may repeated by resuspending it in the some buffer and reprecipitated in the same manner.

The pellet obtained.

7th Step : The pellet ultimately dialysed against a suitable buffer to remove excess of ions. The dialysate is then centrifuged at 6000 x g and the pellet obtained resuspended in the same buffer.

8th Step : The considerably purified virus preparation is then further purified on a suitable chromatographic material in a column like that of $CaPO_4$ or Dowex I, and then reclaiming them by means of elutant or washing solution. Most of the impurities removed by this technique. Protein impurities, of course get absorbed on the column.

9th Step : The virus particles present in the washing solution may then be passed through a membrane filter capable of retaining viruses.

10th Step : Next to that such retained particles can easily resuspended in a small volume of the buffer. This suspension may again reprecipitated with ammonium sulphate. The precipitate collected by centrifugation has considerably purified virus particles.

Repeated and careful precipitation in cold temperature conditions are likely to yield crystalline preparation these may be further be purified by density gradient ultra-centrifugation.

If should be remembered that the same processes is not equally effective in the isolation of plant viruses from different sources.

CULTIVATION OF PLANT VIRUSES
Through Plant Tissue Culture

Dr. P.R. White (1934) was the first to grow plant viruses in tissue culture. He could successfully cultivate tobacco mosaic virus in excised tomato root tips. It has since been widely practised by plant virologists all over the world. Method is as follows :

(1) A systematically virus infected tomato plant is taken. Its stem is cut up into segments which are washed thoroughly and are suspended by threads in conical flasks containing little water.

(2) Care will be taken to see that the segments toughed neither the water nor the side walls. The segments are allowed to develop roots.

(3) After a period of time the root tips are removed and placed in a flask containing 50 ml of a suitable nutrient medium. The chemical composition of one such medium is as follows :

$Ca(NO_3)_2$	_____	0.60 millimoles
$MgSO_4$	_____	0.30 millimoles
KNO_3	_____	0.80 millimoles
KCl	_____	0.87 millimoles
KH_2PO_4	_____	0.09 millimoles
$Fe_2(SO_4)_3$	_____	0.006 millimoles
Sucrose	_____	2 percent
Yeast extract	_____	0.01 percent

(4) The tips are allowed to grow for about a week. Afterwards, the surviving cultures are cut into uniform length of about 1 c.m. These are then subcultured in fresh medium as before.

(5) The root tips of the subcultures are removed and one of two of these are selected and stocked for further subculturing viruses are found to be multiplied in these tips and could be used for studies taken up subsequently.

A more recent development has been the use of isolated plant protoplasts for culturing plant viruses. This method has the additional advantages in that protoplasts viruses can be uniformly and synchronously cultivated.

Certain conditions must be satisfied in order to achieve successful protoplast infection:

(i) Protoplast should be freshly washed with 0.7M mannitol immediately before adding the inoculum.

(ii) Poly-I-ornithine should be added to the inoculum.

(iii) pH and osmolarity during inoculation should be with in acceptable limits.

(iv) After infection takes place the protoplast should be washed to remove excess virus and inoculation medium, and then resuspended in incubation medium.

A simplified method of obtaining tobacco protoplast for infection of TMV has been described by Kassanis and White (1974).

Method is as follows :-

Tobacco Leaf

↓

Removed lower epidermis

↓

Incubate the left
over night with 0.3-0.4%
Maceroenzyme and 0.6 – 1.2%
Cellulase

↓

Good yield of Protoplast

↓

Susceptible to infection by TMV.

ENUMERATION OF VIRUSES

(i) **Counting by Direct Electron Microscopy :**

Counting is done by Latex droplet method. The viral suspension to be counted is mixed with polysterene latex particles of known concentration. The mixture is then sprayed in droplets on a supporting membrane, dried and shadowed. The droplet will be visible as many small particles and a few larger ones. The smaller one are virus and the larger ones the latex particles. (Fig.6).

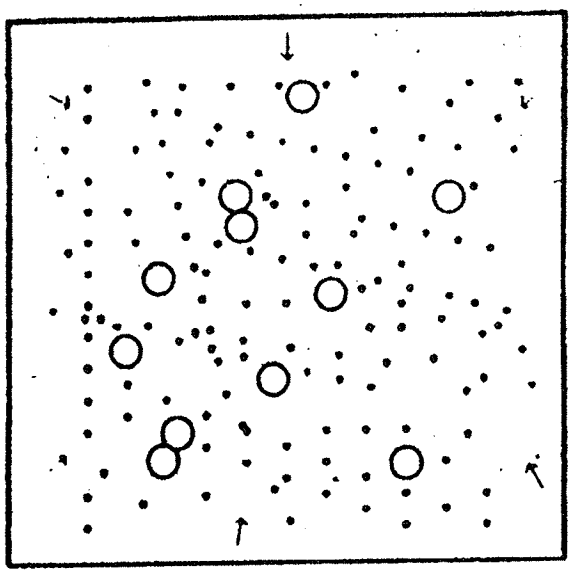


Fig. 6

Figure.6 *Enumeration of viruses by latex method. The viral suspension to be counted is mixed with polysterene latex particles of known concentration. The mixture is then sprayed in droplets on a supporting membrane, cried and shadowed. The figure shows one such droplet. This contains many small particles and a few larger ones. The smaller ones are viruses and the larger ones the latex particles.*

There are 220 viral and 11 latex particles in the droplet. The latex concentration per ml in the original sample was $3.2 + 10^{10}$. Therefore, the number of virus particles in the sample is $220/11 \times 3.2 \times 10^{10} = 6.4 \times 10^{11}$ particles per ml. Average of several droplets gives a reliable estimate.

(ii) Plaque Assay :-

In this method a suspension culture of appropriate host cell is prepared initially and later cultured in petri plates containing suitable agars based media such cultures are then inoculated with aliquots of the titre to be enumerated. On proper incubation virus particles start infecting the host cells. One susceptible host cell is infected by single virion. After some time, disintegration of the host cells occur resulting in liberation of virus particles. These, in turn, infect host cells in the immediate vicinity and destroy them as well. But the other host cells those are not infected continue to grow and divide normally. Thus, soon transparent, visible, circular areas appear on the beneficial lown in the plate. These are called plaques. Number of plaques produced per ml. of viral broth indicates its population concentration. It is most effective in the counting of bacterial viruses. (Fig. 7).

Figure 7. *Plaques formed by bacteriophage T2. Courtesy of G.S. Stent.*

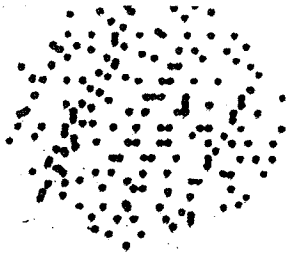


Figure 7

(iii) Acid End point Method:

It is well known that vigorously grown tissue or host cell suspension turns acidic the medium in which they are growing. This feature is taken advantage of in this method. It involves measurement of any change in the acidity of the suspension and correlation of the data with standards prepared earlier. Here, the idea is to determine the highest dilution of a suspension which would be capable of causing infection. Thus a medium which is not acidic to the normal level would indicate the presence of dead cells. When virus particles are cultivated with the tissue suspensions, death could be due to viral infection alone.

For counting infections virus particles present in a particular suspension, initially, it is diluted, let to say, 10^4 and 10^5 times. Both the dilutions are then made to infect identical host cell suspensions. In former case no acidity developed whereas with the latter the medium become acidic to the normal level. So it indicates 10^5 times dilution of virus particles does not contain even a single virion, on the other hand 10^4 dilution contained at least one virion. Therefore the original suspension had between 10^4 and 10^5 virions.

(iv) Haemagglutinin Assay:-

Many Viruses are capable of agglutinating red blood cells. This phenomenon is known as agglutination and the agent responsible, Haemagglutinin. A virus particle attaches simultaneously to two red blood cells and bridges them. Such formation of aggregates can be detected in a number of ways and afford a simple method of enumeration.

Most commonly used haemagglutinin assay procedure is the pattern method.

In this method sample of viral solution is diluted serially by using twofold steps (10, 20, 40, 80, 160 and so on). .5 ml of each dilution was mixed with an equal volume of red blood cells suspension (10^7/ml.) Each mixture was placed in a cup drilled in a clean plastic plate and left for 30 minutes at room temperature. Each assay is done in duplicate. Viral sample caused complete haemagglutination until dilution 80. This meant that these dilution had just enough viruses to agglutinate the blood

cells present. Now the member of red blood cells per ml of the reaction mixture is 10^7. Since one virus particle is capable of agglutinating two blood cells simultaneously, the number of viruses required for agglutinating 10^7 cells would 5×10^6. Therefore, sample of viral solution has $80 \times 5 \times 10^6$ virus particles. However the population are expressed simply in haemagglutinin units, i.e. viral samples has 80 units (Fig.8).

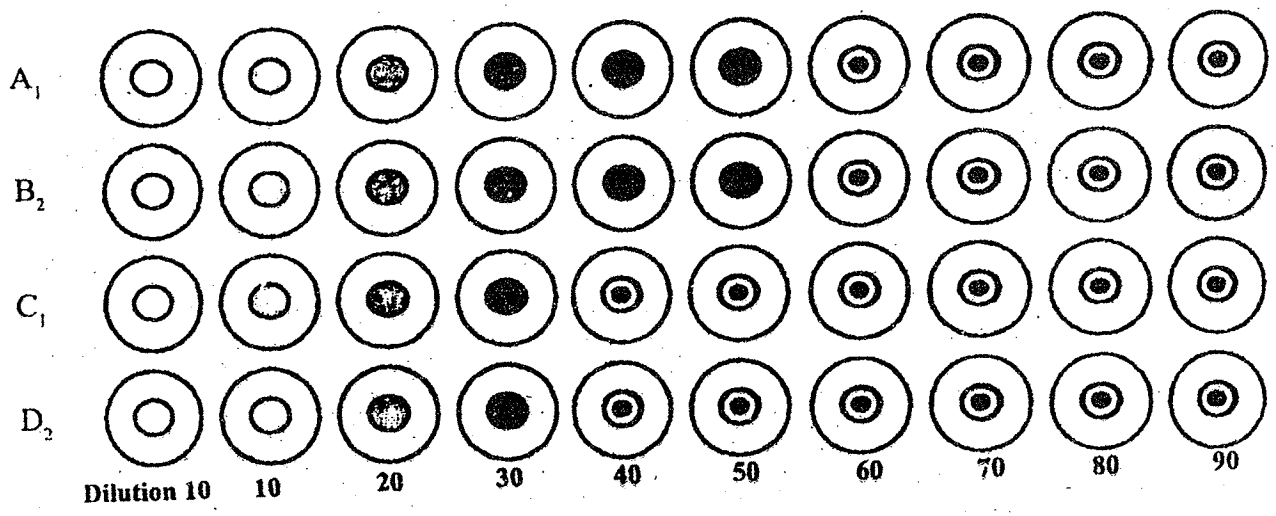


Figure – 8. *Counting of viruses by haemagglutination assay. The method depicted here is the pattern method which is one of the several in use. Two samples A and B were diluted serially by using two-fold steps (10, 20, 40, 80 and so on.). 0.5 ml of each dilution was mixed with an equal volume of red blood cells suspension (10^7/ml). Each mixture was placed in a cup drilled in a clean plastic plate and left for 30 minutes at room temperature. Each assay was done in duplicate. Sample A caused complete haemagglutination until dilution 320. Sample B did the same until dilution 80. This meant that these dilution had just enough viruses to agglutinate the blood cells present. Now the number of red blood cells per ml of the reaction mixture was 10^7 cells would 5×10^4. Therefore, sample A had $320 \times 5 \times 10^4$ virus particles and sample B, $80 \times 5 \times 10^4$ particles. However, the populations are expressed simply in haemagglutinin units. Accordingly, samples A and B had 320 and 80 units respectively.*

REPLICATION OF VIRUSES

- Viral replication cycle.
- Replication of chromosome of DNA viruses.
- Replication of chromosome of RNA viruses.
- Replication of Retroviruses.
- Lysogeny.
- VIROIDS
- PRIONS.

Virus particles outside a host cell have no independent metabolic activity and are incapable of reproduction by processes characteristic of other micro-organisms. Maltiplication takes place by replication, in which the virus protein and nucleic acid components are reproduce within susceptible host cells.

The entire process can be generalised as follows :

ADSORPTION	Attachment of virion at specific site of susceptible host cell.
	↓
PENETRACTION	Either whole virus or viral nucleic acid penetrates to the inside of the cell.
	↓
UNCOATING	Uncoating of the virus and release of the nucleic acid.
	↓
REPLICATION & BIOSYNTHESIS	Replication of the viral nucleic acid & Bio synthesis of other component (with in the cytoplasm, the nucleus or both).
	↓
ASEMBLY	Protein & nucleic acid components are assembled into virus particles.
	↓
RELEASE	Release from the host cell.

ABSORBTION : It occurs in two steps.

1st Step : Preliminary attachment by ionic bonds or charges and easily reversed by a shift in pH or salt Concentration.

2nd Step : Appears to involve firmer more specific attachment and to be irreversible.

Molecular entities on the surface of cells acts as receptors for viruses, interacting with specific proteins on the capsids of naked virions or on the envelops of enveloped virion. In contrast to the marked specificities of adsorption of animal and bacterial viruses, plant viruses do not seem to require specific receptor sites.

Penetration and Uncoating:

In case of animal viruses engulfment of the whole virions by the cell in a phagocytic process called **Viropexis** followed by uncoating or removal of the capsid. This takes place in the phagocytic vacuoles and is due to the action of enzyme called lysosomal proteases. In other mechanism the virallipoprotein envelope fuse with the host cell membrane. The fusion results in the release of the viral nucleocapsid material in to the cytoplasm of the cell. Uncoating again occurs with in the host cell.

Plant viruses penetrate host cell through transient pores (called ectodesmata) which protrude through cell wall at intervals and communicate to the exterior of the cell. Whole virus particles are engulfed at this points. Also insects can inoculate plant viruses into cells during feeding.

Some times this is a purely mechanical process, at other times the viruses is found in the insect tissue and may even multiply there. Insect feeding is probably the most important means of plant virus transfer in nature.

Replication and biosynthesis of virus specific molecules

Replication of DNA Viruses:-

Viruses contain either DNA or RNA. In those with DNA, it is present within the virion either as a circular or a linear molecule, which is termed the viral chromosome. In some groups of viruses, chromosomes are single stranded DNA, in others they are double-stranded DNA. Most DNA viruses double stranded DNA chromosome but one group of animal viruses the parvoviruses possess single stranded linear DNA.

In most cases linear DNA chromosomes are circularized within the host cell by one of two distinct mechanism depending on whether the linear molecule have cohesive ends or terminal returdancy. Cohesive ends are short single stranded regions that are complementary to each other (Fig.9) with in host cells these anneal to form a circular molecule with single nick in each DNA strand. Nicks are then sealed by DNA ligase. Linear DNA chromosomes with terminal retundancy circularize the recombination within homologous terminal regions **(Fig.10).**

Initial replication of the circular viral chromosome begins at a specific site and proceeds in both direction around the molecule by a process similar to that by which the bacterial chromosome is replicated.

Subscquent replications in many cases occur by a process termed **rolling circle replication, (Fig.11).** A nick in one of the DNA strands is made by a specific endonuclease and 3'OH end of the nicked strand (the primer) is extended by addition of nucleotides, the intact complementary strand serve as the template. The 5' end thus displaced and later duplicated.

In this manner a double stranded molecule is produced that can be much larger than the circumference of viral chromosome. Such molecules termed **concatamers**, are cleaved later to produce the chromosome of progeny of virions.

In some cases circularization does not occur prior to replication. The linear molecule is repetitively replicated to produce a number of identical molecules which recombine to generate **concatamers**. These are cleaved by an endonuclease in to terminally redundant chromosome.

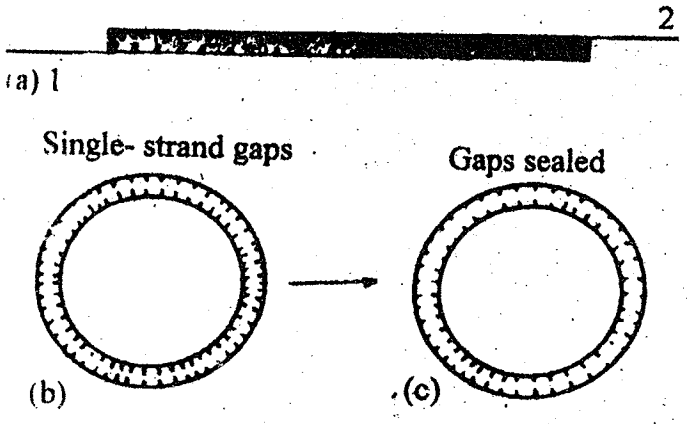


Figure 9

Figure 9. *The conversion of the λ genome from the linear form to the circular form (a) The linear genome has single-stranded regions at each and that are complementary to each other. (b) The single-stranded regions are joined by hydrogen bounds between complementary bases. (c) The sugar-phosphate backbones are joined by polynucleotide ligase forming a fully covalent circle.*

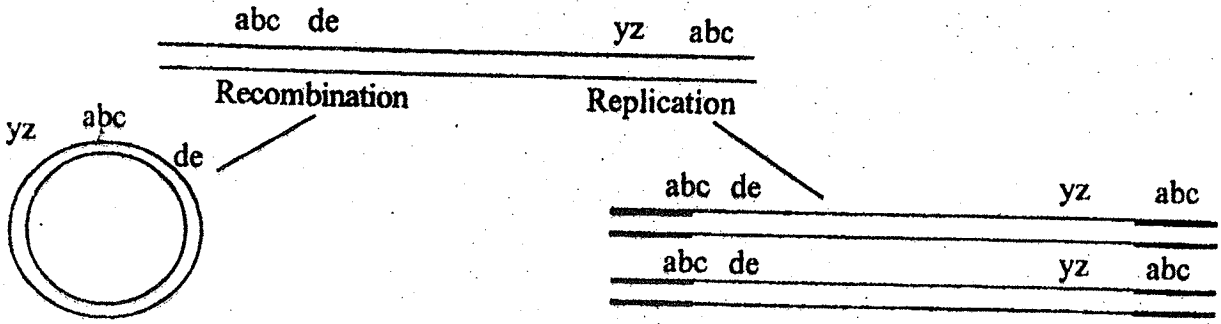


Fig. 10

Figure 10. Alternative strategies for replication of linear double-stranded DNA chromosomes that possess terminal redundancy. In pathway (a) recombination occurs within the region of redundancy. In pathway (b) the chromosome is first replicated producing molecules that can recombine to generate concatemers.

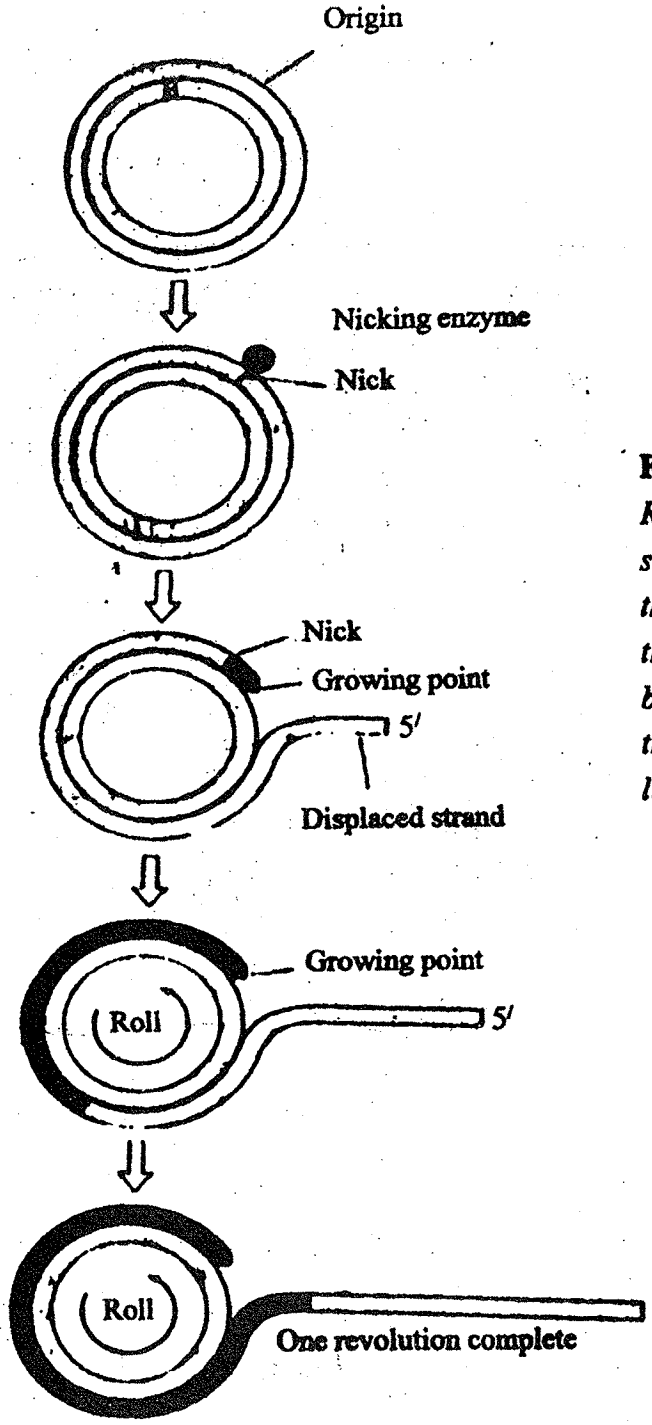


Figure 11. ***Rolling circle replication. Replication bagins at the origin by nicking one strand of DNA in Ø x 174, the nicked strand is the plus strand and the gene A protein makes the nick). After one new progeny strand has been synthesized (one revolution of the circle), the gene A protein cleaves the new strand and ligates its two ends.***

Fig. 11

Replication of chromosome of RNA Viruses

The chromosome of RNA Virus are replicated through double stranded RNA intermediates, [exception – In retroviruses the chromosome of which are replicated through DNA intermediates].

In the case of virions containing (+) strand RNA, the chromosome is translated immediately after it enters the host cell, one of the proteins thus produced is a **replicase** (RNA dependent RNA polymerase) which is not present in the uninfected host. Replicase catalyzes the synthesis of an RNA molecule term a **minus strand** that is complementary to the chromosome. The viral chromosome is thus replicated in two stages (Fig.12). First the (-) strand is synthesize, forming a double stranded molecule termed replicative form (RF). Then using the minus strand as a template, **replicase catalases** the synthesis of new copies of the plus strand. Virus chromosome composed of (-) strand or double stranded RNA cannot be translated because they lack ribosome binding sites. All virions that contain such chromosomes also contain replicase molecules, which enter the host cell along with the chromosomes. These enzyme catalyze the replication of the viral chromosome through a double stranded replicative intermediate. In these viruses additional plus strand are synthesized from the replicative intermediates to serve as mRNA for synthesis of viral proteins.

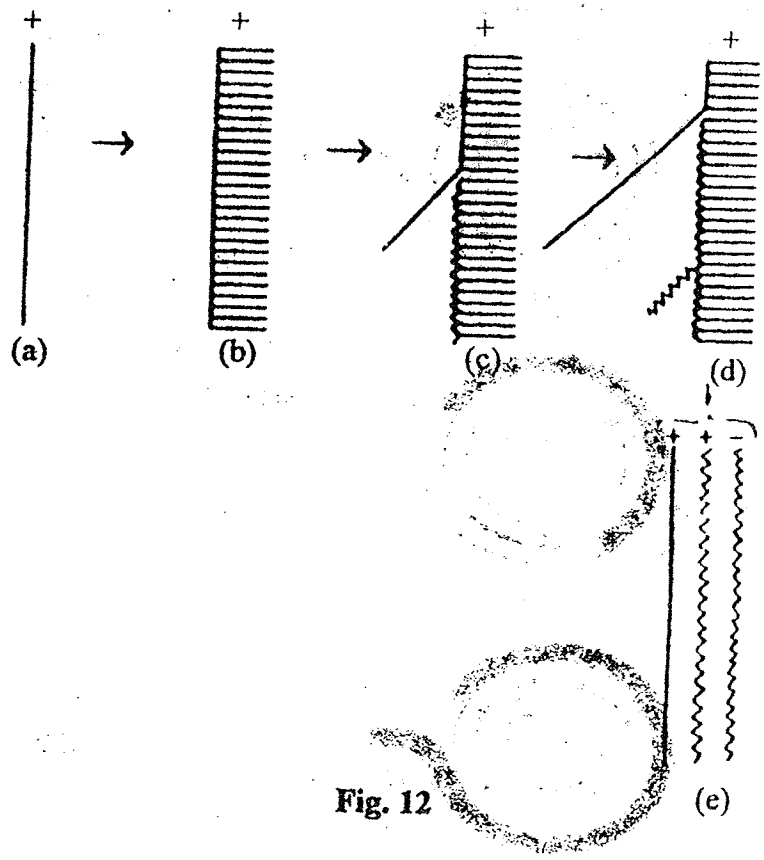


Figure. 12. *The replication of an RNA virus. (a) The infecting parental strand is labelled (+). (b) Viral RNA replicases converts the parental strand to a double-stranded intermediate. (c,d) Then replicase uses the double-stranded intermediate as a template to synthesize a succession of (+) progeny strands. (e) The displaced parental and progeny strands are either incorporated into virions or used by the replicase to form new double-stranded intermediates.*

Fig. 12

Replication of Retroviruses:

Retroviruses are enveloped RNA virus with highly unusual replication cycle. Upon entering the cytoplasm of a host cell the enzyme reverse transcriptase which is brought into the cell inside virions, first synthesizes a strand of DNA that is complementary to the viral (plus strand) RNA. Next reverse transcriptase synthesizes a second strand of DNA, but this one is complementary to the first DNA Strand synthesized (Fig. 13). The two strand form a circular double stranded chromosome that migrates to the nucleres and

becomes integrated into host chromosome. Then the RNA polymerase of the host transcribes the integrated viral genes, producing viral RNA for incorporation into virion [example – Human T cell leukemia Virus (I, II, III) (HTLV)].

HTLV-III is at least in part the cause of acquired immunodeficiency syndroms (AIDS).

Figure 13

Replication of the retroviral chromosome (a) in the cytoplasm, the RNA chromosome is copies by the enzyme, reverse transcriptase, that is contained within the virion (b) Reverse transcriptase then synthesizes a strand of DNA that is complementary to the first DNA strand. In the process the RNA chromosome is displaced, and the double-stranded DNA chromosome becomes circular. (c) The viral DNA chromosome migrates into the nucleus where it becomes linearly integrated in a host chromosome by a mechanism that may be similar to integration of the temperature bacteriophage mu (Chapter – 9).

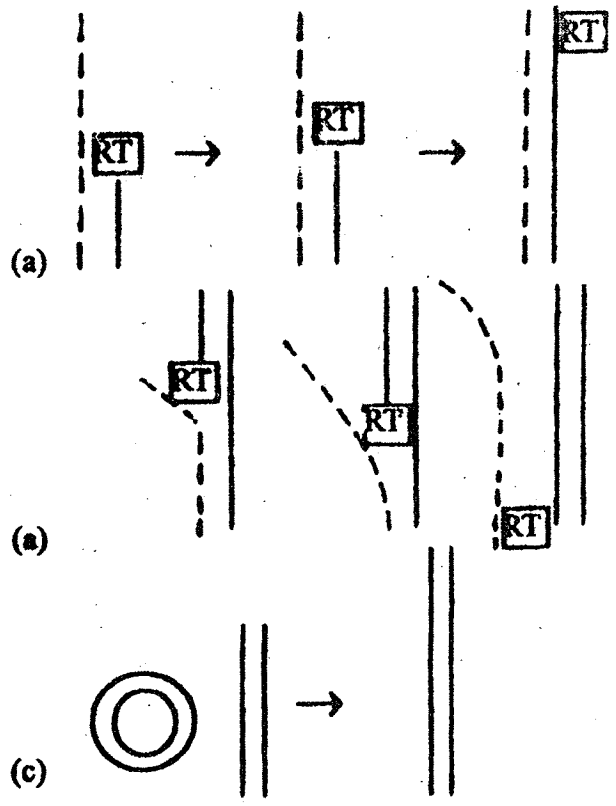


Fig. 12

Virion Assembly:

Assembly of virions begins after capsid proteins and viral nucleic acid accumulate in the host cell. In TMV and related helical viruses assembly is a relatively simple process in which capsid proteins associate with the viral chromosome and wind into a helix. No assistance from the host cell is required for TMV assembly as it will occur spontaneously in vitro when capsid proteins and nucleic acid are mixed.

Assembly of icosahedral and binal viruses is some what different from that of helical virions, in most cases, capsid proteins assemble to form an empty structure termed Procapsid that has the shape and size of the capsid (or of the head in the case of binal phages). Then viral nucleic acid enters this structure

and becomes condensed into a densely packed state. In the case of icosahedral viruses the procapsid is then sealed becoming impenetrable to large molecules. In the binal phages tails are assembled separately and are then joined to filled heads.

In most cases, the final step in assembly of an enveloped virus is the acquisition of a pieced of host membrane that wraps around the viral nucleoprotein core as it passes through the intact host membrane. Before this step can occur, virus encoded proteins aggregate in the cytoplasmic or the inner nuclear membrane. In the case of enveloped animal viruses, viral membrane proteins are synthesized by ribosome bound to the rough endoplasmic reticulum and become embeded in the adjacent membrane as they are synthesized. These proteins are usually glycosylated by enzymes present within the endoplasmic reticulum. In the case of enveloped RNA viruses, viral membrane protein migrates to the cytoplasmic membrane by way of the golgi apparatus. The nucleoprotein core of the virion then migrates to the inner surface of the membrane and becomes enveloped in membrane containing viral proteins as it leaves the cell by a process termed budding **(Fig. 14).**

In the case of enveloped DNA viruses, the membrane protein migrates to the inner nuclear membrane where neucleocapsids assembled in the nucleus associate with areas of inner nuclear membrane that are embeded with viral membrane proteins. This membranes envelops the nucleoprotein core, and the virion leaves the nucleus. The mature virion migrates into the endoplasmic reticulum.

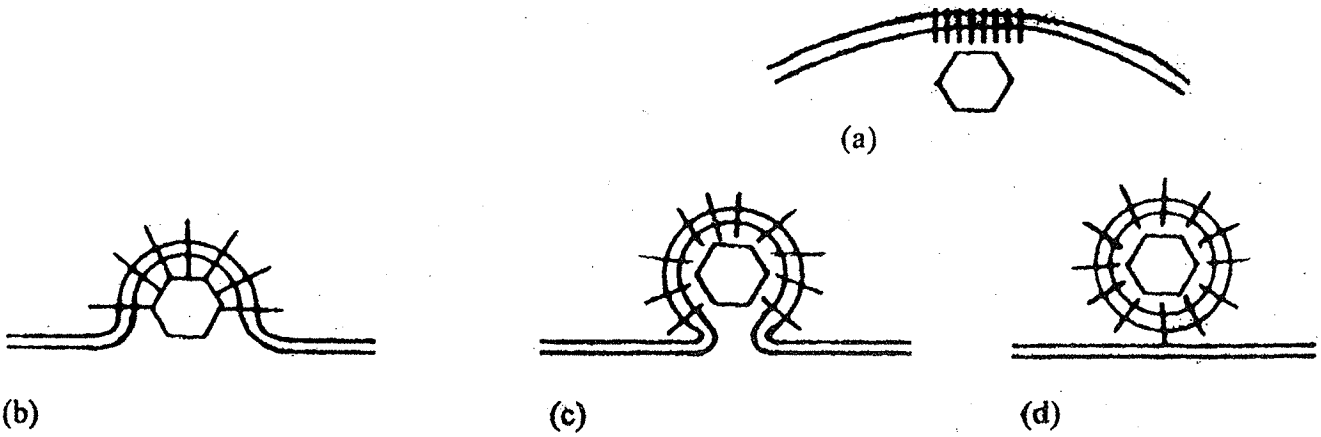


Figure : 14.

Diagram of the budding process as virion nucleoprotein associated with a region of membrane embedded with viral envelope protein. The viral core is wrapped entirely in the membrane, which pinches off to release the mature virion.

Escape:

Enveloped RNA viruses and filamentous bacteriophages escape from the cell as a part of the final

assembly of the virion. Enveloped DNA viruses can migrate from the endoplasmic reticulum into vesicles from which they escape as a consequence of exocytosis. Some non enveloped animal viruses (adenoviruses) pass directly through the cytoplasmic membrane without apparent injury to the host cell. However, many animal and plant viruses kill the host cell and are thereby released by the autolysis that follows cell death. Most phages escape by causing the host bacteria to lyse. In some cases, a phase gene expressed during late phases encodes lysozyme, an enzyme that cleans glycoside bonds in peptidoglycan. In some other cases, a phage gene encodes an enzyme that cleaves the peptide cross line in peptidoglycan. But in many cases the cause of lysis is not known.

Lysogeny:

After the discovery of bacteriophages, some bacterial strains termed **Lysogenic strains** or **Lysogens** were found that produced phage virions spontaneously during growth of the culture, but the majority of cells in the culture were unaffected by these virions, phages produced by **lysoghic strains** are termed **temperate phages** and the relationship between the phage and the bacterium is termed **Lysogeny**. In Lysogeny the viral DNA of the **temperate phage**, instead of taking over the functions on the cell's gene, is incorporated into the host DNA and becomes a **prophage** in the bacterial chromosome, acting as gene. In this situation the bacterium metabolize and reproduces normally, the viral DNA being transmitted to each daughter cell through all successive generations (Prophage behave as plasmids). Some times however, for reasons unknown, the viral DNA is removed from the host's chromosome and the **lytic cycle** occurs. This process is called spontaneous induction. A change from lysogeny to lysis can sometimes be induced by irradiation with ultraviolet light or by exposure of some chemical.

Phages like T_2 that do not cause lysogeny are termed **virulent**. When a temperate phage virion infects a susceptible **nonlysogenic cell**, one of two possible development cycle ensues either phage replication occurs or the infected cell is converted into a lysogen. Temperate phage normally cannot replicate in the lysogenic strain that produces it, but it can in a closely related strain that is not lysogenic for the particular phage. Resistance of lysogens to the phage that they produce is termed **immunity to super infection**. When a lysogenic bacteria are produced with in the infected and are able to grow, resulting information of a **turbid plaque**.

Lysogeny : Phage λ type

The λ prophage integrates in the host chromosome between two sets of bacterial genes, the 'gal' genes (encode enzyme that degrade galactose) and the 'bio' genes (encode biotin synthesis). Most temperate phage resemble phage λ in that they integrate into the bacterial chromosome at specific site but there are exceptions. Temperate phage mu, has the unusual ability to integrate anywhere in the bacterial chromosome.

Integration of the λ chromosome requires the action of protein termed integrase encoded by a λ gene and occurs at a 13 base pair region of DNA sequence homology between the bacterial and phage

chromosome. Following alignment of these regions of homology, integrate catalyzes a reciprocal cross over between the chromosomes resulting in an integrated prophage (Fig. 15).

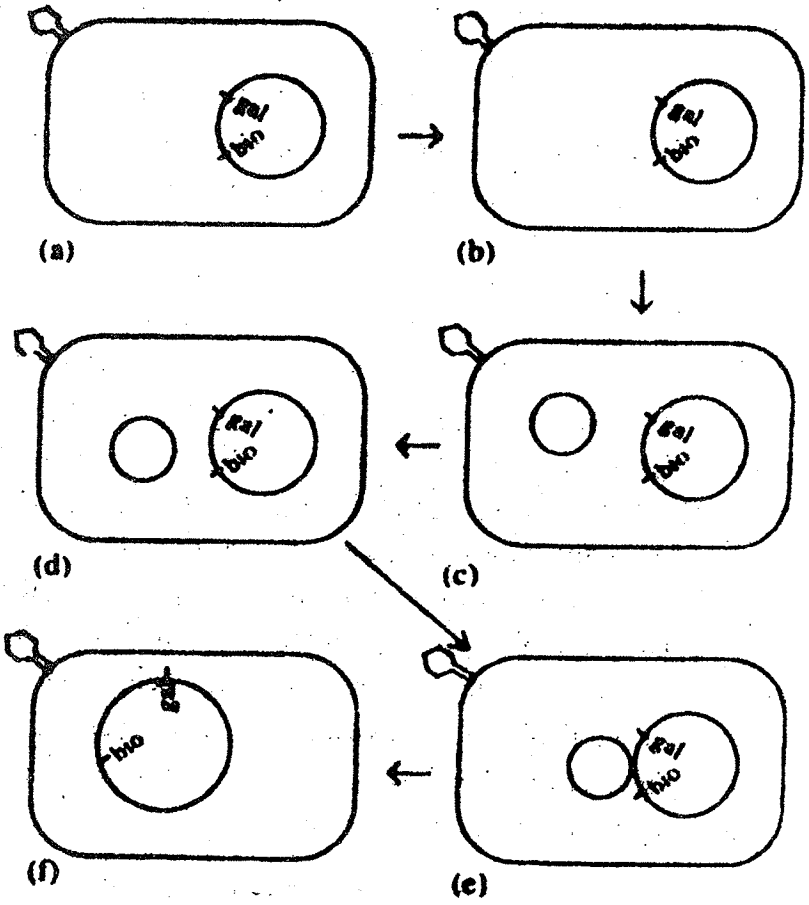


Figure 15.
The formation of λ prophage. (a) Adsorption of the virus. (b) Injection of viral DNA. (c) Circularization of the viral genome. (d) Pairing of homologous regions on the viral and bacterial genomes. (e) A crossover even occurs within the region of pairing. (f) The two genomes have been integrated, forming a single circle. Note that the attachment site for λ is at a specific location, between the loci gal and bio. The specific attachment site on λ is indicated by four short vertical lines on the DNA strand.

Fig. 15

Lysogeny ; Phage P1 type

In lysogeny of the P1 type, viral DNA does not normally become integrated into the host chromosome. Instead the prophage exists as a circular, self replicating double stranded DNA element termed plasmid.

Regulation of lysogeny in Phage λ

Whether lysis or lysogeny follows infection of a susceptible cell by phage λ depends on the relative concentration of two viral-encoded proteins, λ repression and cro protein, produced during early phages of infection. Both proteins can bind to λ DNA at specific sites, termed the right operator (OR) and the left operator (OL), thereby interfering with binding of RNA polymerase to adjacent proteins. By this mechanism, both repression and cro protein can stop early phases of gene expression (Fig. 16).

Binding on one site with in the right operator (ORI), prevents expression of a set of genes including the one (Cro) that encodes the croprotein, and binding to another, OR3, prevents expression of the gene that encodes repressor. However repressor binds preferentially to OR1 and cro protein binds preferentially to OR3. Thus cro protein and repressor compete for their own bio synthesis, each inhibits synthesis of the other. More over binding of a repressor to OR1 facilitates binding of a second repressor molecule to an adjacent site, OR2. When OR3 is unoccupied, repression bind to OR2 facilitates expression of the repressor gene. Thus in the absence of cro protein, repressor stimulates its own synthesis. In addition it shuts off expression of genes required for phage replication.

Following the intracellular accumulation of receptor and cro protein during early gene expression, repression at OR temporarily stops further synthesis of both proteins. At this stage, repressor is usually bound to OR1 and OR2 but cro protein is bound to OR3. Mean while, phage DNA synthesis proceeds, and one or more phage chromosomes usually become integrated into the host chromosome by the action of λ integrate, which is produced during this period. As the host cell continues to enlarge, the intracellular concentrations of both cro protein and repressor and decreased. Thus if cro protein is in relatively low abundance, it will dissociate from OR3 while repressor remains bound to OR1 and OR2 which cause more repressor to be synthesized and lysogeny to be established on the other hand, if the repressor is in relatively low abundance, it dissociated from OR1 allowing expression of cro and all viral genes, the products of which catalyze viral replication and eventual lysis of the host cell.

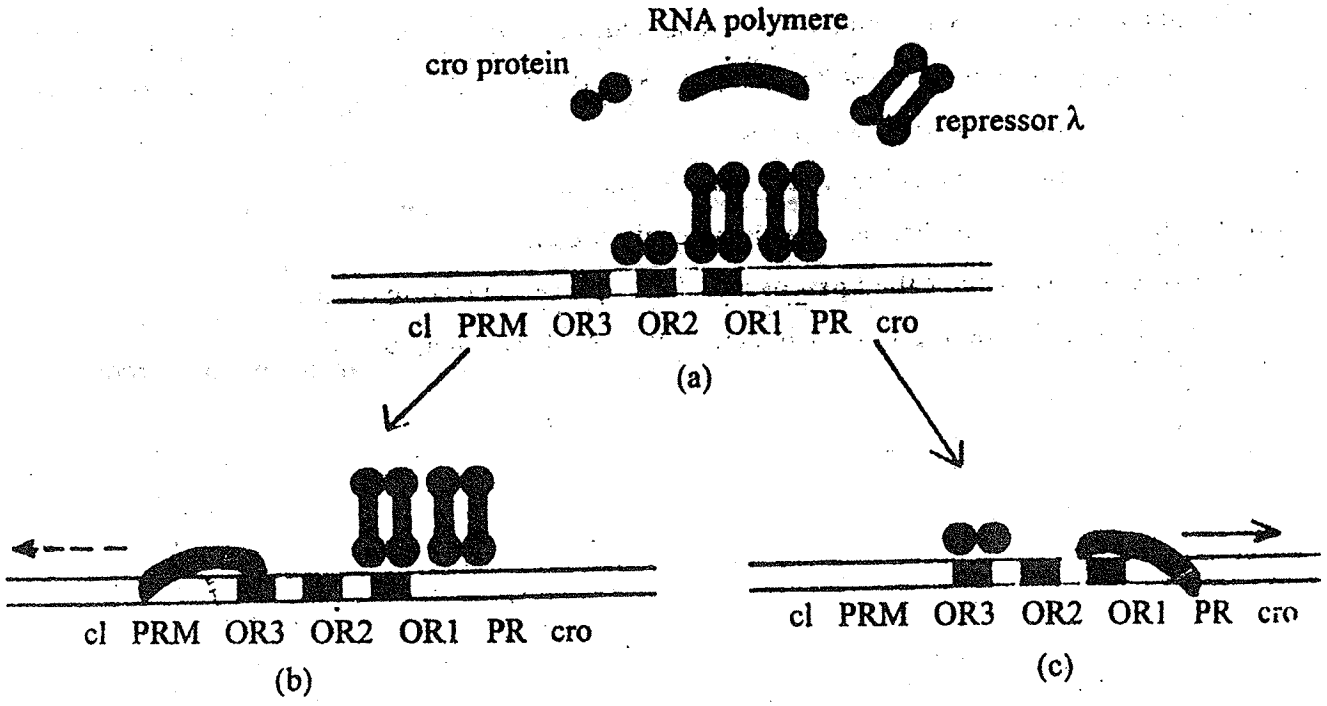


Figure 16.

Regulation of λ by repressor and cro protein (a) Repressor bound to the λ chromosome at site OR1 and OR2, and cro protein bound at OR3, prevent RNA polymerase from binding to this region of DNA. (b) If cro protein dissociates first, RNA polymerase binds to the promoter for repressor maintenance (PRM) and transcribes the repressor gene, cI. This results in an increased concentration of repressor and the establishment of lysogeny. (c) If repressor dissociates first, then RNA polymerase binds to the right promoter and begins transcription of genes cro through J (Figure 9.15), leading to the production of phages and lysis of the cell.

VIROIDS:

A class of extremely simple infections agents, termed viroids, was discovered in 1967. They are merely infections single stranded RNA unassociated with any virion structure. To date, they have been found only to cause disease of plasts. They exist both intracellularly and extracellularly as circular single stranded RNA molecules that contain fewer than 400 nucleotides. Therefore, they are smaller than any known viral chromosome. They are large enough to encode a single small protein, but it is doubtful that they do, because the RNA of viroids lacks the signals (ribosome binding sites, start codons and stop codons) that are necessary for translation of RNA into a protein. It seems probable that a host cell RNA polymerase replicates the viroid chromosome.

PRIONS:

Two human diseases, kuru, and Jakob-creutzfeldt disease and one disease of sheep, scrapic are caused by macromolecules that are small than any known virus. These diseases are characterised by progressive, fatal degeneration of brain tissue. Attempts to purify the causative agents have not been successful, but a preparation that is highly enriched for the causative agents of kuru and scrapic have been obtained. it is largely composed of proteins with molecular weights near 30,000. Infections macro molecules causing these disease have been called prions and if they prove to be composed entirely protein, they will constitute a fundamentally new group of infectious agents. How such an agent might reproduce is, of course, a mystery.

CHAPTER – 3

Methods of transmission and control

In general two principal categories of means of transmission are noted to be operative.

(1) Those mediated through an intermediatary organism called the "**vector**".

(2) The ones transmitted without the intervention of any vectors. (**Table-8**).

TABLE – 8. MODES OF TRANSMISSION NOT MEDIATED BY VECTORS

Disease	*Mode*
Bean Mosaic	Seed
Lettuce mosaic	Seed
Potato leaf curl	Contact
Tobacco mosaic	Man implements
Tobacco necrosis	Soil
Tomato bunch top	Bulbs, rhizomes
Hepatitis	Water
Influenza	Nasal & oral discharge
Myxoma of rabbit	Dust, contact
Poliomyelitis	Faeces, sputum
Rabies	Bites of infected dog, fox
Small pox	Close contact, scales, sputum

Transmission of viral Diseases not Mediated by Vectors

(i) **Air Borne transmission** : for example – (Dried epidermal scale from small pox pustules, Influenza viruses).

(ii) **Transmission Through Contact** : (Potato Virus-x through root)

(iii) **Hereditary or vertical transmission** : for example – (Murine Leucaemic Virus (MLV) Vertically transmitted in mice through mothers milk, chicken pox through the placenta of mother).

Other Modes of Transmission

(i) **Mechanical inoculation** : (Sap or extract of the infected host is used as the inocculum).

(ii) **Grafting between susceptible plants**: (Alfa alfa mosaic virus, TMV Potato Viruses).

(between scion & stock)

Vector mediated transmission

The Vectors of plant viruses mostly belong to the insect order **Hemiptera** (e.g. – Myzus persicae (subgroup Aptridae) act as a vector for more than 50 viruses. These vectors feed by sucking the infected plant sap directly and transmit it to healthy one. (Bean mosaic viruses transmitted in this way).

[for Details consult Book An introduction to viruses by Biswas & Biswas. Page 212-220].

Control of Viral Diseases

Methods of controlling viral diseases could be categorised as (i) Preventive and (ii) Therapeutic or curative.

Prevention of viral diseases : It can be achieved by –

(1) Exclusion of source of infection.
(2) Isolating the source of infection.
(3) Protecting the host from getting infected.
(4) Making the hitherto susceptible host a resistant one.

(1) **Exclusion** :

This method aims at preventing entry of the source of infection into an area where it is naturally non-existent. It can be achieved by –

(i) Application of quanantine laws by the Govt.
(ii) Duty attested health certificates have to be carried by persons moving from one country to another.
(iii) Isolate the person who might be a potential carrier.
(iv) Dispensable plant tissue or plant source must be destroyed.

(2) **Isolating the source of infection**

(a) Isolation of the source of infection prevents its further spread e.g. persons suffering from small pox quaratined at hospitals.
(b) Eradication of dispensable hosts.
(c) Contact with air borne diseases could be avoided by avoiding crowded place like cinema Halls, theatre etc.

(3) **Protection of the host getting infected**

(a) Protect the host organism against any potential infection.
(b) Avoidance of infection.

(4) Immunization

The most widely practised method of preventing a disease from getting established is immunization. This method however, is effective in case of vertebrates. This method aims at making the host system temporarily resistant to disease. [Production of antibodies in response to antigen, vaccination.]

Immunization of plants:

This procedure are hardly applicable to plants as they do not give rise to antibody molecules (i.e. lack of immune response mechanism).

However plants infected by a particular viral strain are known to become resistant to attack virus of the same strain as well as from other strains of the same virus. These procedure however are still in experimental stages.

Eradication of Vector:

Destruction of insect vectors could be achieved by using suitable insecticides. Insects could also be prevented from acting as vectors by adjusting the dates of sowing or planting so that the period of insect activity is avoided and the vector-virus-plant cycle is disturbed.

Breeding Disease Resistant Varieties

This method is potentially the most full proof of all which aims at preventing a disease. This aims at changing the basic nature of the host thereby altering its relationship with the invading virus. The host becomes virus immune. However, this method is a long term one, as its development needs time.

(e.g. – **mosaic disease of sugar cane**) R. Varieties are extremely successful in reducing the adverse effect of the disease.

TREATMENT OF VIRAL DISEASE

PROPHYLAXIS & THERAPEUTIC

To enadicate viral disease, various methods have been adopted :

(1) by chemical agents (2) Physical agents.

Chemotherapy of Viral diseases

The only group of antibiotics known to possess anti-viral activity are the rifamycins capable of repressing RNA dependent RNA and DNA synthesis i.e. particularly effective against RNA Viruses.

Base analogue like 5-fluorodexiunidine are known to inhibit the enzyme thymidilate synthetase and thereby preventing DNA synthesis. A list of analogue & chemicals give in **Table 9, 10.** Other chemical

agents used in antiviral therapy are substituted benzimidasole and guanine derivatives. These have been found to be effective against RNA viruses.

Another compound like isatin beta thiosemi carbazone is effective against pox viruses.

Another chemical agent used chemically is amantadine and its derivative. These are useful prophylactic agents and effective against Influenza and para influenza.

TABLE – 9. CHARACTERISTICS OF SOME ANTIVIRAL BASE ANALOGUES

Compound	*Substitute For*	*Influence*	*Used against*
5-Fluorodeoxyuridine (FUDR)	Thymidine	Inhibits thymidilate synthetase; prevents DNA synthesis	Pox-viruses
5-Bromodeoxyuridine (BUDR)	Thymidine	DNA synthesis; Interferes with maturation of particles	Pox-viruses Herpes simples
5-Iododeoxyuridine (IUDR)	Thymidine	DNA synthesis; Maturation of particles	Pox-viruses simples
Guanidine hydro-chloride	Uridine	Inactivates RNA polymerase	Polioviruses Enteroviruses
5-aza uracil	Uridine	Capsid formation	Tobacco mosaic virus
2-Thio uracid	Uridine	Capsid formation	Turnip yellow mosaic
8-Azo guanine	Uridine	Capsid formation	Peach yellow.

TABLE – 10. CHARACTERISTICS OF SOME ANTIVIRAL CHEMICALS

Compound	*Substitute For*	*Influence*	*Used against*
Isatin beta-Thiosemi carbazone	Uridine	Synthesis of late viral structural proteins; leads to production of defective particles	Pox-Viruses
1-Adamantanamins	Uridine	Uncertains; probably prevents release of viral nucleic acid	Myxoviruses
Polymocinic acid	Uridine	Induces production of Interferous	Myxo; Paramyxi Viruses.

Chemotherapy of Viral diseases of plants

Solution of hydroquinone, urea, Sodium thiosulphate 6-azauracil, 2-thiouracil and 8-azaguanine, all analogues of the RNA base Uracil have been found to be effective against Tobacco mosaic virus and Turnip yellow mosaic Virus.

Radioactive Phosphorus as antiviral agent

Radioactive Phosphorus (p^{32}) is utilized to inactivate recent viral nucleic acid and the virus particles. (e.g. coliphage T_2, Poliovirus etc.)

Interferon as Antiviral Agents

Interferons are natural host specific antiviral agents and used against specific viral diseases either directly administering them or by inducing their formation in the host. Artificial induction of interferons production in the host prior to actual infection by virus would enable the host to develop resistance against any future attack.

Interferon produced by inducer chemical like e.g. Poly citidilic acid & Poly iosinic acid useful against neoplastic growth in mice.

Heat therapy of plant viral disease

e.g. Potato leaf roll virus can be inactivated by treating the tubers with temperature in the vicinity of 40^0C for several days.

The mechanism of heat therapy is not well understood. If has been suggested that high temperature normally denatures the protein monomers irreversibly, thereby interfering with capsid formation.

Inhibition of Plant Viruses in Situ

Sometimes, when sap transmissible viruses are inoculated on to healthy susceptible host plant, the virus is prevented from multiplying and producing symptoms in the latter. This is a type of inhibition in situ which is probably due to some natural inhibitors.

[for details consult the chapter 9 of **An introduction to Virus by Biswas & Biswas** (Vikas Publishing)]

CHAPTER – 4

- Oncogenes
- VIRUSES AND CANCER
- The animal cell culture model of cancer
- Cellular oncogenes
- Transformation by Sv 40.
- Transformation by Retroviruses
- Molecular mechanism.

ONCOGENES

Oncogenes (from oncos, the Greek word for "mass" or tumor) were originally defined as the genetic elements (genomic sequences) of retroviruses responsible for the malignant transformation of host cells. These oncogenes have also been found in spontaneous tumors. Furthermore DNA sequences homologous to the transforming genes of certain retroviruses have been found in normal, untransformed cells. Thus these oncogenes are not themselves of viral origin but are cellular genes that the viruses picked up by recombination events during the course of infection. This means that most of the oncogenes found in viruses and cancer cells have counterparts either identical or closely similar among the normal genes of the human and animal body. In fact oncogenes are found in all the cells of the mammalian body except the Red Blood cells which do not possess nuclei, but the majority of the cells never become cancerons. Oncogenes have the potential of causing cancerous transformation of cells when appropriately activated some 20 oncogenes have been identified most of which were originally discovered in viruses that cause cancers in the Laboratory animals.

The structures of the cellular counterparts of the viral oncogenes (termed cellular oncogenes have been conserved throughout evolutionary history. This suggests that they have essential roles to play in cellular physiology, probably in cell differentiation or regulation of cell division. At the present time, we still do not know what makes these genes go away and induce uncontrolled cell division and abnormal cell division and abnormal cell differentiation patterns. Oncogenes can be induced to act in the cancer process in different ways:

(1) by undergoing mutation

(2) by being abnormally activated when thrown into association with some other genes

(3) by being removed from natural repressors that regulate its activities

(4) by producing oncogene products that may be growth factors that stimulate cell division and contribute to the uncontrolled growth of cancer cells

(5) by over expression due to the accidental integration of a retrovirus next to it.

The evidence implicating cellular oncogenes in the development of human cancers is still largely circumstantial.

VIRUSES AND CANCER

The characteristic tissue of animals are formed by the regulated limited growth of their component cells. As a rare event, a cell may escape normal regulatory constraints and divide in as uncontrolled manner, forming an abnormal mass of tissue. Such masses are termed **neoplasm** or **tumors**.

Tumors are classified by their pattern of growth into two groups, those that do not invade surrounding tissue are turned **benign**. They grow by displacing adjacent cell but rarely kill the organism unless they occur in the brain. On the other hand, **malignant** tumors, termed **cancers**, invade and destroy surrounding tissue as they grow, they also release cells into the blood stream or into the lymphatic circulation that establish new neplastic foci termed **metastases**.

Tumors both benign and malignant, are usually named by appending oma a term describing their appearance when examined with light microscope (**Table 11**). Cancers formed by layers of cells are called **Cascinomas**, those that arise in connective tissue or blood vessles are termed **sarcomas**. Several cancer are named after the specific cell types in which they arise. For example, **hepatomas** arises in liver hepatocytes, melanomas arise in skin melanocytes and lymphomas arise in lymphocytes. An exception to this system of nomenclature occurs in the case of the cancers termed **Leukemias** : that arise in the bone marrow cells which produce leukocytes.

In 1908, V. Ellerman and O. Bang demonstrated that a type of leukemia that affects chickens could be transmitted to healthy one through cell free filtrate of the blood of a leukemic bird. Latter P. Rous also demonstrated that a chicken sarcoma could be similarly transmitted and he established that the active agent in the filtrate was a virus now called Rous sarcoma virus (RSV) and known to be a member of the retrovirus family of RNA viruses.

TABLE 11.

Examples of Human Neoplasms

Neoplasm	*Description*
BENIGN NEOPLASMS	
Papillomas	Water (caused by a virus) Benign tumors formed
Adenomas	by cells arranged into glandular structures.
Fibromas	Benign tumors formed by connective tissue cells.
MALIGNANT NEOPLASMS	
Carcinomas	Malignant tumors formed by cells organized into sheets or layers.
Sarcomas	Malignant tumors formed by poorly differentiated cells of connective tissues, muscle, bone, or blood vessels.
Leukemias	Malignant neoplasms of bone marrow cells that normally produce leukocytes.
Lymphomas	Neoplasms formed by lymphoid tissue
Hepatomas	Malignant tumors arising in liver cells
Melanomas	Malignant tumors arising in melanocytes, the pigment cells of skin.

Example of DNA viruses in Human Cancer:

Herpes virus, **Epstein-Bar (EB) virus** is responsible for malignant lymphoma termed Burkitt's lymphoma. EB virus is also responsible for naso pharyngeal **carcinoma.**

Example of RNA virus in Human Cancer :

In most cases no regular association between a particular type of cancers & retroviruses. But one notable exception is Adult T-cell leukemia which is associated with a retrovirus called human T-cell leukemia virus (HTLV). However, many individuals who become infected with **HTLV** do not develop cancer. Why some infections result in cancers and others do not is not known.

The Animal cell culture model of Cancer

Methods for growing animal cells in culture have contributed greatly to our understanding of cancer.

In a suitable medium, some types of animal cells grow for a limited period like a population of micro organisms, but when they **come into contact** with one another, grow and cell movement stop.

This phenomenon is termed contact inhibition. It is a fundamental property of normal animal cells. However cancer cells do not exhibit contact inhibition, rather they continue to grow in culture, forming disorganised masses of cells.

Attempts to propagate cells from an animal for long periods of time usually fail. It has been found repeatedly transferred animal cells in fresh medium, rarely continues for more than 50 generations. However occasionally a cell in the culture acquires the ability to grow indefinitely by mutation. The decendants of such as "immortal cell" are termed a **cell line**. Some cell line behave in culture like cancer cell, but others continue to exhibit **contact inhibition**. Culture cells that have lost contact inhibition are said to be transformed. The test for transformation provides a useful means of detecting **carcinogenic chemicals** as well as **oncogenic viruses**.

Cellular oncogenes

If Human Cancer cells possess DNA sequences that are homologous to those of viral oncogenes indicate that all human cancers do have such sequence. However, normal cells also have such sequences, termed protooncogenes that are homologous with parts of oncogenes on the basis of it R. Huebner and G. Todero proposed a theory that viral oncogenes were originally acquired from normal cellular genes and have subsequently evolved to viral oncogenes.

Following the discovery of Protooncogenes, fragments of DNA from human cancers were tested for their ability to transform cultures of animal cells. In this way, cancer cell genes that are able to transform cell lines were identified. These are termed cellular oncogenes. In only one case (T-cell leukemia) was an oncogene from a human cancer shown to be associated with retroviral genes. Hence the majority human cancers appear to arise, at least in part, from the activation of normal cellular genes (Proto oncogenes).

Process for activation of Protooncogenes:

Several processes appear to be involved in activation of proto oncogenes.

(i) A single base pair substitution mutation (GC-TA transition) that converts a normal gene into the oncogene (example bladder carcinomas). This oncogene partially homologous to those of certain strains of murine sarcoma virus.

(ii) By chromosomal abnormalities (translocations) where an arm of one chromosome has been broken and rejoined to the arm of another chromosome (example chronic myelocytic leukemia).

Transformation by SV 40 :

In oncogenic DNA virus [Simian Virus (SV 40)], frequency of transformation is typically between 10^{-3} and 10^{-5} transformed cell per virion. Transformation requires integration of the SV 40 into one of the host cell chromosomes, but there are many sites on all of the chromosomes where this integration can occur. Therefore transformation by SV 40 superficially resemble lysogeny by phage mu.

Transformation by Retroviruses:

During normal replication of a retrovirus, a DNA copy of the viral chromosome becomes inserted into a host chromosome. Hence the cells transformed by retroviruses always contain a copy of the viral chromosome. In some retroviruses the frequency of transformation is 100 percent, but the site at which insertion occurs appears to be random. This viral transformation is not the consequence of inactivating a normal cellular gene but rather is the consequence of the addition of new genetic information. Actually transformation results from they presence of a single viral gene termed as oncogene.

Rous sarcoma virus (V-Src) oncogene encodes a protein (MW 60,000) (denoted pp-60-v-Src) is largely associated with the cytoskeleton, a network of protein microfilaments underlying the cytoplasaric membrane and that phosphorylates tysosis residues in certain protein. One of these vinculin is a membrane protein associated with ~ ..es termed adhesion plaques where the membrane establishes contact with a surface. It is hypothesized that phosphorylated vinculin cannot function in establishing contacts.

Maloney murine sarcome virus (v-mos) encodes a kinase which phosphorylates serine residues in certain cellular proteins and is largely found in the cytoplasm unbound to the cytoskeleton.

The oncogenes of simian sarcoma virus (V-Sis) encodes a protein (MW 28,000) that closely resembles platelet derived growth factor (PDGF) a protein released from platelets that stimulates cells to divide during the normal process of wound healing.

Molecular mechanism:

About Twenty genes (**Table – 12**) can produce cancer in Humans. Acting on the same pathway of ceil proliferation.

Only a small subset of genes is responsible for the induction of the uncontrolled cell proliferation that leads cancer. These genes were first identified from oncogenic RNA viruses (Retro viruses). Life cycle of these viruses occurred in the retrograde direction (RNA → DNA) by reverse transcriptase and then inserted in the genome.

A retro virus has only three genes require for its life cycle and has terminal repeats at its end which resemble very much found at the ends of transposons. (Fig. 17).

Some retroviruses, the oncogenic ones carry an extra gene that induces cancer in animals.

The Src gene of Rous Sarcoma Virus (**Fig-17**) codes for a plasma membrane bound protein kinase that specifically phosphorylates the amino acid tyrosin (normally occur in serine or threonine) on proteins on the cell membrane (one of them being vinculin at the cell adhesion plaques).

Huebner & Todaro (1968) predicted that normal cells contained enemies with in the form of oncogenes similar to the viral ones, which could produce cancer by activation. When C. DNA probes from

viral Src gene became available, it was found that all cells contain a gene that is homologous in v-Src but that differs in that it has multiple exons & introns. Retroviruses pick up new genes by recombining with them at the RNA level, so that the introns have already been removed by mRNA splicing.

All 20 viral oncogenes derive from cellular genes in normal cells. The normal cellular version of the gene is called a proto-oncogene. Retroviruses pick up into their genome sequences from the mRNA population that will increase viral production. These viruses can transmit genes from one species to another thus breaking evolutionary barriers.

GENE REGULATION

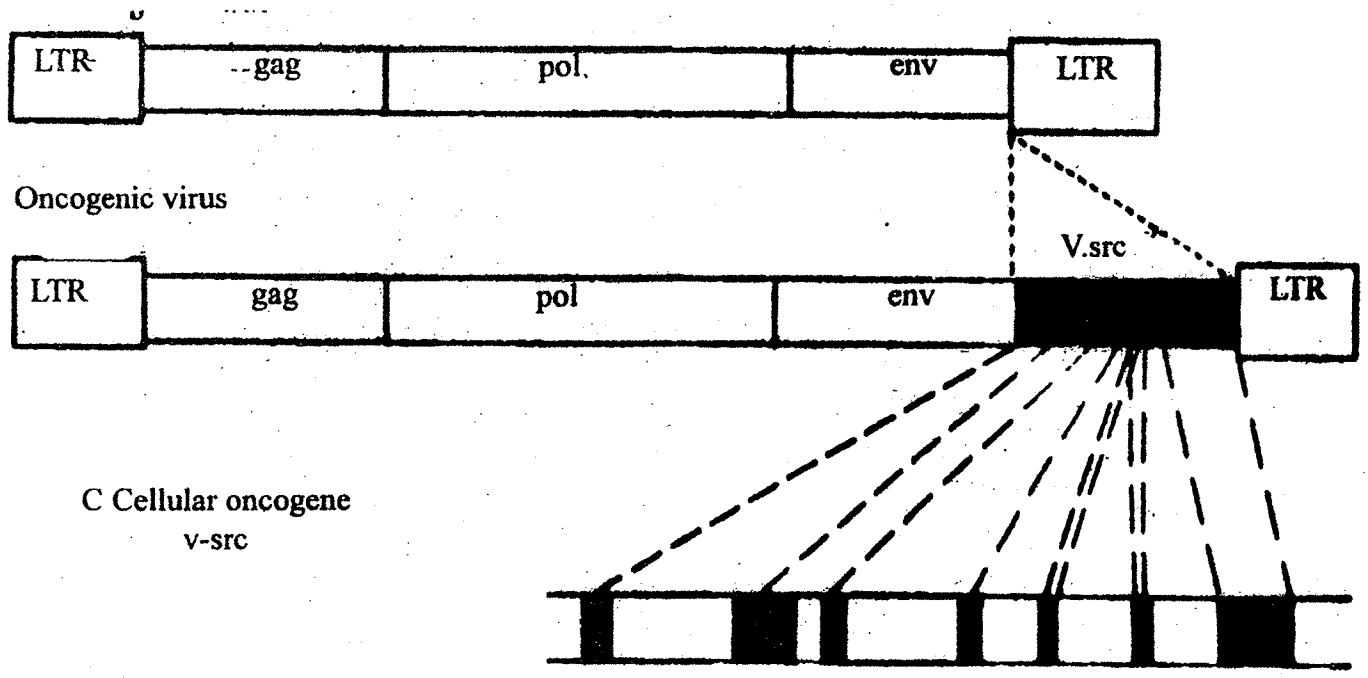


Fig. 17

Fig. 17. *Structure of (A) a retrovirus, (B) a retrovirus that has picked up oncogene v-src (Rous sarcoma virus and on the cellular version of the same oncogene (c-src), containing multiple introns. The genes of the virus are gag (for group specific antigen), pol (for reverse transcriptase polymerase), and env (for the envelop glycoprotein). LTRs, or long terminal repeats, are involved in integration into the genome and transposition. A single gene product src can produce sarcomas in chickens.*

[for further detail consult cell Biology ch. 22 of De Robertis p-594].

TABLE 12. ONCOGENES ARE NAMED AFTER THE VIRAL STRAIN FROM WHICH THEY WERE ISOLATED

Oncogene	Virus Stain	Probable Animal Origin of a Gene	Mode of Action
rel	Avian reticuloendotheliosis virus-T	Turkey	
src	Rous sarcoma virus	Chicken	Tvr Protein kinase
myb	Avian myeloblastosis virus strain BAI-A		Nuclear Protein
myc	Avian myelocytomatosis virus MC29	Chicken	Nuclear Protein
erb-A	Avian erythroblastosis virus gene A	Chicken	Nuclear protein, DNA-binding region similar to glucocorticoid receptor
erb-B	Avain erythroblastosis virus gene B	Chicken	Try protein kinase gene for epidermal growth factor receptor
fps	Fujinami sarcoma virus	Chicken	Tyr protein kinase
ves	Y73 avain sarcoma virus	Chicken	Tyr protein kinase
ros	UR2 avain sarcoma virus	Chicken	Tyr protein kinase
mos	Moloney murine sarcoma virus	Mouse	
Fas	Rasheed rat sarcoma virus	Rat	GTP-binding protein in plasma membrane
abl	Abelson murine leukemia virus	Mouse	Tyr protein kinase
fes	Snyder-Theilen feline sarcoma virus	Cat	Tyr protein kinase
fms	Mc Donough feline sarcoma virus	Cat	
sis	Simian sarcoma virus	Woolly money	Platelet derived growth factor gene

The viral oncogenes are preceded by a v (e.g. v-src) and their cellular counterparts by a c (e.g. c-src).

Suggested Questions

(1) (i) How will you isolate and purify plant viruses?

(ii) How will you enumerate viruses?

(2) (i) What is concatamers?

(ii) How will you control viral diseases?

(3) Describe the mechanism of replication of chromosome of RNA viruses.

(4) (i) What is viriod?

(ii) What is lysogen?

(iii) What is temperate phage?

(5) (i) Why animal cell are used as culture model of cancer?

(ii) Majority of human cancer appear to arise at least in part from the activation of normal cellular gene-explain.

BOTANY

Module No. - 6

Part - I, Paper - I (1st Half)

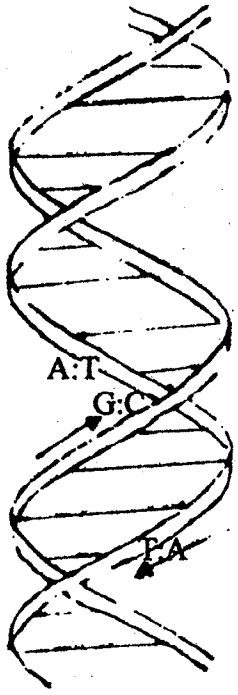
Mocrobiology

CONTENTS

MICROBIAL GENETICS:

DNA REPLICATION :

FIGURE A (left)



Schematic representation of the DNA double helix. The outer ribbons represents the two deoxyribosephosphate strand. The parallel lines between them represent the pairs of purine and pyrimidine based held together by hydrogen bonds. Specific examples of such bonding is shown in the center section, each dot between the pairs of bases representing a single hydrogen bond. The direction of the arrows correspond to the 3' to 5' direction of the phosphodiester bonds between adjacent molcules of 2' deoxyribose.

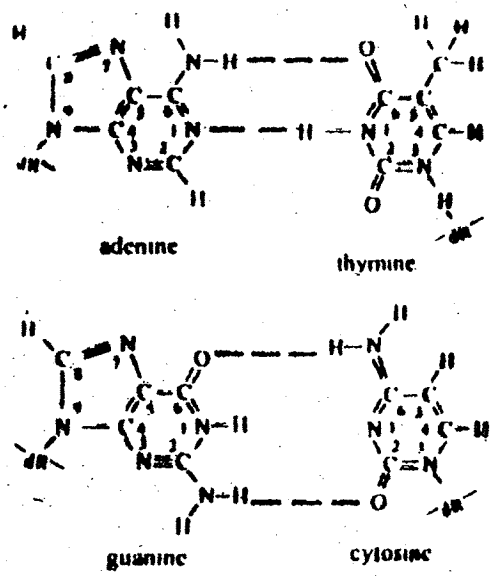


FIGURE B (right)

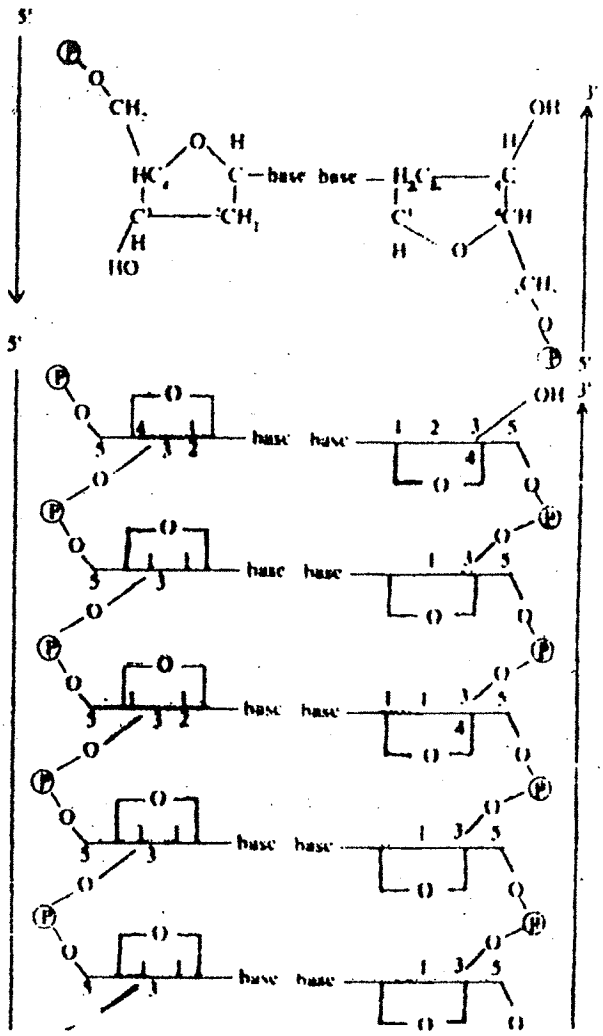
The pairing of adenine with thymine and guanine with cytosine by hydrogen bonding. The symbol – dR– represents the deoxyribose moieties of the sugar-phosphate backbones of the double helix. Hydrogen bonds are shown as dotted lines.

FIGURE C (below)

The antiparallel nature of the double helix. Above, the complete structural formula of one base pair is shown. Below, a segment of duplex is shown diagrammatically. Note that the left-hand strand runs from 5' to 3', reading from top to bottom, while the right-hand strand runs from 3' to 5'.

THE POLYMERIZATION OF NUCLEOTIDES INTO DNA

The structure of the DNA molecule, elucidated by Watson and Crick in 1953, immediately suggests how it can be accurately replicated. It is a douuble helix, each strand of which consists of 2-deoxyribose molecules linked together by phosphodiester bonds molecules linked together by phosphodiester bonds between the 3'-hydroxyl group of one and the 5'-phosphate group of the next. The purine and pyrimidine based (attached to the 1 position of deoxyribose) project toward the centre of the molecule, holding the two strands together by hydrogen bonding between specific purine-pyrimidine pairs. Guanine is paired with cytosine (G – C) and adenine is paired with thymine (A – T) (Figure – A). When the bases are present in their energetically most favourable forms (the keto, rather than the enol, form of the oxygenated bases, and the amino, rather than the imino, form of the aminated bases), only these pairs can fit within the hydrogen-bonding distances. The two hydrogen bonds that form between adenine and thymine, and the three hydrogen bonds that form between guanine and cytosine, are shown in Figure B. The entire molecule can thus be described as a *linear sequence of nucleotide pairs*: the exact order of these pairs constitutes the genetic message, which contains all the information necessary to determine the specific structures and functions of the cell.



THE ANTIPARALLEL STRUCTURE OF THE DNA DOUBLE HELIX

Each strand of the double helix is a polarized structure; its polarity results from the sequential linkage of polarized subunits, the deoxyribonucleotides. As shown in Figure C, each nucleotide has a 5'-phosphate end and a 3'-hydroxyl end; when a series of nucleotides are connected by phosphodiester linkages, a polarized chain is formed which also has a 5'-phosphate end and a 3'-hydroxyl end; when a series of nucleotides are connected by phosphodiester linkages, a polarized chain is formed which also has a 5'-phosphate end and a 3'-hydroxyl end.

The two strand of the double helix are *antiparallel* (i.e., they have *opposite polarity*). If we scan the diagram (Figure-C) from top to bottom, we see that the left-hand strand has 5'→3' polarity, whereas the right-hand strand has 3'→5' polarity. One consequence of the antiparallel structure of DNA will become apparent when we consider the process of DNA replication.

DNA POLYMERASES

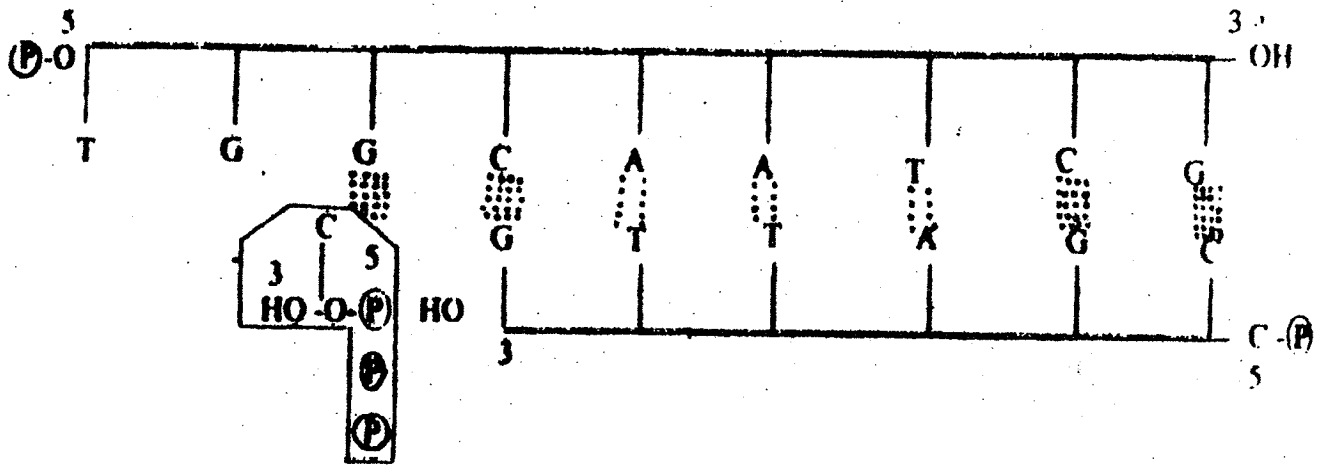
The polymerization of DNA is catalyzed by enzymes called *DNA polymerases*. In addition to the four deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), which are substrates for the reaction, two molecules of nucleic acid are required; one is the DNA *template* to which the substrate deoxynucleoside triphosphate molecules pair according to the rules of hydrogen bonding (G with C and A with T); and a second is the primer, to which the nucleotides are attached as a consequence of the polymerization (Figure D); the primer can be DNA or RNA. DNA synthesis proceeds in the 5' to 3' direction by the sequential formation of phosphodiester bonds between the α-phosphates of the 5'-deoxynucleoside triphosphates and the terminal 3'-hydroxyl group of the primer, with the release of one pyrophosphate molecule (P – P) for each deoxynucleotide added. The template DNA determines the sequence of additon of deoxynucleotide, to the primer DNA molecule.

REPLICATION

Three different DNA polymerases are present in *E. coli*: polymerase I (Pol I), polymerase II (Pol II), and polymerase III (Pol III). Pol III catalyzes the addition of nucleotides to an RNA primer; Pol I can hydrolyze and RNA primer and duplicate single-stranded regions. The role of Pol II remains nuclear.

Although the action of the DNA polymerases is simple and well understood, replication of the intact double-stranded bacterial chromosome is much more complicated and many questions remain to be answered. DNA polymerases require a single-stranded template; thus the double-stranded chromosome must be separated, at least locally, before replication can occur. Such separate forms a bubble in the chromosome at a specific site termed oriC, at which replication always initiates, but the bubble lacks a primer, which is required for DNA polymerase to function. Replication of a closed circular double helix of DNA like the chromosome would soon generate loops, termed supercoiled twists, in the molecule that would soon stop the process of replication. The required functions of strand separation, primer synthes s and elimination of twists are mediated by a variety of proteins that, along with the DNA polymerases, comprise a loose multienzyme complex termed the *replication apparatus*. This apparatus, which is composed of at least 13 different proteins, functions with remarkable speed and accuracy: approximately 3,000 nucleotides are polymerized per second (at 37°C) and only about one mistake (incorrect pairing) is made per 10^{10} nucleotides copied.

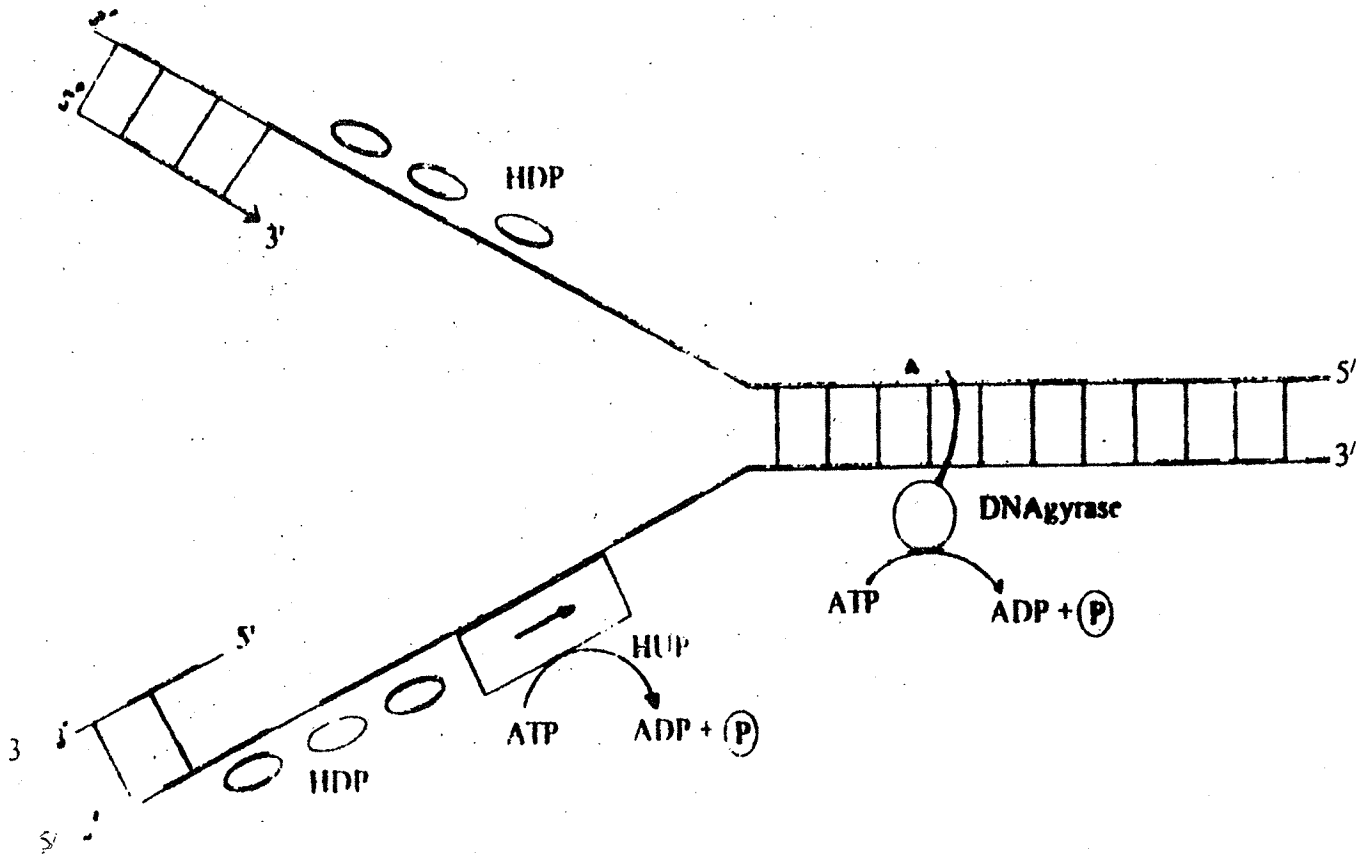
Figure D. *Schematic representation of a short fragment of double stranded DNA with a single-stranded region (the template) at the left end. The lower strand (the primer) is in the process of being lengthened by the action of DNA polymerase, which catalyzes a reaction between a deoxynucleoside triphosphate (in this case CTP) and the 3'-hydroxyl group of the primer strand. The arrow shows the direction of sequential additions of deoxyribonucleosides with the concomitant splitting off of pyrophosphate (P-P).*



Strand separation, the first step in the process of replication requires the participation of several proteins and the hydrolysis of ATP because energy is required to break the hydrogen bonds between the complementary bases and to unwind the strands. The otherwise high energy cost of separation is reduced by the action of one of the proteins, the *helix-destabilizing protein* (HDP) that binds cooperatively to the separated single strands. Other proteins, including DNA-unwinding enzyme I (topoisomerase I) and *Rep protein*, collectively termed unwinding proteins, actively separate the DNA strands with the concomitant hydrolysis of ATP to ADP and inorganic phosphate. Another protein, *DNA gyrase*, prevents the formation of twists by periodically breaking a phosphodiester bond in one of the strands, thereby allowing free rotation of the opposite strand. Later this same enzyme reforms the same bond. The activity of DNA gyrase also requires the hydrolysis of ATP. The activities of the various proteins that mediate the processes of strand separation and elimination of twists are summarized in Figure E.

Once the strands are separated, short pieces of RNA complementary to a protein of the single-stranded regions are synthesized on each of the strands. This RNA, which serves as a primer for DNA polymerase, is synthesized by a special RNA polymerase in the replication apparatus. Like other RNA polymerases it does not require a primer in order to function.

Figure E. *Schematic representation of strand separation at the replication fork. The action of DNA gyrase of allowing free rotation within the double helix at the expense of hydrolysis of ATP is indicated by the circular arrow. a helix-unwinding protein (HUP) is shown at the point of strand separation, moving in the direction indicated (arrow) and hydrolyzing ATP. Molecules of helix-destabilizing protein (HDP) are shown attached to the single-stranded regions.*



The opened chromosome with attached pieces of RNA forms two *replication forks* at which replication catalyzed by DNA polymerase proceeds in opposite directions around the circular chromosome until they meet at the *terminus* : bases of appropriate nucleoside triphosphates pair by hydrogen bonding with exposed bases in the single stranded region and phosphodiester bonds form between the terminal 3'-hydroxyl group of the primer molecule and the a-phosphate of the nucleoside triphosphate. Thus, replication on each strand always occurs in the 5' to 3' direction. Following on one of the replication forks (Figure F). Owing to the different polarities of the complementary single strands, their replication occurs in different directions. The strand that is replicated in the same direction as the movement of the replication fork is termed the *leading strand*; the other is termed the *lagging strand.*

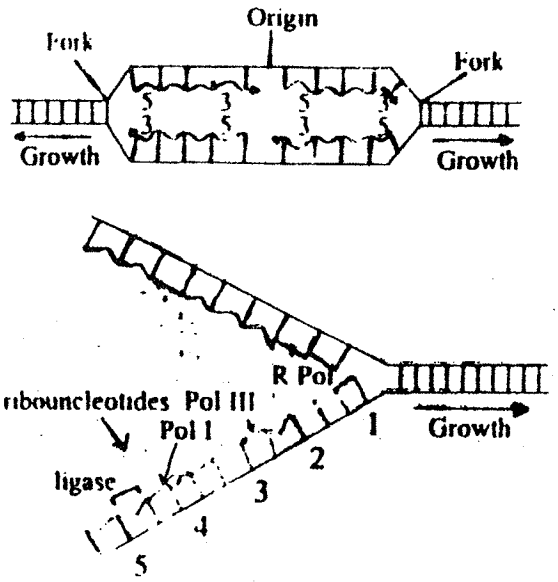


Figure F.

Schematic representation of the steps of replication of the bacterial chromosome. Part (a) represents a portion of a replicating bacterial chromosome at a stage shortly after replication has begun at the origin. The newly polymerized strands of DNA (wavy lines) are synthesized in the 5' to 3' direction (indicated by the arrows) using the preexisting DNA strands (solid lines) as a template. The process creates two replication forks which travel in opposite directions until they meet on the opposite side of the circular chromosome, completing the replication process. Part (b) represents a more detailed view of one of the replicating forks and shows the process by which short lengths of DNA are synthesized and eventually joined to produce a continuous new strand of DNA. For purposes of illustration, four short segments of nucleic acid are illustrated at various stages. In the first (1) primer RNA (thickened area) is being synthesized by an RNA polymerase (R Pol). Then, successive in (2) DNA is being polymerized to it by DNA polymerase III (Pol III); in (3) a preceding primer RNA is being hydrolyzed while DNA is being polymerized in its place by the exonuclease and polymerase activities of DNA polymerase I (Pol I); finally, the completed short segment of DNA (4) is joined to the continuous strand (5) by the action of DNA ligase (ligase).

It will be noted that replication of the entire leading strand requires only a single molecule of primer RNA at *oriC* but replication of the lagging strand requires repeated synthesis of RNA primers as the replication fork moves. Reinitiation occurs on the lagging strand at intervals of about 1000 nucleotides, thus transiently creating short pieces of DNA attached to RNA (called *Okazaki fragments* for their discoverer, R. Okazaki). As synthesis of these fragments proceeds into the RNA primer of the previously synthesized one, the exonuclease activity of DNA polymerase I hydrolyzes the primer RNA as it replaces it with DNA. Finally the short pieces of DNA thus synthesized are joined to form a continuous complementary copy of the original strand by the action of an enzyme, *DNA ligase*, which catalyzes the two successive reactions:

NAD^+ enzyme →

Enzyme-AMP + nicotinic mononucleotide

enzyme -AMP+5′-Phosphate end of DNA+3′ - hydroxyl end of DNA

→ enzyme-AMP + 5'-3' phosphodiester bond + AMP

It is interesting to speculate why DNA polymerases did not evolve (as RNA polymerases did) to be primer-independent, thus obviating the need for synthesis of an RNA primer on each Okazaki fragment. The answer might light in the special requirements for accurate replication of DNA because of its role as a repository of the cell's genetic information. It has been estimated that the intrinsic mistake frequency of replication is about one incorrect base pairing per 10^5 nucleotides. This is reduced to the observed mistake frequency (one per 10^{10}) by an activity of DNA polymerase III, known as *prooffreading*. This is accomplished at least in part by the enzyme's built-in 3' to 5' exonuclease activity, which removes a previous mismatch by moving backward. The enzyme does not catalyze replication in the forward direction unless it is followed by a properly matched base pair. Thus such a self-correcting polymerase cannot initiate chains *de novo*.

GENETIC EXCHANGE AND RECOMBINATION

The ability to exchange genes within a population is a nearly universal attribute of living things. Although the details of the process vary enormously, all systems of genetic exchange among eucaryotes involve the same cellular event: two haploid cells (*gametes*) fuse to form a diploid *zygote*; i.e., a complete complement of genes is contributed by each gamete. This almost never occurs during exchange of genes among procaryotes. In all those cases that have been studied sufficiently to reveal the molecular details of the process, only a small portion of the genome from one procaryotic cell (the *donor*) is transferred to another (the *recipient*), thus forming an incomplete zygote (termed a *merozygote*) that contains the complete genetic complement of the recipient (the *endogenote*) but, with very few exceptions, only a portion of the genetic complement of the donor (the *exogenote*) contained in the fragment of the chromosome that is transferred to the recipient.

In no known case is genetic exchange among procaryotes an obligatory step (as it often is among eucaryotes) in the completion of an organism's life cycle. Rather, genetic exchange seems to be an occasional process that occurs by three quite different mechanisms in various procaryotes. The three mechanisms of genetic exchange are called *transformation, transduction,* and *conjugation*.

In the case of transformation, DNA is released from cells into the surrounding medium, and recipient cells incorporate it into themselves from this solution.

In the case of transduction, DNA is transferred from one procaryotic cell to another as a consequence of a rare formation of an aberrant phage virion in which some or all of its normal complement of DNA is replaced by bacterial DNA (donor DNA). When such a phage virion attaches to and introduces this DNA into another bacterial cell the recipient), genetic exchange is effected.

In the case of conjugation, genetic exchange occurs between cells in direct contact with one another by a process that is, in all known cases, encoded by plasmid-borne genes. Usually only the plasmid itself is transferred from donor to reciptent by this process, but sometimes chromosomal genes are transferred as well.

BACTERIAL TRANSFORMATION

In 1928 F. Griffith showed that injection of mice with an avirulent strain of *Streptococcus pneumoniae* together with heat-killed cells of a virulent strain killed the mice, although injection of mice with either culture alone caused no disease. On autopsy, these mice were found to contain live virulent cells of *S. pneumoniae*. These and subsequent experiments established that surviving cells were recombinant; they exhibited certain properties (including virulence) that were typical of the killed cells and others that were typical of the avirulent culture. Thus a genetic exchange had occurred between the dead cells and the live ones. Subsequent experiments by other investigators established that this type of genetic exchange could occur *in vitro*, and it was presumed that a particular substance, termed the *transforming principle*, mediated it.

In 1944, O.T. Avery, C.M. MacLeod, and M. McCarty purified the penumococcal transforming principle and identified it as being DNA. Indeed, these experiments were the first to establish in any biological system that DNA is the macromolecule in which genetic information is encoded.

TYPES OF TRANSFORMATION MECHANISMS FOUND AMONG PROCARYOTES

Cells that are in a state in which they can be transformed by DNA in their environment are said to be competent. In a significant number of bacteria (Table 1); entry into the competent state is encoded by chromosomal genes and signaled by certain environmental conditions. Such bacteria are said to be capable of undergoing *natural transformation*. Many other bacteria do not become competent under ordinary conditions of culture but they can be made competent by a variety of highly artificial treatment such as exposure of cells to high concentrations of divalent cations. Such systems of transformation have been termed *artificial transformation*.

Until quite recently, the pattern of transformation exhibited by *Streptococcus pneumoniae* was considered to be typical of all naturally transformable Gram-positive bacteria, and that exhibited by *Haemophilus influenzae* to be typical of all naturally transformable Gram-negative bacteria. Comparison of the two processes have been illustrated in Table 2. More recently, studies on the natural transformation of plasmids have shown that this is not the case; differences in mechanism of transformation do not necessarily correspond with the nature of the cell wall as revealed by the gram reaction.

TABLE 1.

Bacteria Known to Encode a Capacity for Natural Transformation
Gram-Positive Bacteria:
Streptococcus pneumoniae, S. sanguis
Bacillus subtilis, B. cereus, B. lichiniformis
B. stearothermophilius
Thermoactinomyces vulgaris
Gram-Negative Bacteria:
Neisseria gonorrheae
Acinetobacter calcoaceticus
Moraxella osloensis, M. urethalis
Psychrobacter spp.
Azotobacter agilus
Haemophilus influenzae, H. parainfluenzae
Pseudomonas stutzeri, P. alcaligenes
P. pseudoalcaligenes, P. mendocina

TABLE 2.

Differences between the Natural Transformation Systems Encoded by *Streptococcus pneumoniae* and *Haemophilus influenzae*

Property	Streptococcus	Haemophilus
Competence factor triggers competence	Yes	No
Form in which DNA enters cell	Single-stranded	Double-stranded
Source of DNA that can enter cell	Any	Only homologous
Form of DNA bound to cell surface	Double-stranded	Double-stranded
Physical state of DNA within cell	Protein bound	Transformasome-contained
Exhibits eclipse period	Yes	No

NATURAL TRANSFORMATION SYSTEMS : Streptococcus pneumoniae

Cells in cultures of the Gram-positive bacterium, *Streptococcus pneumoniae* rapidly become competent during the exponential phase of growth (Figure 1). This conversion of noncompetent cells into competent ones is mediated by a small protein termed the *competence factor*. The competence factor is constantly produced and excreted into the medium by cells of *S. pneumoniae*, but only when the density of the population of the cells in the culture, and hence the concentration of competence factor in the suspending medium, rises to a certain critical value does competence develop. A set of about 12 proteins is synthesized

that mediate the process of transformation. With these proteins competent cells can absorb double-stranded DNA to their outer surface at several sites and cleave it through the action of surface-bound enzymes into smaller fragments. Then one strand of the fragment is digested by the nuclease and the other enters the cells while being bound to a competence-specific DNA-binding protein (Figure 2).

Streptococcus pneumoniae will take up and process DNA regardless of its source : for example, DNA from salmon sperm is taken up as readily as DNA from another *S. pneumoniae* cell. However, only if the DNA is homologous with the endogenote will it become integrated and thereby genetically alter the recipient cell. Fragments of nonhomologous DNA, not themselves constituting a replicon and not becoming part of one by integration into the endogenote, are not replicated and are eventually degraded; they cause no heritable change in the recipient cell.

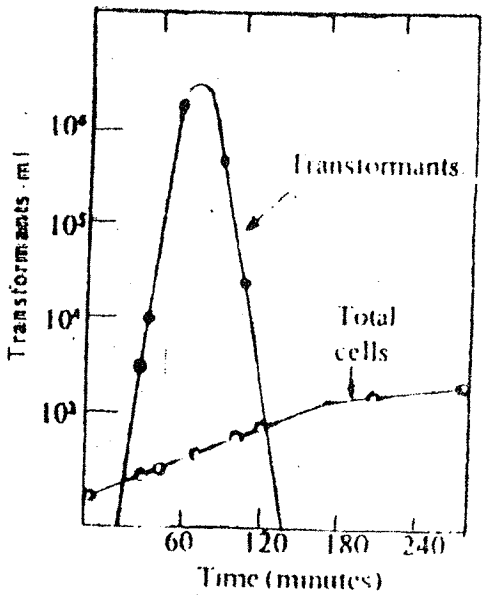


Figure 1. *Course of development of competence during growth of a culture of Streptococcus pneumoniae. The number of competent cells in the culture was estimated from the number of cells that could be transformed (transformants) in samples taken at various times. The total number of cells in the culture (Total cells) is plotted on a different scale; the maximum number present at 240 minutes was 6.5 x 10^8/ml.*

Integration of homologous DNA occurs by a process of strand replacement (Figure 2) forming as an immediate product a *heteroduplex* region, one strand of which is newly entered one and the other of which is the homologous region of the endogenote. Owing to their different origins, the two strands comprising the heteroduplex might not be identical (certainly the case if the consequence of transformation is a heritable change in the recipient), in which case there will be regions in which the heteroduplex is not held together by hydrogen bonding. The existence of such regions can trigger the operation of the recipient cell's DNA repair system through the action of which the mismatched regions of the exogenote sometimes are removed and replaced by bases complementary to the endogenote. (Furiously, it appears that the bases of the exogenous strand, rather than bases from the resident endogenote strand, are preferentially removed). If the mismatched bases of the transformed strand are removed (a process sometimes termed *correction*) before that portion of the chromosome is replicated no heritable change in the recipient results. If, on the other hand, replication occurs first, two homoduplex copies are made, one of which is identical to the DNA taken from outside the cell; the consequence is genetic change of the recipient cell by transformation.

Eearly studies on transformation of *S. pneumoniae* were genetic, in that the progress of the process was followed by extracting DNA from the recipient and testing its ability to transform another recipient.

For example DNA from a streptomycin-resistant strain might be used to transform a sensitive strain and, at various times after the DNA encoding streptomycin resistance is added to the recipient; extracts from the recipient are used to transform another culture of the sensitive strain. The result of such experiments were at first quite puzzling : for a certain period of time after addition of the DNA encoding streptomycin resistance, extracts of the recipient failed to transform another recipient to streptomycin resistance. The period during which the transforming DNA seems to disappear as judged by transformability has been called the *eclipse period.* With our current state of knowledge of the process of transformation in S. *pneumoniae*, the reasons for the occurrence of an eclipse period are understood. Only double-stranded DNA is bound to the cell surface in the first step of the transformation process, so during that period in which the entering DNA is in a single stranded form it is inactive when assayed for ability to transform another cell.

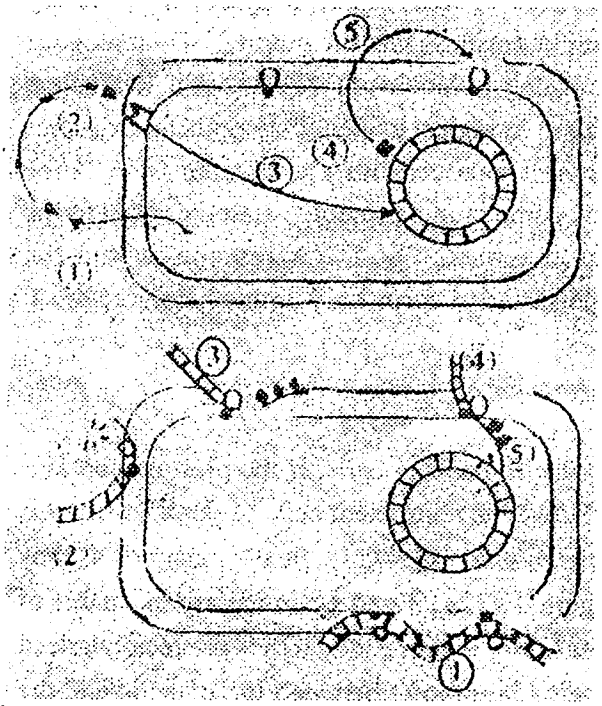


Figure-2 *Schematic representation of the steps leading to transformation of Streptococcus pneumonia. The Development of competence : (1) Cells in the culture produce a souble protein termed competence factor (▲) that (2) adsorbs at a site on the cell surface, M, causing (3) certain genes to be expressed. Among these (4) is an autolysin (◆) that exposes (5) a DNA binding protein (O) and a nuclease (●). Transformation : A long strand of double-stranded DNA a bound to the cell surface (1) where the nuclease (●) degrades (2) one of these strands. The remaining strand is bound (3) to a DNA bind- ing protein (◆). In this form it enters the cell (4) and become integrated into the chromosome (5) by single-sound replacement.*

NATURAL TRANSFORMATIONS SYSTEMS :

Haemophilus influenzae

The process by which the Gram-negative bacterium *Haemophilus influenza* is transformed differs in many fundamental respects from the streptococcal system just discussed. No competence factor is produced that triggers development of the competent state. Rather, cells become competent as a consequence of growth in rich media. In laboratory studies, specially formulated media have been devised for this purpose that are remarkable effective, making it possible to obtain a culture in which all cells are competent.

The *Haemophilus* system of transformation differs from the Streptococcus system in two additional ways : (1) only homologous DNA (DNA from the same or a closely related species of haemophilus) is bound to and taken into cells with any significant efficiency, and (2) transforming DNA enters the recipient in double-stranded form, remaining that way up to the time of its integration into the endogenote.

The past few years have seen the clarification of the general mechanisms by which double-stranded DNA is bound to the cell and taken into it and by which this binding and uptake of DNA is restricted to homologous DNA. The outer membrane of competent cells (but not noncompetent ones) contains, on the average, about 10 vesicular structures that appear to be localized extensions of that membrane or *blebs*, at the base of which are small pores. Imbedded in this membranous region is a protein that binds specifically with an 11-base pair sequence of DNA (5"AAGTGCGGTCA-3') that occurs at about 600 sites on the *Haemophilus* chromosome or about one site per 4,000 base pairs. Since at random a sequence this long would be expected to occur only once in 4×10^6 (or 4^{11}) base pairs, there can be little doubt that the frequent occurrence of this sequence on the chromosome has been selected as a means of restricting the uptake of extracellular DNA to homologous DNA.

Shortly after homologous DNA is added to a competent culture of *Haemophilus* the outwardly extending vesicles disappear and internal ones appear. One might presume that the binding of homologous DNA on the surface of the vesicle causes it to invaginate, trapping the molecule of DNA within it. Regardless of mechanism, strong biochemical evidence in addition to the morphological evidence supports the existence within *Haemophilus* cells undergoing transformation of membranous vesicles that contain DNA. They have been termed *transformasomes*. DNA within a transformasome is in a protected state, resistant to the action of DNase, restriction enzymes, and modification enzymes. The DNA remains within the transformasome as it traverses the cytosol existing in single-stranded form only immediately before it recombines with the endogenote.

NATURAL TRANSFORMATION BY PLASMIDS

Intact plasmids are taken up by competent *Haemophilus influenzae* cells, if the plasmid contains the proper 11-base pair sequence found on the chromosome. However, competent cells of *Bacillus subtilis*, which are transformed by chromosomal DNA much as *S. pneumoniae* is, always cleave plasmids as they enter the cells and reduce them to single-stranded form (Figure 3.) Linearized plasmids are incapable of being replicated; they can become a heritable part of the recipient cell's genome only if they are recircularized within the cell, and recircularization is dependent on their being homology between the plasmid and the endogenote (Figure 3). If the cleavage occurs within this region of homology, pairing of it with the endogenote brings the ends in juxtaposition so that a double-stranded circular plasmid can be reconstructed by the action of DNA polymerase and DNA ligase. If the cleavage occurs within the nonhomologous region of the plasmid, recircularization is not possible, and, as a consequence, the plasmid cannot be replicated. The homology between endogenote and plasmid need not be preexisting the transformed plasmid itself can introduce the required homology. If it exists as a tandem dimer or if two copies of the plasmid (cut at different sites) are introduced into the same cell. There is considerable evidence that plasmid transformation of the other well-studied transformable Gram-positive bacterium, *S. pneumoniae*, occurs by the same sequence of events as are shown in Figure 3. And this mechanism also applies to transformation of plasmids of the Gram-negative bacterium, *Pseudomonas stutzeri*.

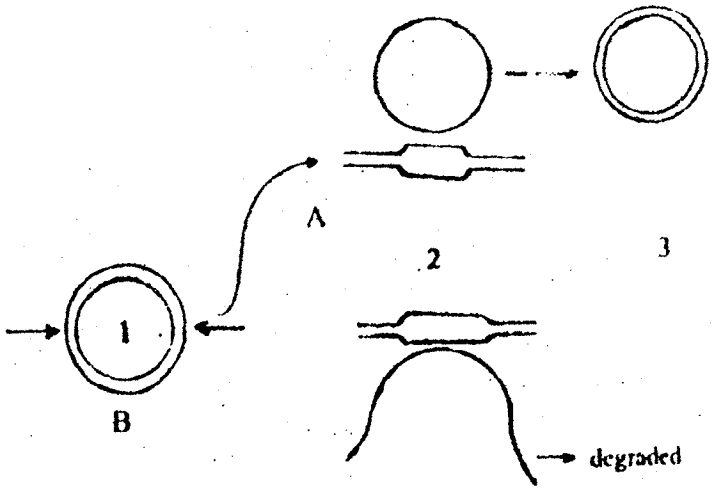


Figure 3. *Model explaining the dependency of the transformability of Bacillus subtilis by plasmids on homology between a region of the plasmid and the endogenote. At the surface (1) of the cell the plasmid is cleaved by a nuclease. If the cleavage occurs within the region of homology, A, the single-stranded form of the linear molecule that enters the cell can pair with the homologous region of the endogenote (2) thereby holding the cut ends in proper position to be joined by the action of DNA ligase. By duplication (3) a double-stranded molecule is generated that can be stably replicated in the recipient. However, if the cleavage occurs within a region, B, that is not homologous with the endogenote, pairing (2) will not promote ligation. This linear molecule cannot be replicated and is eventually destroyed by intracellular nucleases (3). "Plasmid Transformation in Bacillus subtilis' DNA into Plasmid pC 1974:," Mol. Gen. Genet. 181 434 – 440 (1981).*

A

Grow culture to A_{590} 0.85

↓

Chill rapidly to 0°C and centrifuge

↓

Wash once in 0.5 volumes of 10 mM NaCl

↓

Suspend cells in same volume of 30 mM $CaCl_2$

↓

Hold suspension at O°C for 20 minutes

↓

Centrifuge and resuspend in 0.1 original volume of 30 mM $CaCl_2$

↓

Competent cells

B

To a 0.2 ml sample add 0.1 ml DNA solution

↓

Hodl at O°C for 60 minutes

↓

Heat suspension at 42°C for 2 minutes

↓

Chill suspension rapidly to O°C

↓

Plate and incubate cells appropriately

↓

Transformant Clones

Figure 4. Scheme for artificial transforming cells of *Escherichia coli*. A series of manipulations (A) make the cells competent and another series (B) cause the transforming DNA to enter them.

Artificial Transformation

The development of competence and uptake of DNA by naturally transformable bacteria is chromosomally encoded. In *Streptococcus pneumoniae* at least 12 genes govern the process. Interestingly, as we shall see, transformation is the only mechanism of genetic exchange among procaryotes that is chromosomally encoded (transduction occurs as a consequence of aberrant phage development; conjugation is plasmid encoded) and it must have evolved as a mechanism of genetic exchange a biological function that occurs in all major groups of organisms and one that is highly selected. In spite of this, many bacteria, including *Escheichia coli*, do not possess a system of natural transformation. But almost all that have been tested can be made competent for transformation by plasmids by a variety of highly artificial treatments of the culture (as might be expected, these methods are ineffective for naturally transformable bacteria that cleave the incoming plasmid). A scheme for making *Escherichia coli* competent is shown in Figure 4.

Plasmids enter such cells as intact double-stranded molecules. Double-stranded linear molecules also enter but in many cases these are rapidly degraded by intracellular nucleases. In the case of *Escherichia coli* artificial transformation by linear DNA is possible if a mutant strain that lacks two DNA-cleaving nucleases is used as a recipient.

The Role of the Donor Cell in Transformation

In the laboratory, purified solutions of DNA are usually employed in studies on transformation. This begs the question of how DNA becomes available for transformation in nature. Curiously, the role of the donor cell has received very little study. It has been assumed by many bacterial geneticists that the role of the donor cell is completely passive that donation of DNA depends on the occasional and random lysis of certain cells in the population. However, recent experiments suggest that DNA might be actively extruded from certain competent cells by a genetically encoded pathway.

BACTERIAL CONJUGATION

In 1946, J. Lederberg and E.L. Tatum discovered a genetic exchange occurring between certain strains of *E. coli* that eventually proved to be different from transformation in a number of respects; exchange was dependent on direct contact between cells; it occurred even if DNase was present in the medium; and it was polarized – i.e., certain strains designated as F^+ (fertility plus) always acted as donors (*males*) and others designated as F^- (fertility minus) always acted as recipients (*females*). On the basis of these observations it was assumed correctly that genetic material was transferred directly from male to female cells without passing through the suspending medium. The process was termed *conjugation*. In subsequent years it was discovered that F^+ strains all contain a plasmid (termed the *F plasmid*) that carries all the genes that encode conjugative genetic transfer. Indeed, the F plasmid is not known to encode any additional function other than this one and its own replication (Figure 5).

Properties of the F Plasmid

The F plasmid encodes transfer of itself to other cells that lack an F plasmid. Thus, if F^- cells are added to an F^+ culture, all of them rapidly become F^+; i.e., transfer of the F plasmid occurs at a high frequency. At a considerably lower frequency, chromosomal genes are transferred to the F^- cell along with the F plasmid.

The F plasmid shares certain genetic properties with all other plasmid. (1) It carries certain genes (*rep*) that allow it to be replicated by the host cell and, as directed by the genetic region *inc*, (2) it exhibits the phenomenon of incompatibility; i.e., if a certain plasmid is present in a cell, replication of closely related plasmids is inhibited..Thus, closely related plasmids are said to be incompatible because only one member of such a group of plasmids (termed an ***incompatibility group***) can be stably replicated in the same cell. The biochemical mechanism by which incompatability is expressed is not yet understood. The F plasmid belongs to an incompatability group termed IncFI.

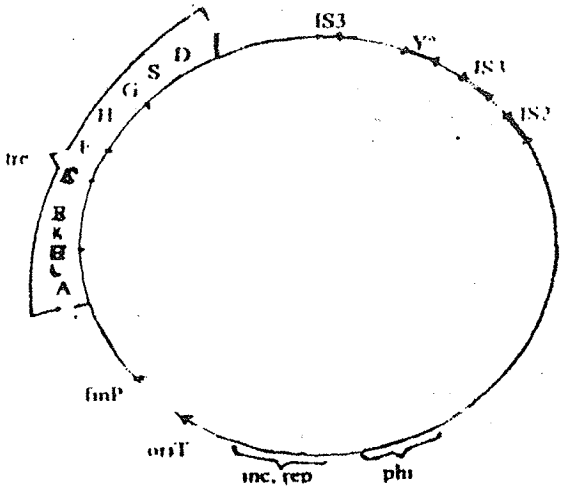


Figure 5. *Genetic map of the F plasmid showing the relative position of genes encoding transfer functions (tra)m fertility inhibition (firi) origin of transfer replication (OrlT), incompatibility (inc)m replication (rep), and phage inhibition (phl). The positions of insertion sequences IS2m IS3, and γδ, are also shown. The length of the genome is 94.5 kilobases.*

Not all plasmids encode self-transfer, as do the F plasmid and a number of others, collectively termed ***conjugative plasmids***. But of those that do and that occur in Gram-negative bacteria, the mechanism of gene transfer appears to be similar. In the case of the F plasmid, transfer is encoded by 13 genes, *traA* through *traL* and *traS*, that form an operon. Among their various **functions, some encode the synthesis of special pili, termed *F pili*** or *sex pili*. Others encode a **special type of replication termed *transfer replication*** of the F plasmid that occurs during transfer and mediates it.

Unlike certain other conjugative plasmids in which expression of the *tra* genes is repressed, the ***tra* genes of F plasmids are always derepressed.** Thus, F^+ cells always have F pili on their outer surface (Figure 6) unless they have been subjected to vigorous shaking, an action that breaks off these long (several mm) brittle appendages. An F plus binds specifically to a protein in the outer membrane (OmpA) of F^- cells, thereby initiating transfer replication and the process of gene transfer by conjugation (Figure 7). A nick (the cutting of a single strand of DNA) is made in the F plasmid at the site termed *oriT* (*origin* of transfer), then a type of replication sometimes termed the *rolling circle* mechanism (see Module-5) ensures in which the intact strand is used as template and the 3' end generated by the nick is used as primer. By this action the 5' end of the nicked single strand is displaced and is transferred to the F^- cell. It is not clear how this transfer occurs. The single-stranded molecule might pass through the hollow core of the F plus or it might pass from the F^+ cell to the F^- at some other point of contact between them. If the latter case is true, the function of the F plus would only be to hold the two cells together so that another direct *conjugative bridge* can form. Within the F^- cell the single-stranded molecule of DNA is duplicated by a chromosomally encoded DNA polymerase and recircularized.

Figure 6. *F plus of E. coli. The donor bacterial cell covered by numerous appendages termed Type I pili (which play no role in conjugation) is connected to the recipient cell (without appendages) by an F pilus.*

The question of how recircularization occurs is also an open one. It has been presumed for some time that the length of single-stranded DNA transferred to the recipient exceeded that of the intact plasmid, and recircularization of the transferred DNA occurred as a consequence of a recombinational crossoverevent (see the section on recombination later in this chapter) between its redundant ends. However, recent studies have shown that recircularization can occur in cells that are genetically incapable of mediating recombination, and there is no evidence for more than a genome's worth of plasmid DNA being transferred to the recipient. Possibly the ends of the transferred piece of DNA become attached to a protein located in the cytoplasmic membrane, thereby holding the ends in appropriate juxtaposition to be joined by the action of DNA ligase. Once circularized, the transferred F plasmid can be replicated in the recipient cell, the genes it carries are expressed, and the cell then becomes F^+.

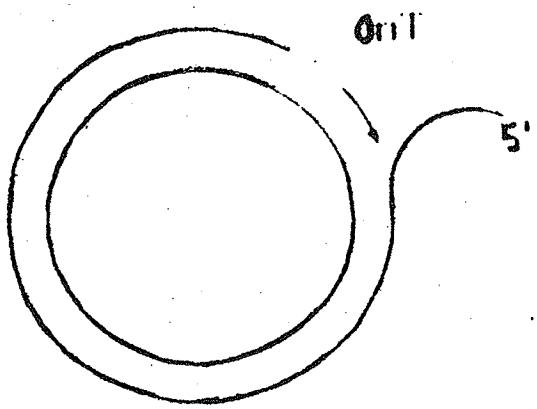


Figure 7. *Transfer replication of an F plasmid. An F-encoded nuclease cleaves one strand of the plasmid at oriT. Then replication at arrow head occurs by a rolling circle mechanism whereby the newly synthesized DNA (coloured) displaces a preexisting single strand, the 5' end of which enters the F cell.*

Hfr Strains

As stated above, when F^+ and F^- cells are mixed, F plasmid is transferred at a high frequency, but at a much lower frequency chromosomal genes are also transferred. This occurs in part as a consequence of the presence in a F^+ culture of cells termed *Hfr* (high frequency recombination) in which the F plasmid and the bacterial chromosome have become integrated into a single large circular molecule. When such a cell comes in contact with an F^- cell, replicative transfer begins within the F plasmid region at *oriT* and continues into the chromosomal region of the large circular molecule. Thus, chromosomal genes as well as F plasmid genes are transferred to the recipient F^- cell. As the bridge between the pair of mating cells is a somewhat fragile one, they frequently brak apart spontaneously, thereby interrupting the genetic transfer. A considerable time (about 100 minutes at 37°C) is required for the entire chromosome to be transferred. The pair of cells rarely stays together long enough for this to happen, so usually the major part of the chromosome is not transferred and, therefore, neither is the portion of the F plasmid at the distal end of the chromosome.

Hfr cells form as a result of a genetic crossover between regions of homology shares by the F plasmid and the bacterial chromosome. These regions are, in all cases studied, identical DNA sequences called *insertion sequences* (see later in this chapter) that also occur at various places in the bacterial chromosome. The F plasmid carries four insertion sequences (Figure 5). The mechanism of formation of an Hfr cell is shown in Figure 8.

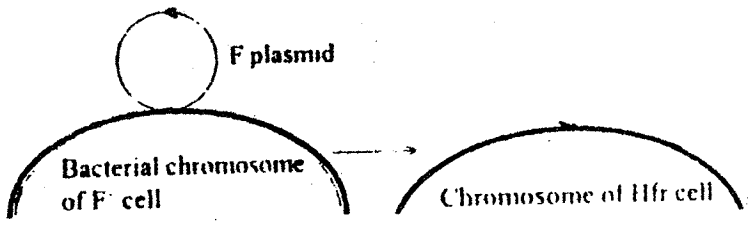
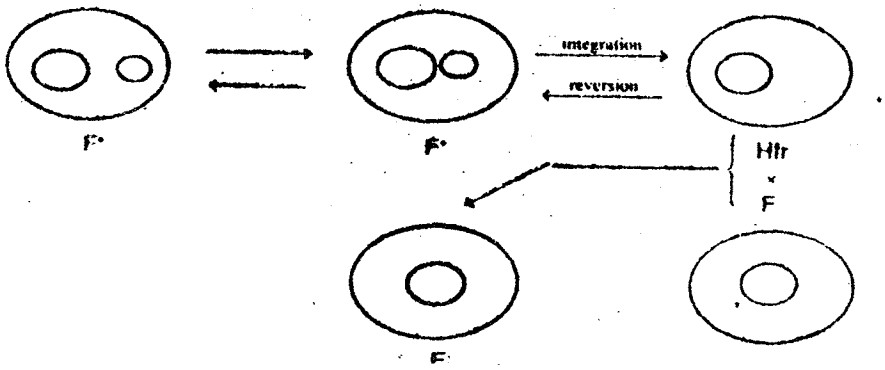


Figure 8. *Mechanism of formation of an Hfr cell from the F⁺ cell. Homologous insertion sequences (colored) pair and by a crossover event the plasmid becomes integrated. The arrow in the F plasmid and the Hfr chromosome indicates the position of ori and the 5' end of the single strand of DNA that enters an F⁻ cell.*

From an F⁺ to an F⁻ cell during conjugation, although the DNA may be passed from one cell to another at sites of contact between them.

The study of conjugation in bacteria was made easier when new strains of cells were isolated from F⁺ cultures which underwent sexual recombination with F⁻ cells at a rate at least 10^3 times greater than F⁺ x F⁻ cells.

FIGURE 8A



An Hfr Cell crises from an F⁺ cell in which the F factor becomes integrated into the bacterial chromosome. During mating of an Hfr and F⁻ cell, the F⁻ cell almost always remains F. This results because Hfr rarely transfers an entire F factor to the F cell. But the recombination frequency is high.

These new donor strains were thus called **high-frequency recombination**,or **Hfr, strains**. Hfr cells arise from F⁺ cells in which the F factor becomes integrated into the bacterial chromosome. They differ from F⁺ cells in that the F factor of the Hfr is rarely transferred during recombination. Thus in an Hfr x F⁻ cross, the frequency of recombination is high and the transfer of F factor is low (Fig. 8A); in an F⁺ x F⁻ cross, the frequency of recombination is low and the transfer of F factor is high.

The order in which chromosomal material is transferred from an Hfr donor to an F⁻ recipient was determined by the *interrupted mating* experiments of **Elie Wollman** and **Francois Iacoh**. An Hfr strain was mixed with an F⁻ strain, and at various times the conjugation was interrupted by breaking the cells apart in a high-speed blender. The cells were then plated on various types of selective agar media in order to select for recombinant cells which had received donor genes before mating was interrupted.

Interrupted mating experiments reveal the order of genes on a chromosome by the time of entry and the frequency of recombination of each marks, which is a detectable mutation serving to identify the gene at the locus or swhare it occurs each gene enters the F⁻ cell at a characteristic time, and a linkage map of the Hfr chromosome can be constructed using time of entry as a measure. This is the principal method of learning where the genes are on a bacterial chromosome (Fig. 9). This is all possible because the Hfr chromosome is transferred to the F⁻ cell in a *linear* fashion (Fig. 10) even though it is a circular chromosome. During transfer the Hfr chromosome begins replicating at the point of insertion of the F factor. Since the F factor can integrate in different positions of the Hfr bacterial chromosome, the first genes to enter an F⁻ cell will vary with different Hfr strains (Fig. 10). This means that the integrated F factor serves as the point of chromosomal opening, and part of it serves as the origin of transfer. The 5' end of the single DNA strand enters the F⁻ cell first.

It takes about 100 min to inject a copy of the whole Hfr *E. coli* gene (i.e. the chromosome and the integrated F factor). Since conjugation is usually interrupted by accident before this can occur, the distal Hfr genes are rarely transferred. Since *all* the Hfr chromosomal genes must be transferred before all the F factor genes are also transferred, most F⁻ recipients remain F⁻ after conjugation with Hfr cells.

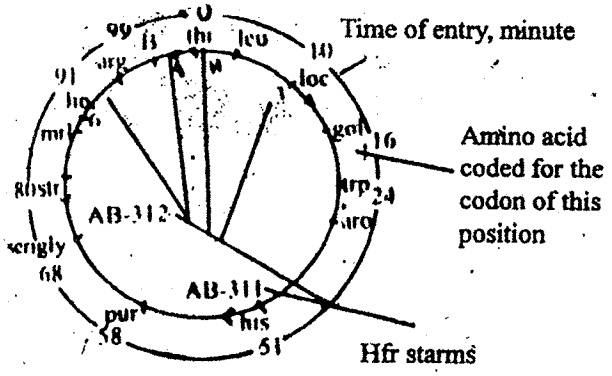


Figure 9. *Simplified linkage map (color) of the circular chromosome of E. coli constructed from interrupted mating experiments using different Hfr strains. The arrows on the linkage map indicate the leading end and direction of entry of the chromosomes injected by each of the Hfr strains the designations of which are shown inside the circle. This is determined by the position of the F factor in each of the Hfr strains. The numbers around the outside of the map show distances as a function of time. in minutes, based on time of entry of each codon in experiments (Note that the map distances in minutes are drawn relative to the Hfr strain H so that the thr gene has been arbitrarily chosen as the origin).*

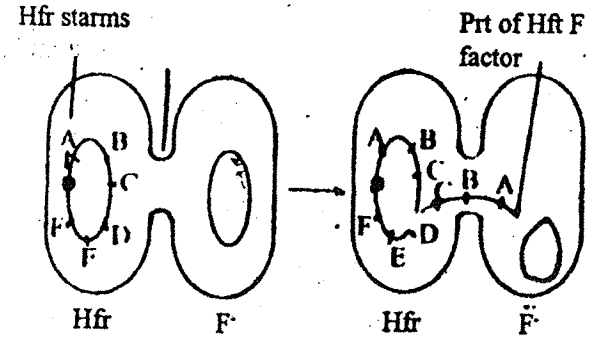


Figure 10. *Mechanism for DNA transfer between Hfr and F⁻ cells. The Hfr chromosome begins replicating at the point of insertion of the F factor. Since the F factor can integrate in different positions of the Hfr bacterial chromosome, the first to enter an F cell will vary with different Hfr strains. As shown the order of genes transferred is ABCDEF. In another Hfr strain, the F factor might be integrated between B and C. In this case the order of genes transferred would be CDEFAB.*

Hfr cells can revert to the F^+ state. When this occurs, the sex factor is measured from the chromosome and resumes its autonomous replication. Sometimes this detachment is not cleanly accomplished, so the F factor carries along with it some chromosomal genes. In this state it is termed an F' factor, and the cell in which this has occurred is called an F' cell (see Fig.11). When such primary F' cells are mated with F^- cells are mated with F^- recipients, the sex factor is transferred very efficiently together with the added bacterial genes. The recipient cell then becomes a secondary F' cell; it is a **partial diploid** for those genes it receives from the primary F' cell. This process whereby bacterial genes are transmitted from donor to recipient as part of the sex factor has been termed **sexduction** by Jacob and Wollman (Fig. 11).

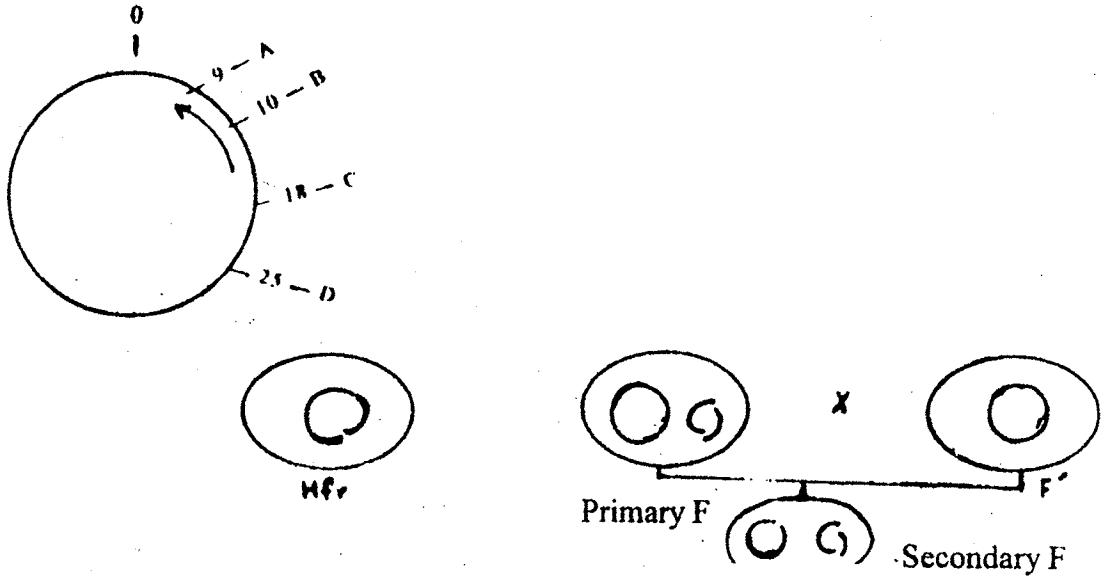


Figure 11. *An F' cell arises from Hfr cells when the F factor and some chromosomal genes are released. During mating of a primary F' cell with an F^- cell, the F^- cell becomes a secondary F^- cell because it now carries the F factor as well as the chromosomal genes from the primary F' cell.*

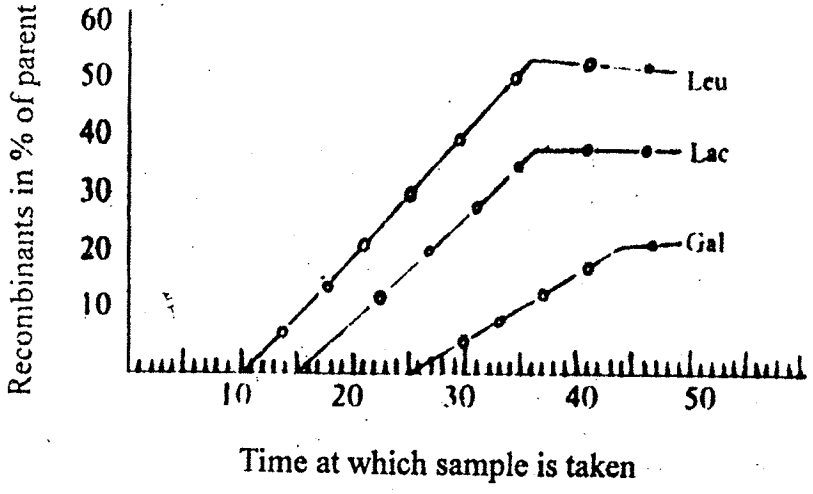


Figure 12.
The kinetics of recombinant formation in an Hfr F cross Sec tex and Table 11.3.

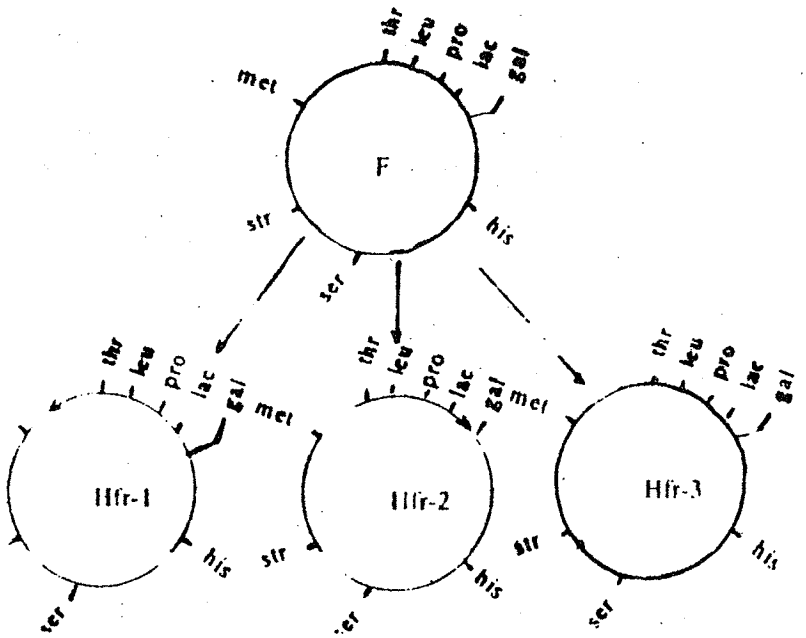


Figure 13.
Schematic representation of the formation of three different Hfr strains from an F strain. Arrowheads on the chromosome indicate the location of integration of the F plasmid and the direction in which the 5' end of a single strand of DNA is donated to an F' cell during conjugation.

F-Mediated Transfer of Other Plasmids

To this point we have considered the ability of the F plasmid to mediate its own transfer to a recipient cell and, under certain conditions, to mediate the transfer of chromosomal DNA. The F plasmid is also capable of mediating the transfer of other plasmids not in themselves capable of self-transfer. Plasmid ColE1 is such a plasmid. If a culture containing ColE1 is mixed with one that lacks it, no transfer occurs, but if the ColE1-containing culture also contains the F plasmid. ColE1 is transferred to the recipient at almost as high a frequency as F itself. The mechanism of the F-mediated transfer of ColE1 is analogous to, but not mechanistically the same as, the transfer of chromosomal DNA by an Hfr strain because ColE1 and F occur in the recipient cell as separate circular molecules of DNA; some more subtle interaction must occur between the F plasmid and the other plasmid. The contransferred plasmid is said to be *mobilized* by F, and the ability of a plasmid to be mobilized depends on its containing a specific region of DNA, termed *mob*. Loss of this region is associated with loss of F-mediated transfer of the plasmid.

Indeed the poorly understood phenomenon of mobilization probably also accounts for some of the transfer of chromosomal DNA in an F^+ F^- cross, because the frequency of such transfers is higher than that which would be expected to result from the number of Hfr cells that an F^+ culture always contains.

Other Systems of Conjugation in Gram-Negative Bacteria

As we have seen in the preceding sections of this chapter, the capacity of *E. coli* to exchange genetic material by conjugation and the way in which this occurs are determined by the presence of the F plasmid in certain strains. This plasmid has the capacity to transfer itself from one cell to another. Occasionally

it interacts with another element of the bacterial genome (the chromosome or another plasmid) causing some or all of this other element also to be transferred.

Therefore, in inquiring about the distribution of conjugative genetic exchange among bacteria, three question arise : (1) in which bacteria can the F plasmid be stable replicated? (2) which other plasmid are capable of self-transfer? (3) do other plasmid that are capable of self-transfer interact with the bacteria genome in such a way as to bring about its transfer? These questions are addressed in the following sections.

The F plasmid can replicate only in enteric bacteria (Chapter 19) so F plasmid mediated conjugation can occur only within this group. But a vast number of other plasmids that are capable of self-transfer exist in Gram-negative bacteria. These plasmids are called *conjugative plasmids*. However not all conjugative plasmids readily mobilize the transfer of chromosomal DNA. In certain cases, those that lack this ability have been shown to gain it when they gain a small bit of DNA shown to be an *insertion sequence*. An example of this phenomenon is seen in the case of plasmid R68, termed a *broad host range* plasmid because it has the capacity to replicate in a large number of, if not all, Gram-negative bacteria. Although plasmid R68 transfer itself at a high frequency from one cell to another, mobilization of the chromosome or another plasmid occurs at only a very low frequency. But derivatives of plasmid R68 have been isolated that mobilize chromosomal and plasmid DNA from *Psudomonas aeruginosa* and a variety of other Gram-negative bacteria at a high frequency. One of these, plasmid R68.45 mobilizes chromosomal DNA from *P. aeruginosa* at about a 10^5-fold higher frequency than plasmid R68 does. It is also able to mobilize efficiently the chromosomes of many other Gram-negative bacteria and has been used to study the chromosomal genetics of a wide range of genera. Plasmid R68.45 differs from plasmid R68 by containing a 2.10 kb insertion sequence designated IS21.

As discussed earlier, the F plasmid also owes its ability to mobilize chromosomal DNA to its complement of insertion sequences. However, the mechanisms of F-plasmid also occur in the chromosome, and mobilization appears to depend on homologous recombination events between these two regions. But IS21 sequences do not occur in the chromosomes of those bacteria that have been investigated in this respect. Thus homologous recombination cannot be the basis for its ability to mobilize. Rather, mobilization in this case appears to be consequence of the ability of the IS21 sequence to undergo transposition, sometimes causing the R68.45 plasmid to become integrated into the chromosome, at least ransiently. Possibly as a consequence of the transient nature of this integration, strains that contain the R68.45 plasmid donate to recipient cells segments of chromosome from multiple origins.

Hfr-like strains derived from plasmids other than F are also encountered in certain Gram-negative bacteria. For example, certain Hfr-like strains of *Pseudomonas aeruginosa* that carry plasmids termed FP have played a major role in mapping the genes on the chromosome of this organism.

Genetic Exchange by Conjugation among Gram-Positive Bacteria

Conjugational genetic exchange has been less frequently encountered among Gram-positive bacteria than among Gram-negative bacteria, and judging from one of the best-studied examples of the former class, that of conjugal transfer of plasmids among strains of *Streptococcus faecalis*, certain aspects of the process differ fundamentally (Figure 14).

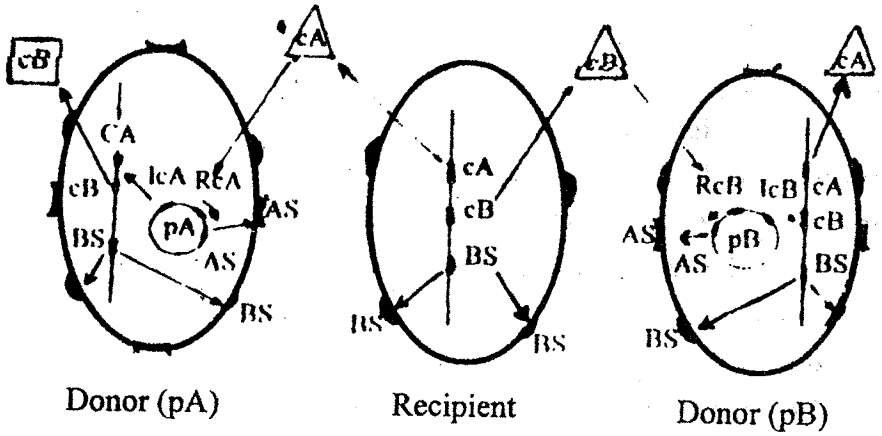


Figure 14. *Conjugation of Streptococcus faecalis. The scheme shows the response of two donor cells (pA and pB) to pheromones (cA and cB) produced by the recipient. These react to chromosomal genes (RcA and RcB) which cause another gene (AS) to produce an aggregation substance (AS) which causes the cells to bind to donors at binding substance (BS) site. Cells that carry a particular plasmid (e.g. pA) do not produce the corresponding pheromone (cA) because the product of a plasmid gene (IcA) inhibits expression of the structural gene (cA).*

Pili do not play a role in this process, rather plasmid-containing cells form clumps with cells that lack the plasmid, and plasmid transfer occurs within these clumps. Clumping results from the interaction between an *aggregation substance* on the surface of the plasmid-containing cell and a ..... of a plasmid-lacking recipient. Binding substance is always present on the surface of cells of *S. faecalis*. Both plasmid-containing and plasmid-lacking cells produce it, but aggregation substance is produced only when a plasmid-containing (donor) cell is in close proximity of a cell that lacks that particular plasmid (recipient cell). The recipient cell produces a chromosomally encoded small molecule (*a pheromone*) that diffuses through the medium, enters the donor cell, and induces a plasmid-encoded gene to synthesize aggregation substance. Although donor cells also carry chromosomal genes that encode synthesis of the pheromone, they do not produce it, and therefore do not produce aggregation substance in the absence of a recipient cell, because the product of a plasmid-encoded gene (*Ica*) represses synthesis of the pheromone.

TRANSDUCTION

Most bacteriophages, the virulent phages, undergo a rapid lytic growth cycle in their host cells. They inject their nucleic acid, usually DNA, into the bacterium, where it replicates rapidly and also directs the synthesis of new phage proteins.

Within 10 to 20 min, depending on the phage, the new DNA combines with the new proteins to make whole phage particles, which are released by destruction of the cell wall and lysis of the cell. However, some bacterial viruses the temperate phages, which ordinarily do not lyse the cell, carry DNA that can behave as a kind of episome in bacteria; like other episomes, such as the F factor, these viral genomes can become integrated into the bacterial genome; they are then known as prophages. Bacteria that carry prophages (lysogenic bacteria) can be *induced* with ultraviolet light and other agents to make the prophages start to replicate rapidly and go through a lytic growth cycle, resulting in lysis of the cell with release of new phage particles. Phage particles may become filled with cell chromosomal DNA or a mixture of chromosomal and phage DNA (rather than completely with phage DNA, as is normally the case). Such aberrant phages can attach to other bacteria and introduce bacterial, rather than just phage, DNA into them. By this means they transfer bacterial DNA from one cell to another. Thus we can define bacterial transduction as the transfer by a bacteriophage, serving as a vector of a portion of DNA from one bacterium (a donor) to another (a recipient).

This phenomenon was discovered by Zinder and Lederberg in 1952 when they searched for sexual conjugation among *Salmonella* species. They mixed auxotrophic mutants together and isolated prototrophic recombinant colonies from selective nutritional media. When the U-tube experiment was carried out with a parental auxotrophic strain in each arm and separated by a microporous fritted glass filter, prototrophs appeared in one arm of the tube. Since the filter prevented cell-to-cell contact but alloee free passage of fluid between the cultures, it could be concluded that some phenomenon other than conjugation was involved. Furthermore, the phenomenon could not be prevented by DNAse activity, thus eliminating transformation as the process for changing the recipient auxotrophs to prototrophy. Further experiments implicated a bacteriophage as the vector or transducing agent in the following manner. The bacteriophage was released from a lysogenic (recipient) culture. The phage through the filter and infected the other strain (donor), lysing it. During replication in the donor strain, the phage adventitiously included parts of the bacterial chromosome with it. It then passes through the filter again, carrying part of the donor's genetic information and imparting it to the recipient strain.

Phages that occasionally produce transducing particles and therefore are capable of effecting transductional genetic exchange are relatively commonly encountered in the microbial world. The host range of bacteriophages, including those capable of mediating transduction, is typically quite narrow. Therefore, in order to make transductional crosses an investigator must, in general, have a different transducing phage for each bacterial species in which crosses are to be made. Nevertheless, it has usually

proven possible to isolate transducing phages for most bacteria when serious attempts have been made to do so.

Two types of trasnsducing particles and therefore two types of transduction exist. One of these is termed *generalized* or *nonspecialized* transduction, because it mediates the exchange of any bacterial gene. The other is termed *restricted* or *specialized transduction* because it mediates the exchange of only alimiited number of specific genes.

Generalized Transduction

If all fragments of bacterial DNA (i.e., from any region of the bacterial chromosome) have a chance to enter a transducing phage, the process is called generalized transduction. In this process, as the phage begins the lytic cycle, viral enzymes hydrolyze the bacterial chromosome into many small pieces of DNA. Any part of the bacterial chromosome may be incorporated into the phage head during phage assembly and is usually not associated with any viral DNA. For example, coliphage P1 can transduce a variety of genes in the bacterial chromosome. (This means that in a large population of phages there will be transducing phages carrying different fragments of the bacterial genome). After infection a small proportion of the phages carry only bacterial DNA (see Fig. 15). The frequency of such defective phage particles is about 10^{-5} to 10^{-7} of the progeny phage produced. Since this DNA matches the DNA of the new bacterium infected the recipient bacterium will not become lysogene for P1 phage. Instead, the injected DNA will be integrated into the chromosome of the recipient cell. Defective P1 phages bearing *E. coli* DNA can be detects by the genetic markers present in that DNA. For instance, if a *thr*$^+$ cell is infected by a phage carrying a fragment of *E. coli* DNA with a *thr*$^+$ gene this *thr*$^+$ gene may be integrated (recombined) into the bacterial chromosome to result in prototrophic recombinant detectable by growth in a medium devoid of ..... nine.

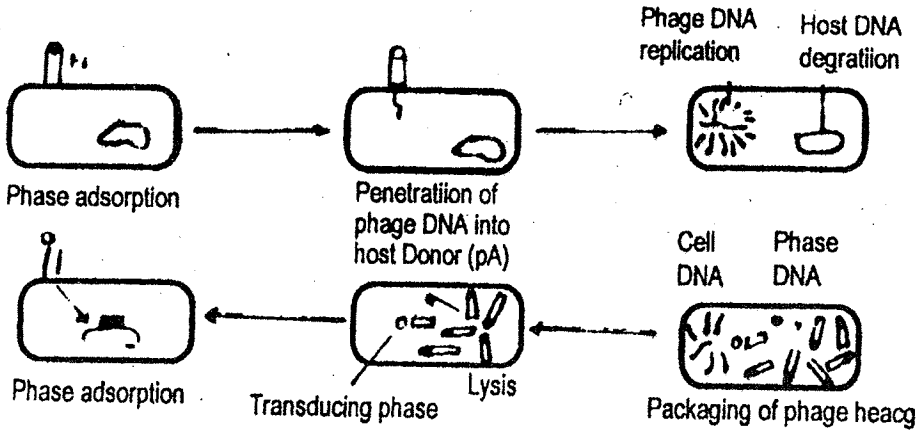


Figure 15. *Generalized transduction. The phage P1 chromosome, after injection into the host cell, causes degradation of host chromosome into small fragments. During maturation of the virus particles, a few phage heads may envelop fragments of bacterial DNA instead of phage DNA. When this bacterial DNA is introduced into a new host cell, it can become integrated into the bacterial chromosome, thereby transferring several genes from one host cell to another.*

Generalized transduction, like bacterial conjugation and transformation also provides a means for mapping bacterial genes since the gragments transferred by a bacteriophage are often large enough to contain hundreds of genes. The mapping technique involves providing to the phage-infected bacteria a growth medium that *selects for* those recombinants that have inherited a given gene ... marker from bacterial DNA carried by a transduction phage. Growth of other media can then be used to test how many of these recombinants have also inherited other donor markers. The closer two markers are together on the bacterial chromosome, the more likely they are to be inherited together by means of a single transducing phage. For example, when coliphage P1 is grown in a *thr*$^+$ *leu*$^+$ *azi*^{R} host and then used to infect a *thr*$^-$ *leu*$^-$ *azi*$^-$ recipient bacterial cell, only 3 percent of the selected Thr$^+$ recombinants are also Leu$^+$ and some are Az^{R}m or azide-resistant. But, if Leu$^+$ recombinants are selected about 50 percent of these are also Az^R. This means that *leu*$^+$ is more closely liked to *czi*^{R} tan it is to *thr*$^+$ and the suggested order is therefore *thr*$^+$ *leu*$^+$ *az*^{R}. Thus the degree of linkage of genes can be measured by the frequencies of contransduction of markers. The fact that only 3 percent of *thr*$^+$ transducing phages also carry *leu*$^+$ shows that these two genes are so far apart hat they are rarely included at the same time in a DNA fragment that goes into the P1 heat. (The P head carries a DNA molecule of slightly less than 10^5 nucleotide pars).

Specialized Transduction

Bacterial genes can also be transduced by bacteriophage in another process called specialized transduction in which certain temperate phage strains can transfer only a few restricted genes of the bacterial chromosome. More specifically, the phages transduce only those bacterial genes adjacent to the prophage in the bacterial chromosome. Thus the process is also called restricted transduction. It occurs when a bacteriophage genone, after becoming integrated as prophage in the DNA of the host bacterium, again becomes free upon induction and takes with it into the phage head a small adjacent piece of the bacterial chromosome. In this way, when such a phage infects a cell, it carries with it the group of bacterial genes that has become part of it. Such genes can then recombine with the homologous DNA of the infected cell.

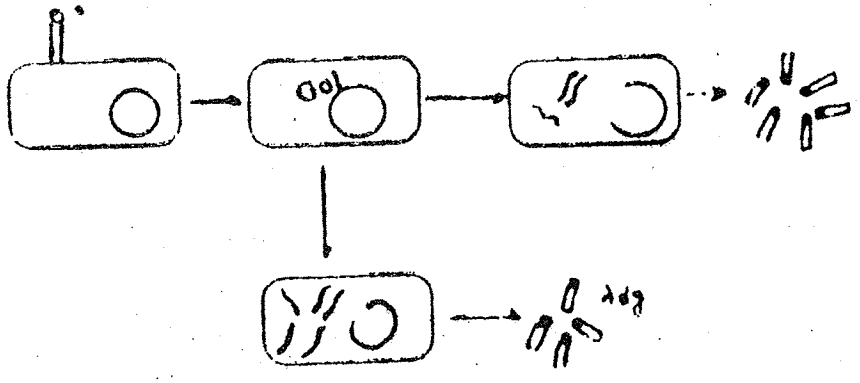


Figure 16. *Specialized transduction. When phage I infects a cell, its DNA is inserted into the bacterial genome next to the genes for galactose metabolism (gal genes). Usually when such a cell is induced, the I DNA comes out, replicates and makes normal phage. However, occasionally the I DNA is excised imperfectly taking gal genes with it and leaving some of itself behind, leading to ldg (defective, galactose transducing phage).*

The best studied specialized transducing phage is the phage lambda (l) of *E. coli*. The location of the l prophage in the bacterial chromosome is almost always between the bacterial genes *gal* and *bio*. Whenever the phage genome comes out of or is *excised* from, the bacterial chromosome, it sometimes takes with it *gal* or *bio* genes. When phages carrying *gal* or *bio* genes infect a new host, recombination with the *gal* or *bio* genes of the host can occur. This process is illustrated in Fig. 16. It should be noted that almost all phages that carry some bacterial genes because of "incorrect" excision are defective in certain viral functions because they are missing a piece of phage genetic information taken up by the bacterial genes. They cannot proceed through their entire replicative cycle, but the cell will yield phages if it is also infected with a complete phage that can code for the missing functions of the defective phages.

Extrachromosomal Genetic Elements (Plasmids)

In addition to the normal DNA chromosome, extrachromosomal genetic elements are often found in bacteria. These elements are called *plasmid* are are capable of autonomous replication in the cytoplasm of the bacterial cell (Figs 11 – 12 and 12 – 23). Plasmids are circular pieces of DNA that are extra genes. Some plasmids are capable of either replicating autonomously or integrating into the bacterial DNA chromosome and are called *episomes*. Thus the F factor of *E. coli* was called an episome because it can alternately exist in the F^+ or Hfr state.

Some bacteria have plasmids that are bacteriocinogenic factors. They determine the formation of bacteriocins, which are proteins that kill the same or other closely related species of bacteria. The bacteriocins of *E. coli* are called colicins; those of *Pseudomonas aeruginosa* are called pyocis, and sme. Bacteriocins have proved useful for distinguishing between certain strains of the some species of bacteria in medical bacteriological diagnosis. Bacteria possess other kinds of plasmids called R plasmids which confer resistance to a number of antibiotics. Some of the R plasmids can be transferred to other calls by conjugation, hence the term infectious resistance. Each form of resistance is due to a gene whose product is an enzyme that destroys a specific antibiotic.

THE MAJOR GROUPS OF PLASMIDS

Recent studies have shown that a high percentage of bacteria contain one or more types of these dispensable genetic elements, and studies on them have distinguished and characterized many hundred different types. This complex array has been grouped or classified on the basis of a number of their properties (Table 3). All plasmids share the properties of being dispensable, self-replicating, circular molecules of double-stranded DNA, but they differ in many other respects, including among others their size, the number of copies of them per cell, their compatibility with other plasmids, the host bacterium in which they can replicate, and the types of functions they encode.

Table 3

Properties of Certain Plasmids Studied in Enteric Bacteria and Pseudomonas

Plasmid[a]	Incompatibility Group	Host Range	Phenotype	Size (kb)
F	IncF1	Many enteric	Tra^+	94
R109	IncH	*Salmonella spp., E. coli*	Tra^+, Ap, Cm, Sm, Sa, Tc	180
RP1	Inc P1	Almost all Gram negative Bacteria	Tra^+, Ap, Km, Nn, Tc	
PBR322		Some enteric bacteria	Tra^+, Ap, Tc	

In the past no system existed to designate plasmids; a variety of letters and numbers were used. Now plasmids are designated by a lowercase "p" followed by two capital letters and a number, e.g. pBR322.
Over 23 incompatibility groups are known for plasmids found in enteric bacteria.
Tra^+, encodes self-transfer, Ap; Cm, Nm, Sm, and Tc encode resistance respectively to the antibiotics ampicillin. Chloramphenicol, kanamycin, neomycin, streptomycin and tetracycline. Su encodes resistance to sulfa drugs belongs to the same group as ColE1 which has not yet been designed.

Detection and Isolation of Plasmids

Often the mean G + C (guanine + cytosine) content and therefore the density of the DNA of a plasmid differs sufficiently from that of the chromosome of the host cell so that these two components of a cell's genome can be separated and subsequently purified by submitting the DNA fraction of a cell extract to ultracentrifugation in a density gradient of cesium chloride.

Plasmids can also be detected and isolated by subjecting cell extracts to *gel electrophoresis*. The extract is placed on the edge of gel slab (the usual gelling agent is a modified form of agar, termed *agarose*) and subjected to an electric field that causes the negatively charged DNA molecules to migrate towards the positive pole. The rate of migration and therefore the position in the gel slab at the completions of the process depends on the size of themolecule and whether it is in linear, open circular, or superhelical form. The location of the separated bands of DNA in the gel can be visualized ... under ultraviolet light when stained by ethidron bromide.

R Factors

Many plasmids carry genes that confer on the host cell resistance to antibiotics and other toxic agents. Plasmids of this type are sometimes called *R factors*. The rapid spread in recent years of R factors among pathogenic bacteria has had profound effects on medical practice.

They spread rapidly by conjugative transfer through a bacterial population and persist under the selective pressure of drug treatment. Some are self-transferable and others are mobilized by other conjugative plasmids (for details consult Module 3).

Other Plasmid-Encoded Characters

Plasmids also encode a variety of other functions. Some encode toxins, the production of which causes certain bacteria to be pathogenic to mammals. Other encode functions that make certain bacteria pathogenic to plants, or able to synthesize pigments or antibiotics, or to degrade certain unusual carbon sources. The ability of *Rhizobium* to invade root hairs of plants as the initial step in forming a nitrogen-fixing nodule is also encoded by plasmids. Examples of some other many bacterial functions known to be encoded on plasmids are listed in Table 4. In spite of the many recent advances made in discovering which cellular functions are encoded by plasmids, some plasmids have been detected that have no known function other than the ability to encode their own replication; these are called *cryptic* plasmids.

Incompatibility among Plasmids

It has been known for some time that certain pairs of plasmids (for example, a F plasmid and an F' plasmid, or two different kinds of F' plasmids) cannot be stably replicated in the same bacterial cell. Two such plasmids are said to be *incompatible*, and a collection of incompatible plasmids constitute an *incompatibility group*. A variety of evidence suggests that plasmid members of the said incompatibility group (designated Inc followed by a capital letter and sometimes also a number), are closely related, so this property is often used as a system of classification. Knowing to which incompatibility group a particular plasmid belongs often reveals other facts about it. For example, members of the IncP1 incompatility group all have the property of being able to replicate stably in a broad range of bacterial host cells.

The Agrobacterium Plasmid Ti

A crown gall tumor found in many dicotyledonous plants is caused by the bacterium Agrobacterium tumefaciens. The tumor-causing ability resides in a plasmid called Ti. When a plant is infected, some of the bacteria enter and grow with the plant cells and lyse there, releasing their DNA in the cell. From this point on, the bacteria are no longer necessary for tumor formation. By an unknown mechanism a small fragment of the Ti plasmid (the T DNA), containing the genes for replication, becomes integrated into the plant cell chromosome. The integrated fragment breaks down the hormonal regulated system that controls cell division and the cell is converted to a tumor cell. This plasmid has recently become very important in plant breeding because specific genes can be inserted into the Ti plasmid by recombinant DNA techniques, and sometimes these genes can become integrated into the plant chromosome, thereby permanently changing the genotype and phenotype of the plant. New plant varieties having desirable and economically valuable characteristics derived from unrelated species can be developed in this way.

Broad Host Range Plasmids

Most plasmids can exist in only a limited number of closely related bacteria, these are called narrow host range plasmids. However, the self-transmissible R plasmids of incompatibility group IncP of E. coli and of group IncP or Pseudomonas aeruginosa are notable in that they can be transferred to and maintained in bacteria of a large number or species. These are called broad host range plasmids. Why some plasmids have a narrow host range and others a broad host range is unknown. The broad host range plasmids have recently become exceedingly useful since they integrate (albeit at low frequency into the host chromosome of numerous species and have thereby enabled the establishment of genetic systems in species in which mapping had not previously been possible. In this way, genetic maps have been obtained for several economically important bacteria; such mapping facilitates the construction of strains with desirable characteristics.

Most of these plasmids are able to mobilize the chromosome only at very low frequency (ca. 10^{-8} per cell). However, by various genetic and recombinant DNA techniques variants of these plasmids have been constructed in which chromosome mobilization is enhanced by a factor $10^3 - 10^5$.

PLASMIDS

Others Plasmids

Several plasmids render fairly innocuous plasmid-free cells pathogenic. For example, the Ent plasmids of E. coli synthesize enterotosins that are responsible for travelers' diarrhea. A plasmid called Hly (for hemolysis) has been found in E. coli strains isolated from pigs. Whereas the hemolysin destroys red blood cells in blood samples, the Hly plasmid does not seem to cause and pathogenicity. Certain plasmids residing in the human pathogen, staphylococcus aureus, enhance pathogenicity. A penicillinase (penicillin-destroying) S. aureus plasmid has been carefully studied. Many species of Pseudomonas can utilize several hundred organic compounds as carbon sources-in particular, such toxic substances as chmphor, toluene, and octane. This metabolic ability resides in a set of plasmids collectively known as degradation plasmids. Each plasmid provides one or more metabolic pathways to degrade these compouns. Since many enzymes are needed, the plasmids are fairly large (molecular weight of 50 – 100 x 106). These plasmids enable bacteria to degrade many synthetic compounds and hence are making an important contribution in the removal of environmental pollutants. For example, some strains can degrade persistent chlorinated hydrocarbons, herbicides, and various detergents. Many laboratories are attempting to use genetic engineering to construct "super plasmids", which can be used for pollution control and chemical syntheses.

Plasmids have also been isolated that confer resistance to toxic metal ions. Such plasmids, whose origin is unknown, are found in environments containing these ions, such as the sludge produced by industrial reprocessing of photographic film (resistance to the Ag^+ ion). In only one case has the biochemistry of resistance been elucidated : resistance to Hg^{2+} ions results from a plasmid encoded reductase that converts Hg^{2+} to metallic mercury, which is sufficiently volatile that it evaporates away.

Table 4. Some Cellular Functions Known to be Encoded by Plasmids

A. IN ALL PLASMIDS
   1. Self-replication

B. IN SOME PLASMIDS
   1. Self-transfer
   2. Resistance to antimicrobial agents.
      a. Antibiotics; e.g., streptomycin, penicillin, kanamycin, neomycin, chloramphenicol, tetracyclin.
      b. Synthetic chemotherapeutic agents; e.g., sulfa drugs and trimethoprin
      c. Heavy metals; e.g., Ag, Hg and Cd.
   3. Pigment production.
   4. Toxin production
   5. Catabolic functions; e.g., lactose, octane, and camphor degradation.
   6. Phage-sensitivity and resistance
   7. Antibiotic production
   8. Bacteriocin production
   9. Induction of plant tumors
   10. H_2S production
   11. Host controlled restriction and modification.

RECOMBINATION

Recombination occurs in procaryotes, as it does in eucaryotes, between homologous regions of DNA, be unlike the situation in eucaryotes, recombination is almost always as essential component of genetic exchange of chromosomal genes among procaryotes because the fragments of DNA transferred by transformation, or conjugation are rarely capable of self-replication (they do not constitute a replicon). For the genes carried on the transferred fragment to be stably inherited by the recipient clone, the portion of the fragment that contains them must be incorporated into a replicon (the chromosome or a plasmid) within the recipient cell by two recombinational events. A single recombination (or crossover) between a linear fragment and the chromosome does not yield a circular molecule, rather it yields a linear structure which cannot be replicated. In contrast a recombination between a circular element and the chromosome yields an integrated circular structure (Figure 7).

Four different types of recombination can be distinguished : *homologous or general recombination, illegitimate recombination, site specific recombination* and *replicative recombination I* (Table 5).

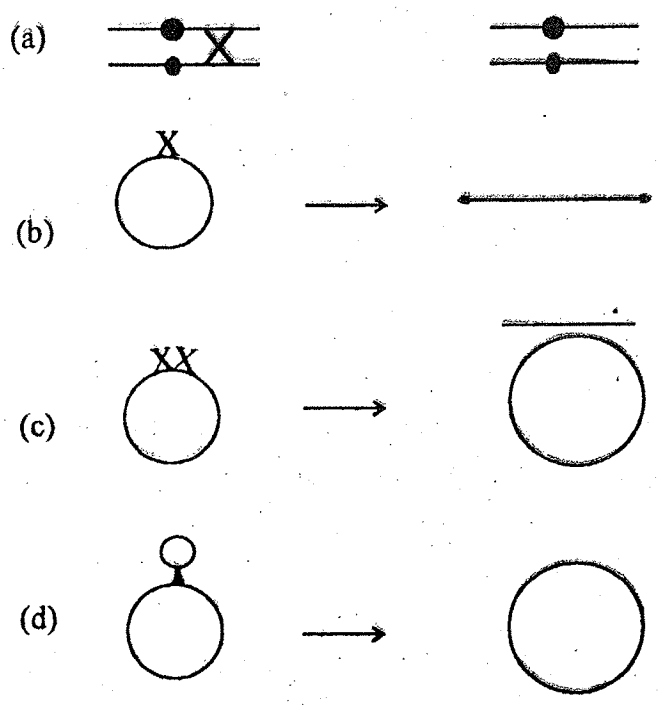


Figure 17. Consequences of crossover between various genetic elements. (a) A single crossover between two eukaryotic chromosomes produces two recombinant chromosomes both of which are replicons. (b) A single crossover between a linear fragment and a bacterial chromosome produces a single linear molecule that is not a replicon. (c) two crossovers between a linear fragment and a bacterial chromosome integrates a portion of the fragment into chromosome and produces a fragment that is not a replicon. (d) A single crossover between a plasmid and a bacterial chromosome produces a slightly larger chromosome with the plasmid integrated into it.

Most recombination including those associated with genetic exchange between cells, occur between homologous regions of DNA by general recombination; these are catalyzed by products of a set of chromosomal genes, *recA* through *recF*. The mutational loss of activity of some of these causes only a decrease in the frequency of general recombination, suggesting that general recombination can occur by at least two paralle pathays. But loss of *recA* function completely eliminates general recombination, implying that both pathways require the participation of the RecA protein as the product of the *recA* gene is called.

Table 5.

Types of Recombination

I. General recombination : can occur between any pair of homologous sequences of DNA.

II. Illegitimate recombination : occurs between nonhomologous regions of DNA.

III. Site-specific recombination : occurs between certain homologous sequences of DNA, e.g., between a temperate phage and bacterial chromosome.

IV. Replicative recombination : occurs between certain genetic elements (transposable elements) and many other consequences.

Illegitimate recombination is a rare event that occurs between nonhomologous regions of DNA. Loss of RecA does not affect the frequency of illegitimate recombination; indeed, recombinations that occur in the absence of RecA can be assumed to have occurred as a consequence of illegitimate recombination.

Site-specific recombination is typified by the event that leads to *reduction* of lambda and certain other temperate phages to the prophage state. Recombination occurs within homologous segments shared by the phage and the chromosome (the *att* site), and is catalyzed by phage-encoded enzymes. As a consequence, site specific recombination does not require the participation of the RecA protein.

The transposition of insertion sequences and transposons is mediated by replicative recombination. Like site-specific recombination, replicative transposition is catalyzed by proteins encoded in genes on the genetic element undergoing recombination; it also occurs in the absence of RecA protein.

Molecular Mechanism of General Recombination

The final consequence of general recombination is the same as breaking two double-stranded molecules of DNA and rejoining each to the other. Several plausible models by which this might occur have been proposed, one of which is shown in Figure 18. The two DNA duplexes pair, nicks are made in homologous regions of two strands with the same polarity, thus allowing them to pair with the complementary strand of the other duplex. It is at this point that the RecA protein functions in an ATP-driven reaction. On resealing the nick, a single strand crossover has been effected. In this form the crossover point is free to migrate in either direction process termed *branch migrations*, creating heteroduplex regions composed of one stand from each of the original DNA duplexes. Rotating this structure enables a planar molecule to be visualized. At this point paired nicks are again made in two strands with the same polarity, and these are resealed to each other. If these nicks are not made in the strands not involved in the original crossover, general recombination is accomplished the net result of which is the joining of one of the original strands to the other at a point composed of a short heteroduplex region. If, on the other hand, the second pair of nicks and subsequent exchange is made in the strands that were involved in the original crossover, no general recombination occurs; only a short heteroduplex region is created.

Insertion Sequences, Transposons, and Replicative Recombination

In this references have been made to transposable genetic elements, termed *insertion sequences* and *transposons*, that have the ability to transpose to various sites on the bacterial genome. When such a transposition occurs, two copies of the element are generated, one at its original site and a second at the new one. Because of this, the mechanism by which these transposable elements are inserted at the new site is termed *replicative recombination.*

Insertion sequences and transposons differ in two respects : size and gene content. Insertion sequences are small elements (slightly smaller or larger than 1 kb) that encode only their capacity for replicative recombination. The latter are larger apparently composite elements (upto about 10 kb in length) terminated by insertion sequences and containing genes within the central region, termed the *core*, that encode various functions, typically resistance to an antibiotic, but sometimes the capacity to degrade a particular compound as a source of carbon. The capacity of transposons for replicative recombination resides in their terminal insertion sequences. The properties of some insertion sequences and transposons are summarized in Table 6.

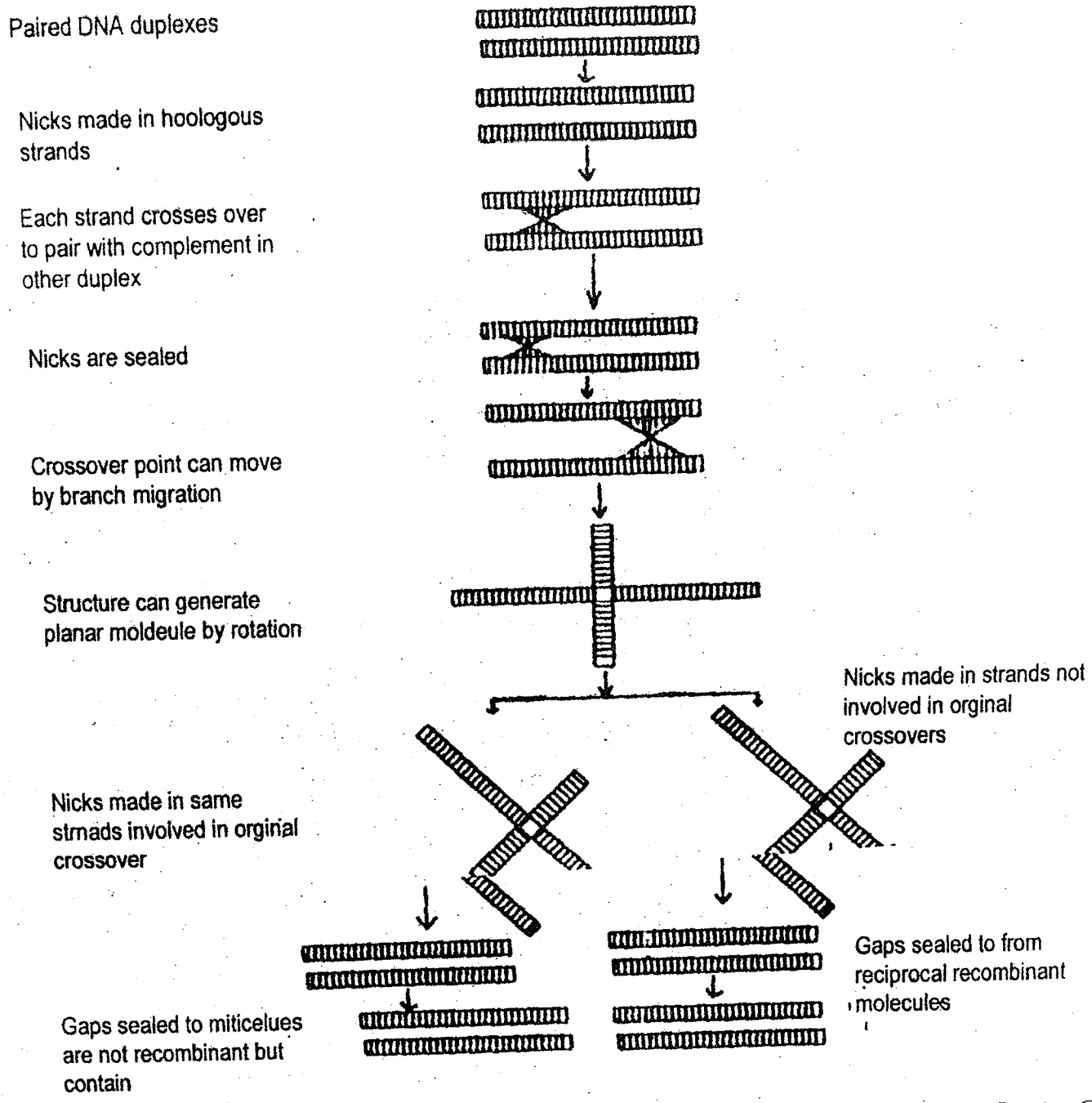


Figure 18. *Model of the molecular events leading to general recombination. After B, Lewin, Gen (New York, Chichester, Brisbane, Toronto and Singalore : John Wiley, 1983).*

Table 6.
Properties of Selected Insertion Sequences and transposons

Element (designation)	Size (bp)	Polarity[E] (orientation)	Properties Encoden[b]
Insertion Sequences			
IS1	768	Both	None

IS2	1,327	One	None
IS3	1,400	One	None
Transposons			
Tn1	4,957	One	Ampicillin
Tn5	5,400	Both	Kanamycin[c]
Tn9	2,638	Both	Chloramphenicol
Tn10	9,300	Both	Tetracycline

[a] Indicates that transcription and therefore expression of distal sequences in a gene or genes in an operon is inhibited if the element is inserted in one or the other orientation.
[b] All these elements encode proteins mediating their own transposition.
[c] In *E. coli*, Tn5 expresses only a protein that confers resistance to kanamycin. In most other bacteria it expresses a second protein conferring resistance to streptomycin.

Certain of the properties of transposable genetic elements are revealed by experiments done by E. Lederberg that leg to the discovery of insertion sequences. Among a group of mutant clones that had lost the capacity to metabolize galactose as a carbon source because they no longer synthesized galactokinase, the enzyme encoded by *galK*, she found that some were found to carry mutations with unusual properties. Although *galK* function had been lost, the mutational events had occurred in a more promoter proximal gene, *galT* or *galE*, of the same operon; these mutations eliminated the activity of the product of the gene in which they occurred and in all downstream genes of the operon. Thus these mutations fell into a class of mutations, termed *polar*. Some nonsense and deletion mutations exhibit polar effects, but the one under investigation had properties that different from nonsense and delectionmutations. They were more polar (they produced lower levels of the products of the downstream genes); they were not suppressed by nonsense suppressor mutations (as nonsense mutations are); and they reverted to wild type at a low but significant rate (as deletion mutations never do). Subsequent experiments established that the mutations were not caused by microlesions in the best sequence of the DNA of the affected gene, nor by a loss of nucleotide pairs, but rather by the gain of new base sequences. Further, it was shown that a number of different mutations were caused by the gain of the same sequence. This class of mutation causing sequences was termed *insertion sequences*. Later it was shown that these elements encoded their own transposition and were responsible as well, for the transposition capacity of transposons. The insertion sequences at the ends of transposition occur in either possible orientation with respect to one another – as the same sequence of bases reaching in the same direction (*direct repeats*), or the reverse (*inverted repeats*). A plausible mechanism of replicative recombination is shown in Figure 19.

The discovery of transposons that undergo replicative recombination at relatively high frequency (Tn5 on introduction into a new cell undergoes replicative recombination at a frequency of about 10^{-5}) provided an explanation for the spread throughout a bacterial population of certain genes such as those that encode resistance to antibricons. Such genes on transposons can transpose to plasmids, thereby forming

multiple drug resistance plasmids. These plasmids can then be transferred to other strains, and if the plasmids have broad lost range properties, the resistance genes can be spread to other species and genera.

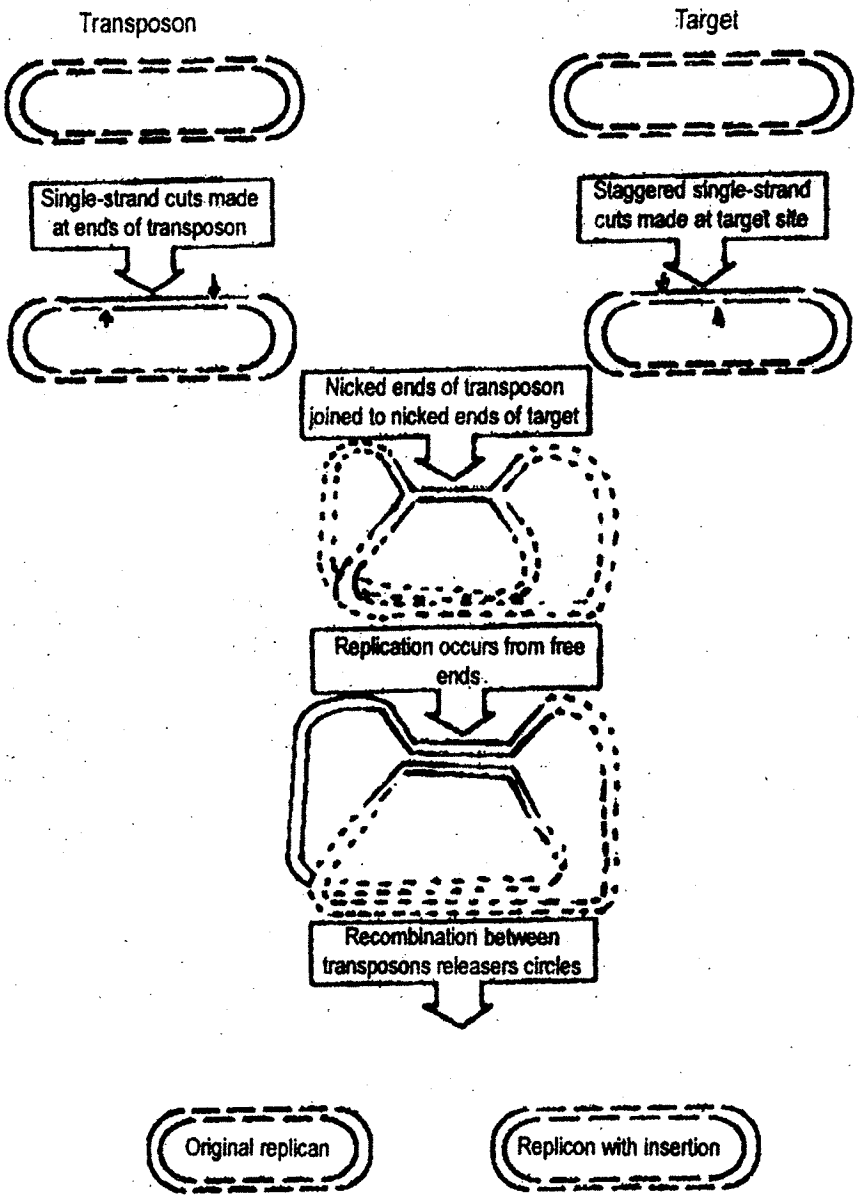


Figure 19. *Model of the molecular events leading to replicative recombination. After B. Lewin, Genes (New York, Chichester, Brisbane, Toronto and Singapore : John Willey, 1983).*

Suggested Question

1. Mention the function of the following enzyme in DNA Replication. (1) DNA polymerages, (2) topoisomerase-1, (3) DNA gyrase.
2. Explain how interrupted mating experiments are
3. Describe the relationship between plasmid and episomes.
4. Differentiate between generalized transduction and restricted transduction.
5. State the art of transformation in *streptococcus pneumoniae*. How does it differ from *Hemophillus influenzae*? Or Write notes on :

   (i) plasmid; (ii) Insertion sequence; (iii) Transposon.

CHAPTER – 2

Genetic Engineering

In addition to advancing the understanding of natural phenomena, genetics is also important in manipulating biological systems, for scientific or economic reasons, an endeavor that has made possible the creation of organisms having new phenotypes or genotypes. The fundamental techniques for accomplishing this have been mutagenesis, gene transfer, and genetic recombination followed by selection for desired characteristics. When using such techniques, geneticists have been forced to work with the random nature of mutagenic and recombination events, which required selective procedures, often quite complex, to find an organism with the required genotype among the many types of organisms produced. Recently, a **new technique has been developed by which the genotype of an organism can inserted be modified in a *direct and predetermined* way. In this technique, which is alternately called recombinant DNA technology, genetic engineering, or gene cloning,** purified DNA fragments are isolated and recombined by in *vitro* manipulations. Selection of a desired genotype is still necessary but the probability of success is usually many orders of magnitude greater than that with traditional procedures. The recombinant DNA technology has greatly enhanced our ability to manipulate organisms and has revolutionized the study of gene structure and regulation. The basic technique is quite simple: two DNA molecules are isolated and cut into fragments by one or more specialized enzymes and then the fragments are joined together in a *desired combination.* Finally, they are restored to a cell by the $CaCl_2$ transformation procedure for replication and reproduction.

THE JOINING OF DNA MOLECULES

The basic procedure of the recombinant DNA technique consists of two stages (1) joining a DNA segment (which is of interest for some reasons) to a DNA molecule that is able to replicate, and (2) providing a milieu that allows propagation of the joined unit (Figure 1). When this is done, the genes in the donor segment are said to be **cloned** and the carrier molecule is the **vector** or **cloning vehicle.**

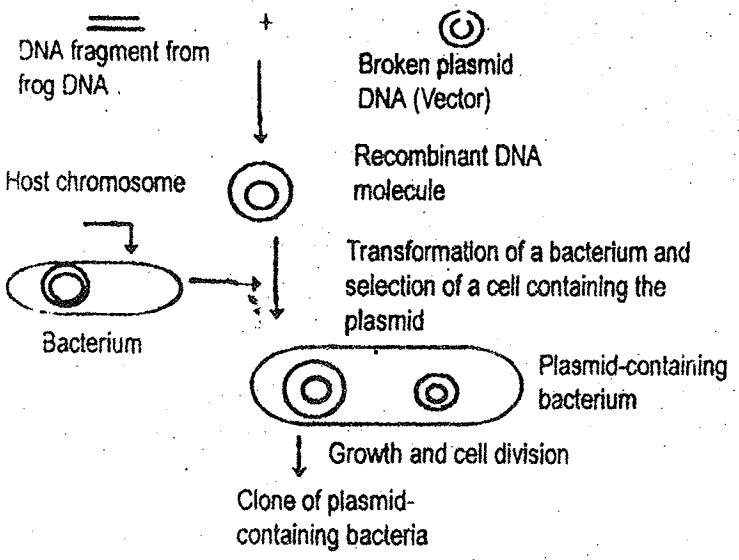


Fig. 1. *An example of cloning. A fragment of frog DNA is joined to a cleaved plasmid vector. The hybrid plasmid transforms a bacterium. Henceforth, frog DNA is carried to all progeny bacteria by replication of the plasmid.*

Vectors

To be useful, a vector must have three properties :

1. It must be able to replicate.
2. There must be some way to introduce vector DNA into a cell.
3. There must be some means of detecting its presence, preferably by a plating test.

The three most common types of vectors in use are plasmids, *e. coli* phage λ, and viruses, because the DNA of each of these vectors has all three of the aforementioned properties. That is, each has a replication origin and carries genes that are identifiable by simple plating or biochemical tests, and the DNA can be made to penetrate particular cells by the $CaCl_2$ transformation technique. Only a small number of vectors are in common use; some of these vectors and their properties are listed in Table 1.

Restriction Enzymes

The existence of restriction enzymes was first postulated by Werner Arber in the early 1960s which studying bacteriophages. Arber found that when virus DNA entered a bacterium it was cut up into smaller pieces and destroyed. He theorized the presence of *Restruction enzymes'* that could destroy the infecting viral DNA without affecting the DNA of the host bacterium. He also proposed that the enzymes recognised and acted at *specific sites* on the viral DNA.

DNA restriction was discovered as an outgrowth of the study of host restrict and modification of phages, described for phase λ in this Chapter. An attempt to account for the site specificity of the DNA degradation led to the isolation of novel nucleases called restriction enzymes or **restriction endonucleases**. These are enzymes that recognize a specific base sequence in a DNA molecule and make two cuts, *one in each strand*, generating **3'-OH and 5'-P termini**. Nearly 1000 different restrict enzymes have been purified from hundreds of different microorganisms. All but a few of these enzymes recognize sequences that are nucleotide **palindromes**.

Table 1. Some cloning vehicles in use at present

Designation	Genotype of characteristics
Plasmid	
pSC101	*tet-r*
colE1	*immE1*
pBR322	*tet-r amp-r*
pUC8/9	*amp-r lac*$^+$
pHC79	*amp-r tet-r λ cos*
Phage	
λgt4-λB	Can lysogenize; is thermally inducible.
λ-Charon	*lacZ*$^+$
λΔz1	Insertion occurs in *lacZ* gene.
M13mp7	Contains *lacP, lacO,* and *lacZ*; single-stranded DNA; useful for sequencing.
Virus	
SV40	Virus infects animal cells. Maximum size of added DNA is no more than $3x10^6$ molecular weight units.

The DNA sequences at which the restriction enzymes make breaks show *two-fold symmetry*, with the same sequence running in opposite directions in the two strands (*palindromic sequences*). Fig. 2.

The endonuclease EncoR1 cleaves the two strands of the circular plasmid DNA between G and A nucleotides of the palindromic sequence.

↓

5' CAATTC 3'
3' CTTAAG 5'

↑

Classification of Restriction enzymes:

There are two major types of restrict enzymes : type I enzymes, which recognize a specific sequence but make cuts elsewhere; and type II enzymes, which make cuts only *within* the recognition site. Type II enzymes are the most important class and we confine our attention to them. All restriction enzymes of this class make two single-stand breaks, one break in each strand. There are two distinct arrangements of these breaks : (1) both breaks at the center of symmetry (generating **flush or blunt ends**), or (2) breaks that are symmetrically placed around the line of symmetry (generating **cohesive ends**). These arrangements and their consequences are shown in Figure - 3. Table 2 lists the sequences and cleavage sites for twelve useful restrict enzymes, nine of which generate cohesive sites and three of which yield flush ends.

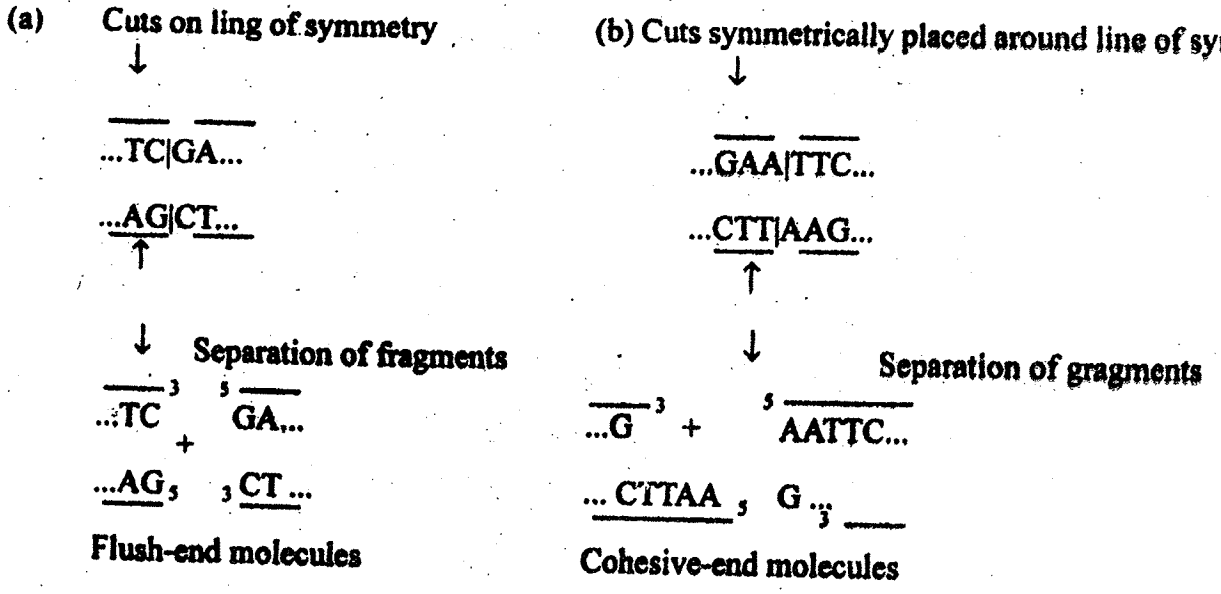


Fig. 3. Two types of cuts made by restriction enzymes. The arrows indicate the cleavage sites. The dashed line is the center of symmetry of the sequence.

Compassion of two type of Restriction enzymes.

	Class I enzyme		Class II enzymes
1.	Non-specific in cleavage	1.	Cleavages is site-specific
2.	MW 300,000	2.	Lower MW (20,000 - 100.00)
3.	Conain non-identical subunits	3.	Contain two identical subunits
4.	Require ATP, Mg^{++} and generally S-adenosyl-methionine as co-factors.	4.	Require only Mg^{++} as co-factor.
5.	Examples : EcoK, EcoPI, EcoB	5.	Example : EcoRI.

Table 2. Some restriction endonucleases and their cleavage sites

Microorganism	Name of enzyme	Target sequence and Cleavage sites
Generate cohesive ends		
E. coli	EcoRI	G A A ; T T C C T T : A A G
Bacillus amyloliquefaciens H	BamHI	G G A : T C C C C T : A G G
B. globigii	BglII	A G A : T C T T C T : A G A
Haemophilus aegyptius	HaeII	Pu G C : G C Py Py C G : C G Pu
Haemophilus influenza	HindIII	A A G : C T T T T C : G A A
Providencia stuartii	PstI	C T G : C A G G T C : G T C
Streptococcus albus G	SalI	G T C : G A C C A G : C T G
Thermus aquaticus	TaqI	T C : G A A G : C T
Generates flush ends		
Brevibacterium albidum	BalI	T G G : C C A A C C : G G T

Microorganism	Name of enzyme	Target sequence and Cleavage sites
Haemophilus aegyptius	HaeI	$\begin{matrix}\downarrow\\ \binom{A}{T}GG:CC\binom{T}{A}\\ \binom{A}{T}CC:GG\binom{T}{A}\\ \uparrow\end{matrix}$
Serratia marcescens	SmaI	$\begin{matrix}\downarrow\\ CCC:GGG\\ GGG:CCC\\ \uparrow\end{matrix}$

Note : The vertical dashes line indicates the axis of dyad symmetry in each sequence. Arrows indicate the sites of cutting. The enzyme TaqI yields cohesive ends consisting of two nucleotides, whereas the cohesive ends produced by the other enzymes contain four nucleotides. The enzyme HaeI recognizes the sequence GGCC whether the adjacenet base pair is A-T or T-A, as long as dyad symmetry is retained Pu and Py refer to any purine and pyrimidine, respectively.

Nomenclature: The enzyme is designated by a three-letter abbreviation for the *host organism*, followed by a fourth letter designating the *strain*. When required, Roman numerals are used to indicate the different *restriction-modification systems* in a strain, when more than one enzyme is obtained from the same organism. Thus the designation *Hind III* stands for *Haemophilus influenzae*, serotype *d*, enzyme III.

Cleavage. Restriction enzymes recognize and cleave at specific sites on DNA. Most of these sequences are *tetranucleotide* or *hexanucleotide* pairs which show a characteristic two-fold rotational symmetry (*palindromic sequences*). The *Eco* RII site is unique in that it contains an odd-number (5) of base pairs, with the axis of symmetry passing through the central A:T pair:

CCTGG

CCACC

Two-types of 5'termini are thus formed, and, therefore, all fragment ends are not necessarily mutually cohesive.

Sometimes enzymes from unrelated organisms can recognize the same nucleotide sequences. For example, restriction enzymes with GGCC specificity have been found in *Bacillus subtilis, Haemophilus aegyptius* and *Haemophilus haemoglobinophilus*. Such enzymes are called *isoschizomers*.

Cleavage positions may be either '*even*' (e.g. *Hind*II) or '*staggered*' (e.g. *Eco*RI). In *even cleavage* the breaks on the two strands are exactly opposite each other. Such breaks do not produce unpaired terminal nucleotides. *Staggered breaks* are separated by several nucleotides and produce complementary single-stranded 'sticky' ends (see section on : 'Splicing and insertion of DNA).

Use of restriction enzymes. Restriction enzymes have been used for *sequence analysis* of DNA and or *cloning and amplifying DNA*.

DNA from animal viruses and bacteriophages contains from 5,000 to 50,000 base pairs. It is important

to how the primary structure of DNA, i.e. the sequence of bases, for decoding the information stored in genes and for understanding gene structure and regulation at molecular level. The discovery of restriction enzymes was a major break-through in *sequence analysis* of DNA. By using combinations of different restriction enzymes it is possible to hydrolyze large DNA molecules into fragments less than 300 base pairs in length. These fragments can then be used for sequence analysis and arranged into a physical map of the chromosome. This is a slow and laborious process. The mapping of the entire 5,000 base-pair DNA of the virus SV40 into some 100 fragments has taken several years. Complete sequence analysis of the fragments would take much longer.

The DNA fragments produce by restriction endonucleases can be covalently linked *in vitro* to linear *plasmid DNA* or to *lambda phage DNA*. The *recombinant DNA* species produced can be inserted into *E. coli* by *transformation*. Each transformed cell can then be grown as a separate *clone*. By this method a complex genome can be broken down into thousands or millions of pieces, and each piece isolated to form a separate clone.

ISOLATION OF DNA

The DNA of an organism may contain from a few to thousands of genes. The problem, therefore, is to isolate the required gene from the entire DNA of the organism. If the property of a particular gene is easily detected, the *'shotgun' technique* can be used to isolate the gene. The other approach is to isolate the *messenger RNA* (mRNA) transcribed by the gene and then obtain DNA from it.

1. The 'shotgun' method (Fig.4)

In this method the total DNA of the organism is subject to the action of *restriction enzymes* and broken down into many fragments. Each of these fragments is then combined with the DNA from a suitable *vector* to produce *recombinant DNA*. The different recombinants are inserted at random into a host cell, usually *E. coli*, the different types of recombinant host cells are cultured separately and grown into colonies. The details of this method will now be taken up.

1. DNA is isolated from the organism of choice (virus, bacterium, plant or animal) and is cleaved by *restriction enzymes* such as *Eco*RI into many fragments. Such DNA is called 'foreign' DNA, and is designated as X DNA in the figure. The DNA restriction enzyme cuts the DNA at specific points, leaving overlapping breaks with 'sticky' (cohesive) ends. The fragments (x 1 to X 4) are of different lengths.
2. The outer membrane of *E. coli* bacteria (host cells) is first dissolved by a detergent-like liquid and its DNA is released. The *plasmids* are separated from chromosomal DNA in an ultracentrifuge. They are now placed in a solution containing the same *restriction enzyme* which cuts the plasmid ring (pDNA) into a linear form. Linear plasmid DNA also has overlapping, single-stranded, cohesive ends.

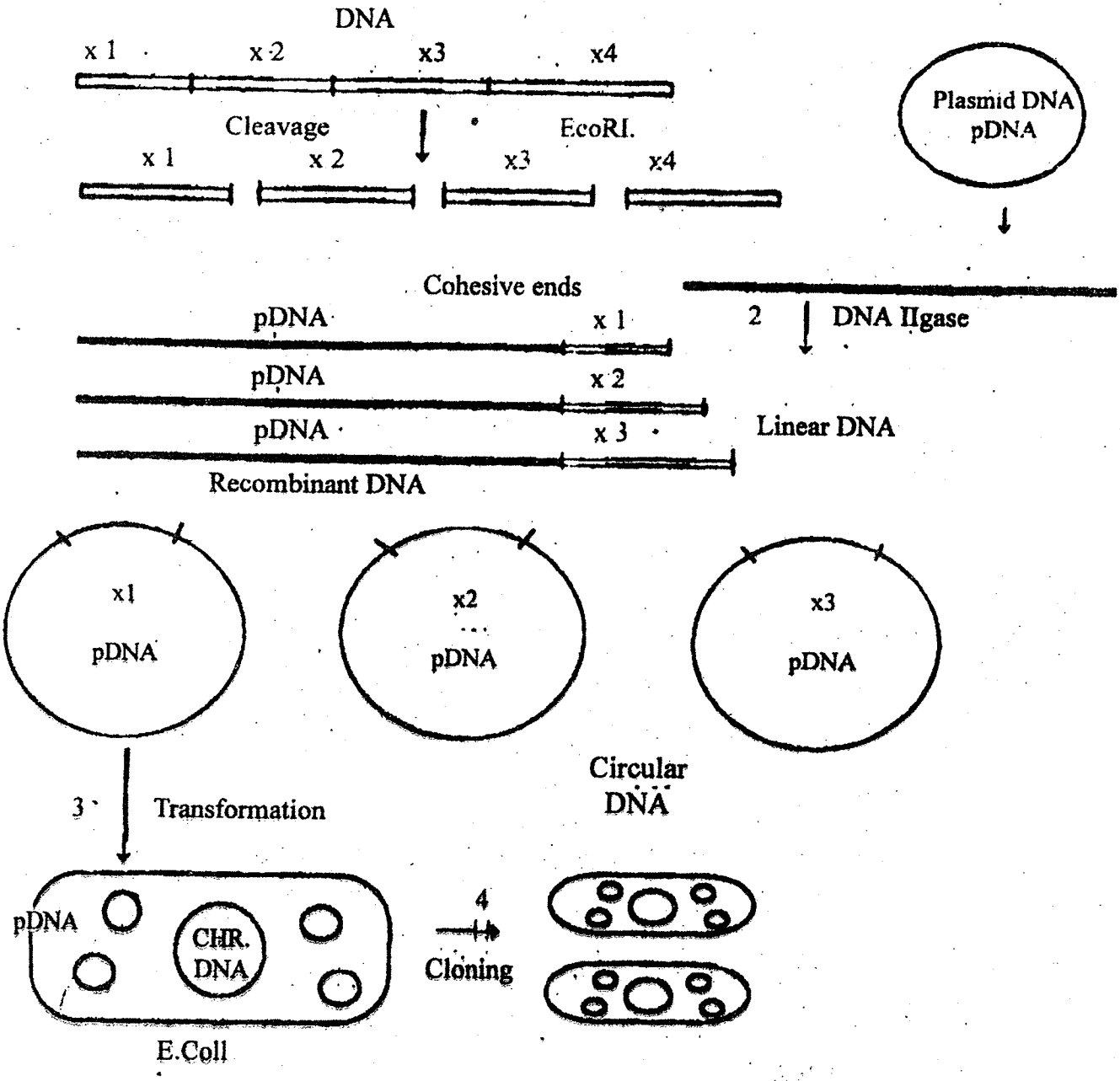


Fig. 4. *The 'shotgun' method of isolating genes.*

1. Cleavage of 'foreign' DNA by the restriction enzyme EcoRI. Circular plasmid DNA cleaved to produce linear DNA.
2. Randone association between plasmid DNA and foreign DNA fragments to produce recombinant DNA which may belinear or circular. Plant DNA is joined to the DNA fragment by DNA ligase.
3. Transformation, Recombinant DNA plasmids taken up by bacterial cell.
4. Cloning of bacterial cell to produce multiple copies.

3. Foreign DNA fragments are mixed with linear plasmid DNA. Random association between the two types of DNA takes place by hydrogen bonding at the cohesive ends. The foreign DNA becomes inserted into plasmid DNA to form a larger ring or a longer linear form. Covalent joining is broght about by another enzyme, *DNA ligase*. The resulting DNA is called a plasmid *chimera* or *recombinant DNA*. Both linear and circular molecules of plasmid DNA are joined to different combinations of fragments.

4. The chimera plasmid are placed in a solution containing cold calcium chloride and normal *E. coli*bacteria. On heading suddenly (2-5 minutes, 42°C) the *E. coli* membranes become permeable to plasmid chimeras, which pass into the cell. Thus recombinant DNA plasmids are taken up by *E. coli* cells.

5. The bacterial cells are spread on a nutrient medium in a petridish. Each *E. coli* cell, containing particular genes inserted in its plasmids, grows by repeated binary fission into a colony. The individuals of each colony are all derived from one *E. coli* cell, and are hence genetically identical. Such groups of genetically identical individuals are called *clones*. Each colony contains individuals with specific segments of foreign DNA in their plasmids. Even under favourable conditions only one cell in 10^5 or 10^6 produces a transformed clone.

By using the shotgun method the genomes of several organisms (rRNA gene of the toad *Xenopus laevis*, the histone gene of sea-urchin, 90% of the genome of *E. coli*, yeast, *Drosophila*) have been cloned into *e. coli*.

2. Obtaining DNA from mRNA

In the shotgun technology foreign DNA is broken up and inserted into bacterial plasmid DNA at random. The chances of implanting a particular gene by this method are very small. The alternative method of isolating genes is to first obtaining the messenger RNA transcribed by the particular gene, and then to obtain DNA from the mRNA. It is relatively easier to obtain mRNA because different cell types are specialized for the synthesis of different proteins. Each protein is translated on its own mRNA.

DNA can be obtained from mRNA by the *hybridization method* or by the *reverse transcriptase method.*

Hybridization method (Fig. 5). This method depends upon the principle that mRNA forms a complex with complementary segments of DNA from which it is transcribed. The total DNA from an organism is first isolated. This double-stranded DNA (dsDNA) is treated with heat or alkali and is converted into the single-stranded form (ssDNA) by denaturation. The strands are then mixed with mRNA transcribed by the gene. The mRNA pairs with the complementary region of DNA to form a *DNA-RNA complex (hybrid)*. This complex can be isolated and the DNA separated from RNA. The ssDNA strand which gets separates

has the same base sequence as that of one strand of the original DNA from which the mRNA was transcribed. The ssDNA can now be converted to dsDNA by the polymerization action of *DNA polymerase I* of *E. coli*. This method is useful for isolating genes which exist in multiple copies, e.g. ribosomal genes.

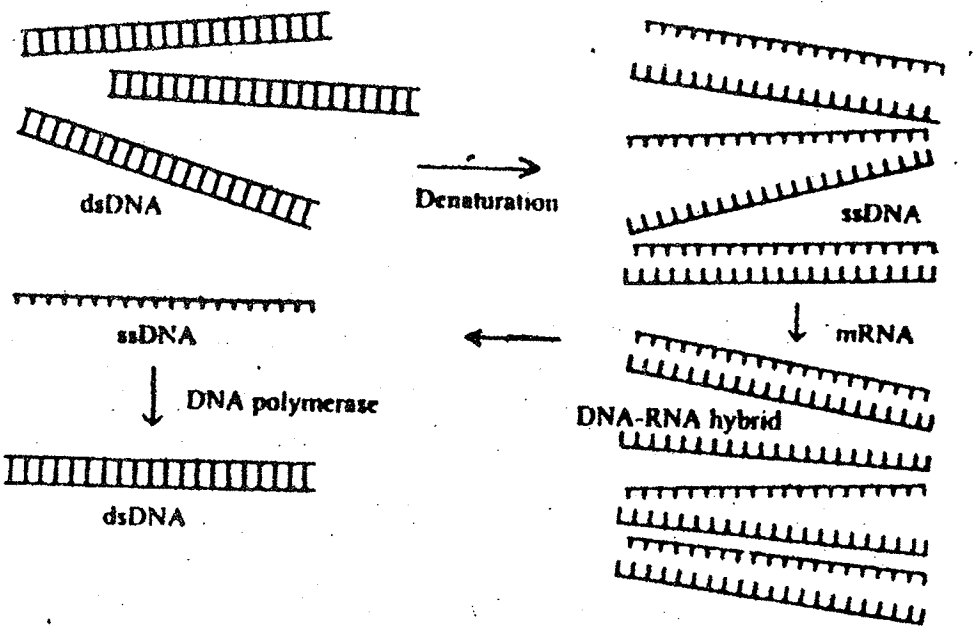


Fig. 5. *Hybridization method of isolating a gene.*

Reverse transcriptase method. The normal sequence for the transfer of genetic information is : DNA → RNA → PROTEIN. Transcription of RNA from DNA is catalysed by the enzyme *transcriptase (DNA-dependent RNA polymerase)*. In the 1960s Temin and Baltimore independently demonstrated that there is a reverse flow of information, i.e. from RNA to DNA, in RNA tumour viruses. The enzyme *reverse transcription (RNA-directed DNA polymerases)* catalyses they synthesis of an ssDNA strand complementary to an RNA template. This discovery provides a method for isolating particular genes (Fig. 6).

(1) Purified mRNA of the required gene is used as a template to transcribe ssDNA by the catalytic action of *reverse transcriptase*. This ssDNA is complementary to mRNA, and is hence also called *complementary DNA* (cDNA) (2) Hydrolysis of the RNA template with an alkali leaves the ssDNA behind. (3) The ssDNA is then coverted to dsDNA by using the enzyme *DNA polymerase I* of *E. coli*. (4) The enzyme *Sl nuclease* breaks the covalent linkage between the two DNA strands. (5) Poly (dT) or poly (dA) tails are added to dsDNA by the action of the enzyme *terminal transferase* in the presence of TTP or ATP, respectively. (6) dsDNA is then spliced to vector DNA and *cloned* to produce multiple copies. The ovalbumin gene of chicken and the globin gene of rabbit have been cloned by this method.

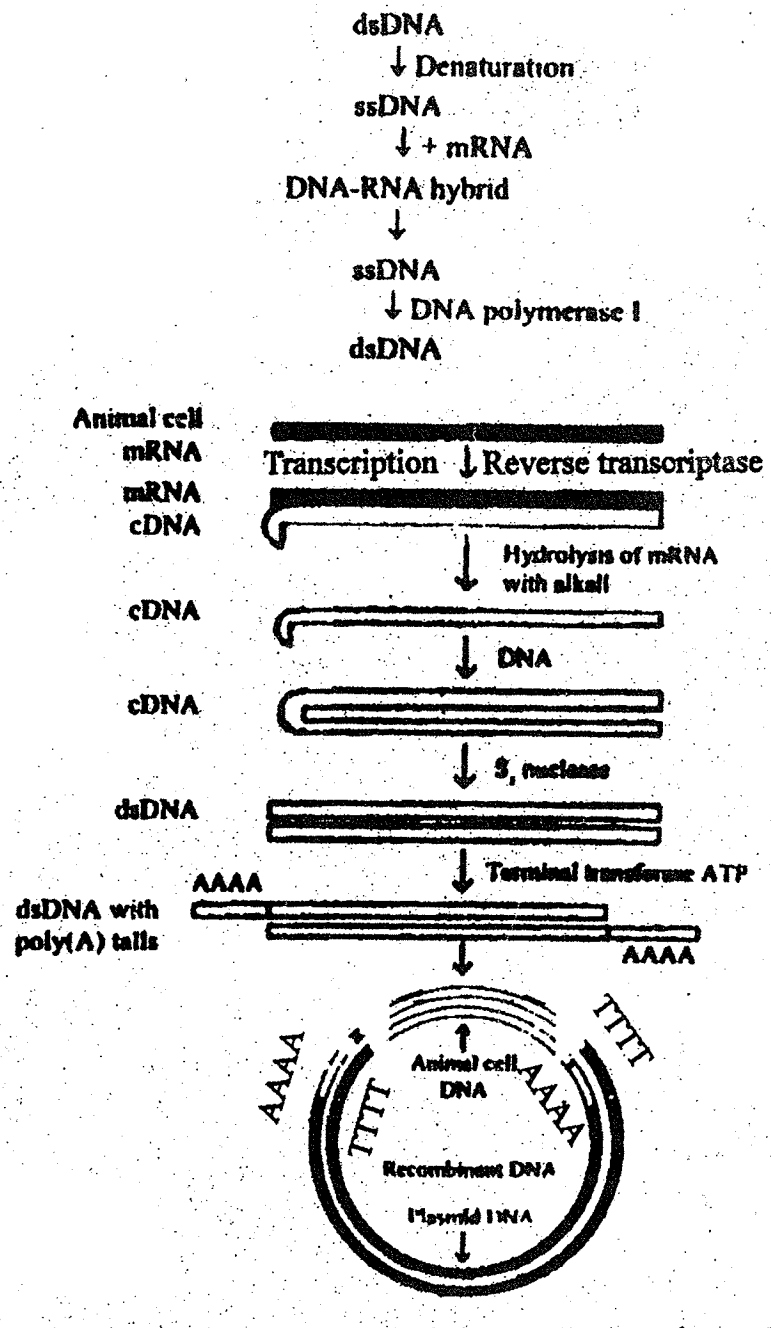
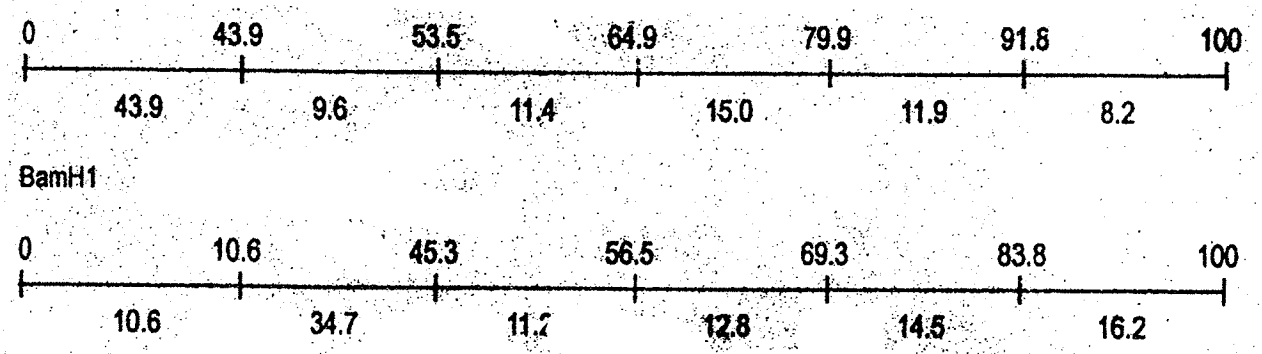


Fig. 6. *Reverse transcriptase method of obtaining a gene from mRNA.*

	Reverse		DNA	
mRNA	→	ssDNA	→	dsDNA
	transcriptase		polymerase I	
				Terminal transferase
	Cloning			
Multiple	←	Vector	←	dsDNA + poly (dT)/poly (dA)
Copies		DNA		

An important point about these restriction enzyme is this : since a particular enzyme recognizes a unique sequence, *the number of cuts made in the DNA from an organism is limited.* For example, a typical bacterial DNA molecule, which contains roughly 3 x 10^6 base pairs, is cut into a few hundred to a few thousand fragments. Smaller DNA molecules such as phage or plasmid DNA molecules may have fewer than ten sites of cutting (frequently, one or two, and often none). Because of the specificity just mentioned, *a particular restriction enzyme generates a unique family of fragments from a particular DNA molecule.* Another enzyme will generate a different family of fragments from the same DNA molecule. (Some enzymes – isoschizomers – have the same specificity and generate identical families) Figure 3(a) shows the sites of cutting of *E. coli* phage λ DNA by the enzymes EcoRI and BamHI. The family of fragments generated by a single enzymes is usually detected by agarose gel electrophoresis of the digested DNA (Figure 3(b)). The fragments migrate at a rate that is a function of molecular weight run concurrently.



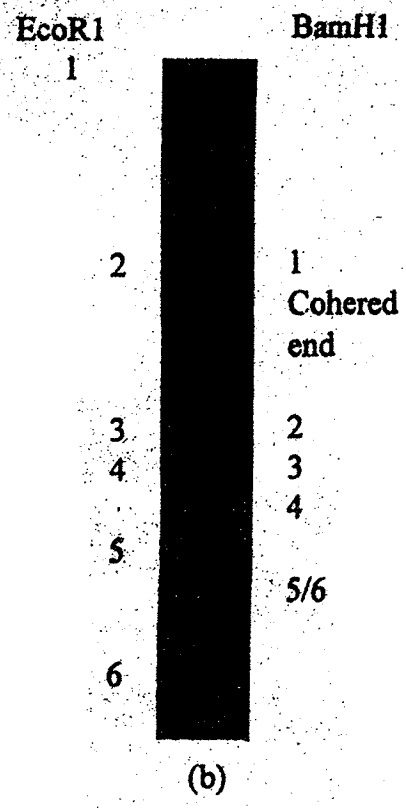


Figure 3(a) *Restriction maps of λ DNA for EcoRI and BamHI nucleases. The vertical bars indicate the sites of cutting. The numbers above line indicate the percentage of the total length of λ DNA measured from the gene A end of the molecule. The numbers below line are the lengths of each fragment, again expressed as percentage of total length. (b) A gel electrophoregram of EcoRI and BamHI restriction-enzyme digests of λ DNA. The bands labelled cohered ends contain molecules consisting of the two terminal fragments joined by the normal cohesive ends of λ DNA. Numbers indicate fragments in order from largest (1) to smallest (6). Bands 5 and 6 of the BamHI digest are not resolved.*

Restriction enzymes serve as a mean of *protection* against foreign DNA in many microorganisms. They functions as a part of the *restriction-modification (R-M) system*. A matched *modification enzyme* recognizes and modifies, usually by *methylation*, the nucleotide sequence recognized by the restriction enzyme. The DNA modified by methylation is thus protected against cleavage by the restriction enzymes of the organism. On the other hand unmodified foreign DNA is degraded. R-M systems are widespread in bacteria where they play an important protective role in eliminating foreign DNA which may enter the cell through viruses, or as unmodified DNA. Restriction enzymes have not been reported from mammalian cells, where the immune surveillance system makes a restriction-modification system unnecessary.

HOST RESTRICTION AND MODIFICATION

Another specificity factor in phage infection is the inability of a "foreign" bacterial RNA polymerase to recognize phage DNA sequences for initiation of RNA synthesis. However, even when adsorptionand mRNA synthesis are possible, with most bacteria there is usually another barrier called host restriction and host modification. This is a phenomenon in which a bacterrium of a type X is able to distinguish a phage that has been grown in type-X bacterium from one grown in a different type such as Y and is able to prevent the phage grown in Y from carrying out a successful infection. The notation used to discuss this phenomenon is the following : a phage P grown in a bacterium X is denoted P-X. Host modification and restriction are illustrated by the data in table-3 Note that λ-K, which has been grown in *e. coli* strain K, forms plaques at a low efficiency in strain B. Thus, λ-K is restricted by strain B. the phage population in these rare plaques (λ-B) has been modified by strain B, so the phage grow efficiently in strain B; however, λ-B now fails to grow in strain K– that is, it is restricted. The molecular explain for this is the following:

Table 3 The restriction and modification pattern of *E. coli* phage λ

Bacterial strain	Phage		
	λ-K	λ - B	λ - C
K	1	10^{-4}	10^{-4}
B	10^{-4}	1	10^{-4}
C	1	1	1

Note Numbers indicate relative plating efficiency.

E. coli contains an enzyme called a restriction endonuclease (Chapter 2) – specifically< it is the EcoB nuclease – a site-specific nuclease that cuts DNA strands only near a specific base sequence (most restriction enzymes cut within a target sequence, but EcoB cuts near the sequence, see Chapter 20). Phage λ-K contains this sequence; when its DNA is injected into *E. coli* B, the phage DNA is broken, *E. coli* B also contains this sequence and would destroy its own DNA were the sequence not modified. A site-specific methylating enzyme (EcoB methylase) methylates an adenine in the sequence, thereby rendering the sequence resistant to the EcoB nuclease. When λ-K infects strain B, a few parental phage-DNA molecules in the large population

of infected cells are methylated before they are restricted. All progeny DNA molecules are already methylated on one strand and the newly synthesized strands are also methylated rapidly (see Chapter 8), and restriction is avoided in these rare phage. They, a small population of phage having the B modification (λ-B) is produced. *E. coli* K also contains a restriction enzyme (EcoK). It attacks a base sequence that is different from the sequence recognized by EcoB. An EcoK methylase also protects *E. coli* K from self-destruction, production the K modification. A λ phage that has always been grown in strain K – namely, λ-K-is methylated in the EcoK-specific sequence and is resistant to EcoK nuclease. However, λ-B has an unmethylated EcoK sequence, so λ-B DNA is usually broken when a strain K cell is infected. Occasionally, a λ-B DNA molecule escapes restriction and replicates, and its replicas have a methylated K-specific sequence. Thus, the rare progeny phage that result when λ-B successfully infect *E. coli* K are λ-K; they now lack the B modification and are restricted when infecting strain B.

Note in Table 3 that λ grown on strain C-i.e., λ-C-also fails to grow well in strains B and K, but neither λ-B nor λ-K is restricted by strain C. The reason for the lack of restriction is that strain C has no restriction nuclease active against any base sequence in λ DNA. The λ-C phage are restricted by both strains B and K because of course strain C does not have the EcoB and EcoK methylases.

Host restriction and modification is a widespread process, probably serving to destroy foreign DnA.

VECTORS (CARRIER DNA MOLECULES)

A carrier DNA molecule or *vector* is essential for introducing new DNA permanently into a cell. Examples of some molecular vectors used in anetic engineering is given in Table 3.

Table 3. Examples of some Molecular Vectors Used in Genetic Engineering

Vehicles	Advantages
Plasmids	
pBR322	Small size; occur as multiple copies in a cell
pRK290	Can replicate in a variety of Gram-negative bacterial
pHV14	Can replicate in *E. coli* and *B. subtilis*
Cosmids	Ensures that a large DNA sequence is cloned
Phages	
Lambda	The cloned DNA can be manipulated as the phage
M13	Cloned DNA can be obtained in single-stranded form and is therefore more easily sequenced.

*Plasmids that contain a gene (*cos*) from phage λ, thereby allowing it to be packaged, *in vitro*, in a phage λ head.

Plasmids : The vector initially used for DNA cloning were bac plasmids. These extra chromosomal genetic elements range in size from 5 to 400 (kb) pairs are commonly present in two to up to several hundred copies per cell. Plasmid present in a large number of copies per cell are most often used as cloning vectors, since they enable the DNA segments linked to them to be amplified many times and the increase the yield of the cloned DNA fragment and the gene products encoded by it.

Example I p^{SC}_{101}

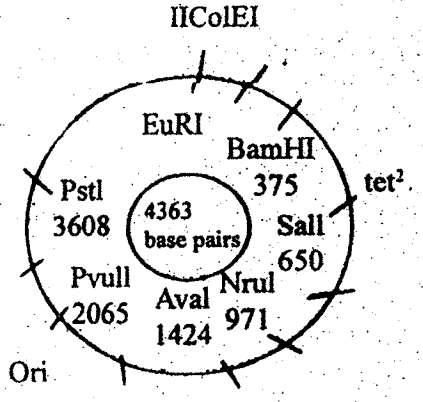


Fig. pBR322

Present in the cell 4-6 copies.

1. A single cleavage site for the EcoRI.
2. Carries tel-r gene.

1. Is normally present in 10 to 20 copies per chromosome.
2. Number of copies can be multiplied/amplified by using chromophenicol antibiotic with the plasmi containing cell.
3. Actually chloromphenicol stop its protein synthesis & host chromosomal synthesis but it **does not inhibit plasmid DNA synthesis.**

4. While ColEI & other naturally occurring (plasmid) multi copy vectors do not normally carry antibiotic resistance genes such genes can be added by the use of in vitro joining produces, thus falilitating the selection of bacteria that take up the plasmids one of the most widely employing cloning vector p BR 322 was made in this way.

III p Br 322

1. **Closed circular DNA is 4363 bp.**
2. Obained from pMB8 a small ColE1 like plasmid + segments of the pSC 101 plasmid and the b lactamacse gene of transporson tn_3
3. Having single cleavage sites for a no of commonly used respiction endnucleases and carries gene **amp², tetr.**

Bacteriophage λ Vectors:

Bacteriophase λ posses a number of advantages so it is attractive as a cloning vector.

i) DNA fragments as large as 24 kb can be propagated using this vector.'

ii) The primary pool of clones can be amplified by limited phage growth as plaques.

iii) The entire collection of phage clones can be stored for long periods in small volume.

iv) Since λ phage will not accommodate molecules of DNA that are much longer than the viral genome, the use of phage λ as a vector requires the removal of a significant portion of the viral DNA. Fortunately the central third of the λ genome contains gene that are not essential for plaque formation and their region can be replaced by an externally derived DNA fragments of approximately the some size without influencing phage growth.

v) The native λ genome (approx. 50 kb) is large in size relative to the size of bacterial plasmids (5 - 10 kb) used as cloning vectors.

vi) Phage λ DNA normally **contains multiple cleavage sites** for even those endonucleases that requre a 6bp recognition sequences.

vii) To use bacteriophage λ as a vector, it ordinarily is necessary to remove all except **one** cleavage site for a particular endonucleases and to ensure that the remaining site is located in a non essential region of the phage genome.

viii) λ phage ensures that all of the plaques produced are derived from phage genomes that contain an inserted DNA fragments.

ix) The recombinant DNA molecules that incorporate some of those vectors can be introduce directly into *E. coli* by means of transfection produce developed earlier for bacteriophage λ_1 using $cacl_2$ treated cells.

x) Recomb. DNA molecules that incorporate some of these vectors can be introduced directly into *E. coli*. by means of transfection procedure.

xi) Recomb. DNA molecule can be packaged into phage particles in vitro.

xii) The availability of systems for the in vitro packaging of DNA into phage particles set the stage for the development of still another type of vector, the cosmid, which incorporates certain desirable of both phage and plasmid vectors.

Cosmids:

1. If cosmids were developed as vectors specifically designed for the cloning of large fragments of DNA.

2. The first part of their name, cos comes from the fact that they contain the cohesive ends or cos sites of normal λ. These ends are essential for the packaging of any DNA in λ phage heads. The last part of their name mid, coes from the facl that cosmid carry a plasmid origin of replication. The first cosmid consisted of a Col E_1 derived plasmid carrying to cos side of phage λ. Such cosmids can be used for cloning in the same way as any other plasmid vector. However, because cosmids contains the cos sites. cosmid DNA along with an inserted DNA fragment can be packaged as a λ phase. The

result after packaging is a defective but nevertheless infectious phage particle. Once the cosmid and the inserted DNA fragment have been introduced by infection into a λ sensitive cell, the plasmid replication system allows the vector to replicate as a plasmid. Since in cosmids virtually the entire bacteriophage λ genome has been deleted, except for the region adjacent to the cos, sites, these vectors can propagate exogenously derived DNA fragments upto 40-50 kb in length.

Bacteriophage M 13 Vectors:

A series of highly useful cloning vectors also has been developed from the single stranded DNA bacteriophage M_{13}. When this *phage infects bacteria*, the strand that is packaged *in the phages filamentous protein capsid* (the plus strand) replicates to form double stranded intermediate, known as the replicative from (RF). The RF of M_{13} is structurally and functionally similar to a plasmid and can be isolated from bacterial cells by the methods used to obtain plasmid DNA. Foreign DNA fragments can be inserted at any one of a numbered of unique restriction sites in the 7200 bp M_{13} genome. Depending on the orientation, one or the other strand of the inserted fragment will be packaged into progeny virus particular along with the plus strand of M_{13}. Since the entire sequence of M_{13} adjacent to the cloning site can be synthesized chemically. This polynucleotide can serve as a primer for the sequencing of any DNA fragment that is cloued at the same site in M_{13}, using the dideoxy method of sanger. A single primer complementary to the segment adjacent to a particular cleavage site can this be used to sequence a variety of different DNA fragments inserted into the site, greatly facilitating analysis of the cloned DNA.

Shuttle Vectors

A shuttle vector is a vector that can replicate in different organisms. The first shuttle vector, which contained *E. coli* and yeast components, was used to clone yeast genes. If a yeast gene were cloned into an *E. coli* plasmid and then *E. coli* cells were transformed by the recombinant plasmid, in general the yeast gene would not be expressed, for the usual reasons - lack of recognition of yeast promoters in *E. coli*, incorrect processing, and several other problems. When cloning of yeast genes was first attempted, these difficulties limited the techniques for detecting colonies containing yeast DNA to in *situ* hybridization. Shuttle vectors, which contain sequences from an *E. coli* plasmid and a particular region of the yeast genome, were designed to avoid this problem (Figure 21-5). Essential features of this shuttle vector are : two replication origins (one active in yeast and one in *E. coli*), two selective markers (*trp*. detectable in yeast, and *amp-r*, detectable in *E. coli*), and restriction sites next to a yeast promoter. Such a vector can be cleaved and a yeast DNA fragment can be inserted, but, most important, the hybrid vector will transform *trp*r yeast cells, production Trp^+ cells. The gene of interest carried on the fragment can also be detected by virtue of its expression in yeast.

A problem with these vectors is that they are not particularly stable in yeast, because they lack a centromere (the portion of a chromosome by which the chromosome is attached to the mitotic spindle);

thus, plasmid replicas are not efficiently segregated into daughter cells. To avoid this problem, the recombinant vector is isolated from a culture obtained from a colony of successfully transformed yeast and then used to transform *E. coli*, in which its presence can be detected by an antibiotic-resistance marker (Figure 21-5). In *E. coli* the recombinant vector can be maintained indefinitely. The sequence of steps in the use of this shuttle vector is listed below :

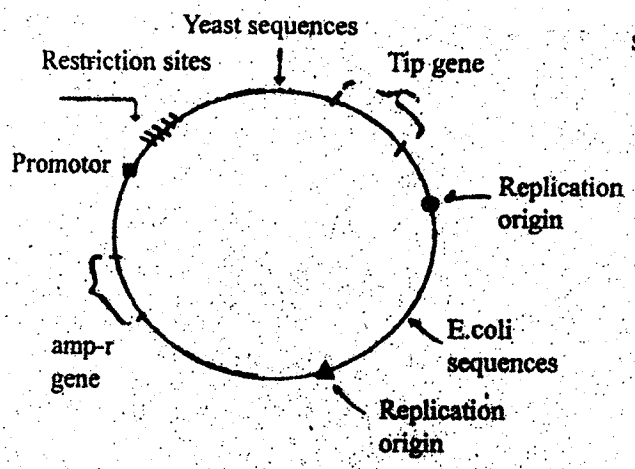


Fig. A *yeast E. coli shuttle vector.*

Inserted a eukaryotic gene in the cleaved restriction site in the yeast segment.

1. Transform Trp⁻ yeast and plate on a medium lacking tryptophan.
2. Select Trp⁺ colonies.
3. Test Trp⁺ colonies for expression of the eukaryotic gene.
4. Isolate a colony with the expressed gene.
5. Isolate the plasmid.
6. Transform Amp-s *E. coli*.
7. Select an Amp-r colony.

The Amp-r colony will contain the shuttle vector with the inserted eukaryotic gene.

COLONING ORGANISMS

Various strains of the human colon bacterium *Escherichia coli* have been used for cloning. They include HB101, h303 and RRI. The cloning organism should preferably be deficient (i.e. *recA*⁻) in the major pathway of DNA recombination. This will reduce the possibility of undesirable recombinations. The cloning organism should preferably lack restriction enzymes that might degrade foreign DNA. Such strains are usually also different in modification enzymes that protect DNA. Strains that bud off "minicells" are desirable if gene expression (transcription and translation) is the object of study. The minicells contain plasmids, enzymes and other components required for gene expression, but lack chromosomal DNA. In such a system mRNA or proteins coded by recombinant DNA can be more easily studied as there is no interference from products of chromosomal DNA expression. In eukaryotic cells primary and secondary tissue cultures of the African Green Monkey kidney cells have been used for recombinant DNA experiments.

E. coli was the cloning organism for most recombinant DNA studies. In 1974 Fink and his co-workers achieved the transplantation of genes from a lower organism into a higher organism when they introduced bacterial DNA into baker's yeast. Yeasts are safer than *e. coli* for recombinant DNA experiments because they do not live in human beings and are not pathogenic to man. Pink and his coworkers introduced the bacterial gene for leucine production into a yeast strain that was deficient in the ability to produce leucine. A snail enzyme was used for inducing the yeast to take up bacterial DNA.

Cloning a Restriction Fragment in a Plasmid

A particular restriction enzyme recognizes only a single base sequence. Furthermore, except for those enzymes producing blunt ends, the cuts generate at each end of a fragment single-stranded termini that are complementary. This is the bases of the joining procedure to be described - namely, the fragments generated by a particular enzyme acting on one DNA molecule have the same set of single-stranded ends as the fragments produced by the same enzyme acting on a different DNA molecule (as long as both DNA molecules have sequences recognized by the enzyme). Therefore, fragments from the DNA molecules of two different organisms (for example, a bacterium and a frog) can be joined by renaturation of complementary single-stranded termini. Furthermore, if the joint is sealed with DNA ligase (ligated) after base-pairing, the fragments are joined permanently.

The joining technique takes on special importance if one of the sources of cleaved DNA is a plasmid. Figure 7 shows a plasmid DNA molecule that has only one cleavage site for a particular restriction enzyme. which is also used to cleave frog DNA. If frog fragments are mixed with linearized plasmid DNA and joining is allowed to occur, a circular plasmid DNA molecule containing frog DNA can be generated. The significance of this technique is that the hybrid plasmid can be reestablished in a bacterium by $CaCl_2$ transformation. Then, the inserted DNA fragment, which replicates as part of the plasmid, is cloned.

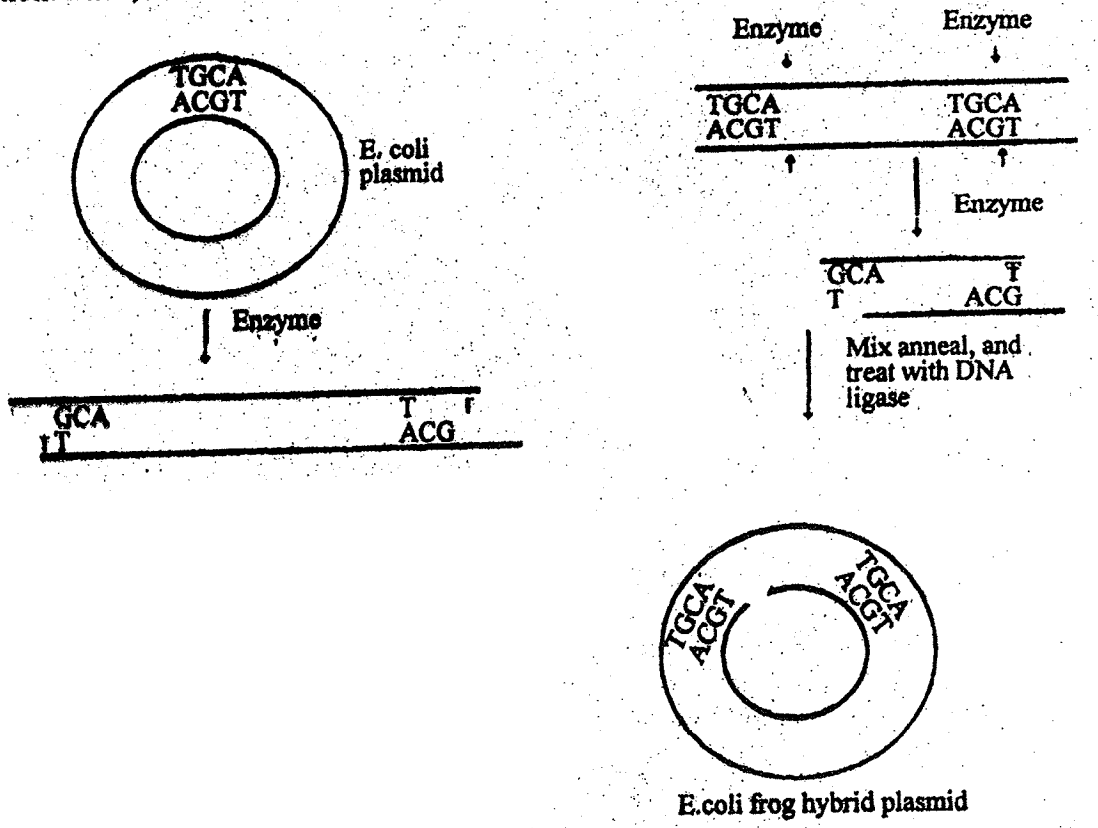


Figure 7. *Construction of a hybrid DNA molecule from fragments derived from different organisms by using restriction enzymes. Such interspecies hybrids are often called chimeras. Short arrows indicate cleavage sites.*

Joining of DNA Fragments by Addition of Homopolymers

The field of recombinant DNA research began in 1972, just before the properties of restriction enzymes were understood, with the development of a general method for joining any two DNA molecules. This method used the enzyme **terminal nucleotidyl transferase**, an unusual DNA polymerase obtained from animal tissue, which adds nucleotides (by means of triphosphate precursors) to the 3'-OH group of an extended segment of a DNA chain. The reaction differs from that of ordinary polymerases in that *it does not need a template strand.* In order to generate the extended single strand with a 3'-OH terminus one need only treat the DNA molecule with a 5'-specific exonuclease to remove a few terminal nucleotides. In a reaction mixture consisting of exonuclease-treated DNA, dATP, and the enzyme, poly(dA) tails will form at both 3'-OH termini (Figure 8). If, instead, dTTP were provided, the DNA molecule would have poly (dT) tails. Two molecules can be joined if poly (dA) tails are put on the DNA molecule and poly (dT) tails on the second molecule and the poly (dA) is allowed to anneal to the poly(dT), as shown in the figure. Completion of the joined molecule is accomplished by gap-filling with DNA polymerase I and sealing with DNA ligase.

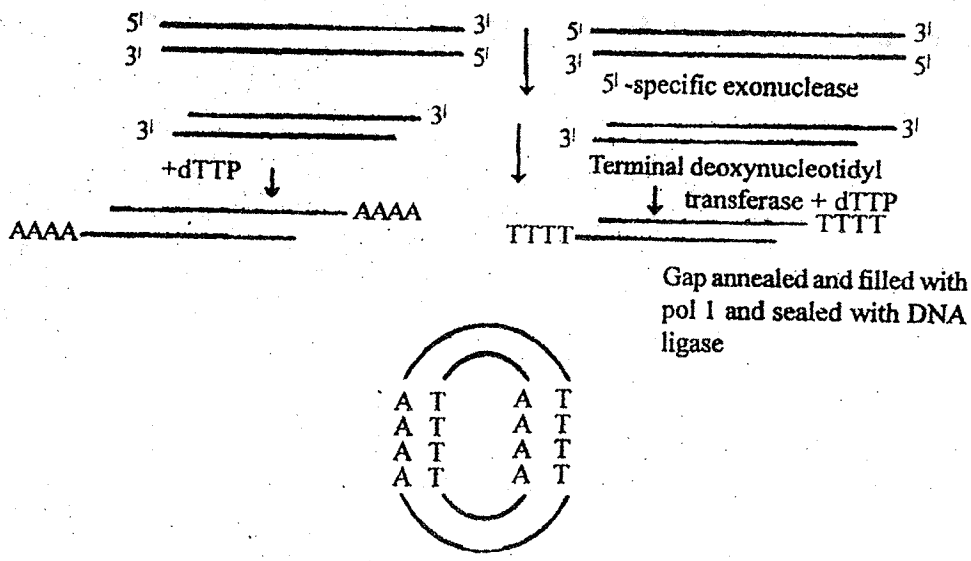


Figure 8. *The joining of two molecules with complementary homopolymer tails.*

This method, which is called homopolymer tail joining, is useful when DNA molecules lacking complementary ends are to be joined. Such molecules may be the result of digestion with restriction enzymes that yield blunt ends or may be prepared by mechanical breakage of large DNA molecules; c-DNA (complementary DNA), which is a DNA molecule prepared in the laboratory by copying an RNA template and which is extremely important in the recombinant DNA technology (to be described shortly), also has blunt ends.

Blunt-End Ligation

E. coli phage T4 encodes a DNA ligase, which is produced in large quantities in an infected cell. This enzyme is a typical ligase in that it seals nicks in double-stranded DNA having 3'-OH and 5'-P termini, but has the additional property of joining two DNA molecules having completely base-pairing ends (Figure 9). How this reaction occurs is unknown. Whereas blunt-end legation is quite useful in many situations. It has a significant disadvantage compared to homopolymer joining, namely, the two ends of any fragment can be joined to form a nonrecombinant (and generally useless) circle. Such joining does not occur with homopolymer tail joining since the two ends are identical rather than complementary. This problem can, however, be avoided by use of a high DNA concentration, as shown in the figure.

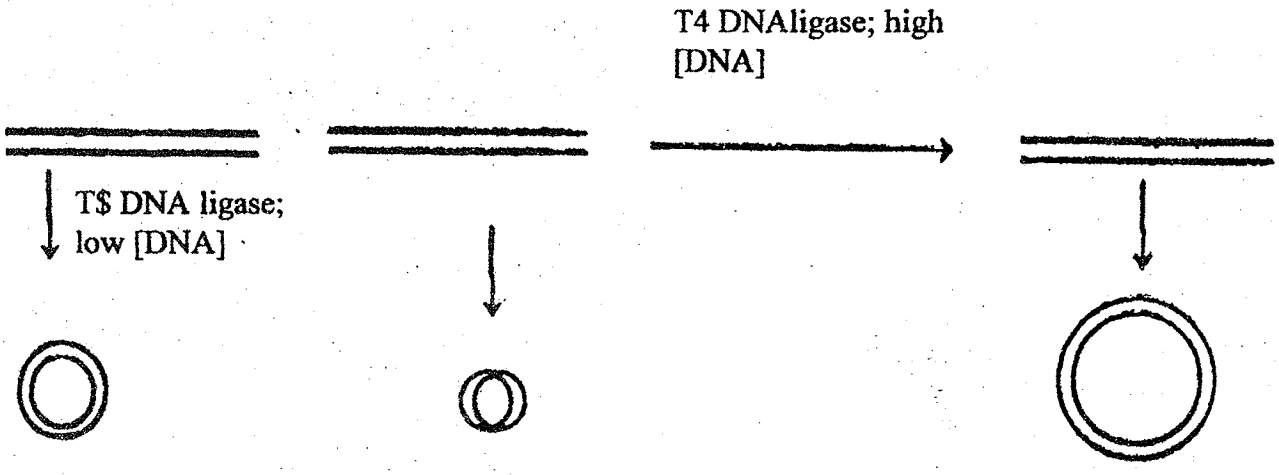


Figure 9. *Two blunt-end joining reactions. 'At low concentration DNA intramolecular circularization is favoured.*

Joining with Linkers

In some cases it is useful to be able to join one molecule with blunt ends to a second molecule produced by a restriction endonuclease that generates single-stranded termini. This is possible if a short DNA segment (a linker) containing a restriction site is coupled to both ends of the blunt-ended molecule. How this is done is shown in Figure 10, in which a blunt-ended molecule is inserted at the EcolRI site of a vector. This procedure is useful because at a later time the sequence contained in the blunt-ended fragment can be recovered from the vector by treatment with a restriction enzyme that cuts the site in the linker. Such recovery is not possible if joining is done with homopolymer tails.

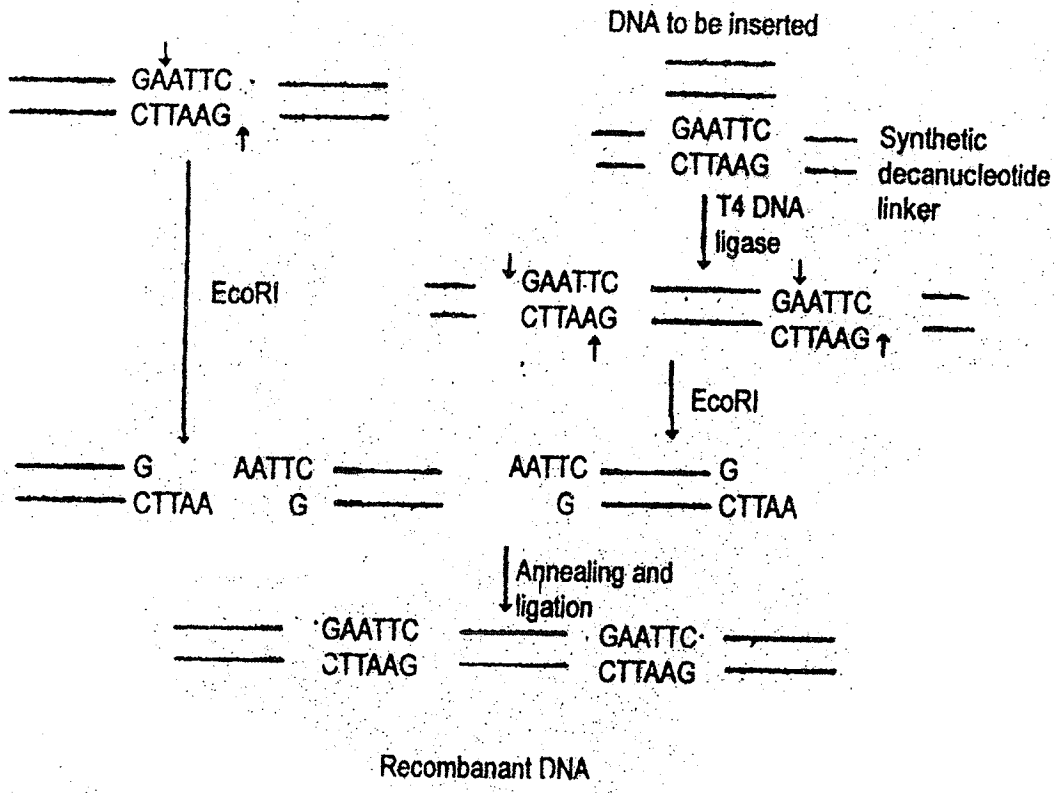


Figure 10. Formation of recombinant DNA through use of a linker. The short arrows indicate the sites of cutting by the EcoRI enzyme.

APPLICATIONS OF GENETIC ENGINEERING

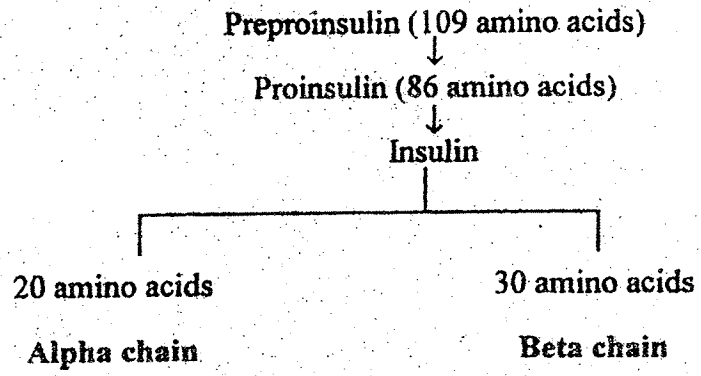
The application of genetic engineering techniques has already begun to yield results, and several products are manufactured commercially. Firms producing recombinant DNA products include Genentech Inc., Cetus Corp., all in the U.S.A., and Biogen S.A. of Geneva. Applications of recombinant DNA technology can be braodly classified into live groups, *medical* applications, *industrial* applications. *environmental* applications, *agricultural* applications and *fundamental understanding of biological processes.*

1. Medical applications

Among the medical applications of genetic engineering are the production of *hormones, vaccines, interferon, enzymes, antibodies, antibiotics* and *vitamins*, and in gene therapy for some hereditary diseases.

1. **Hormones.** The hormone insulin in is currently produced commercially by extraction from the pancreas of cows and pigs. About 5% of the patients, however, suffer from allergic reactions to animal-produced insulin because of its slight difference in structure from human insulin. Human insulin genes have been implanted in bacteria which, therefore, become capable of synthesizing insulin in test programmes, and it appears to be as effective as insulin from animal sources.

Insulin is secreted by the beta cells of the islets of Langerhans in the pancreas. The beta cells first synthesize *a preproinsulin* molecule of 109 amino acids. The first 23 amino acids of preproinsulin serve as a signal for the passage of the molecule through the cell membrane these are cleaved to produce *proinsulin* which consists of 86 amino acids. The central part of the proinsulin molecule is cut out by enzymes, leaving two chains which comprise insuline. One chain consists of 20 amino acids and the other of 30.



Itakura and his associates synthesized two DNA strands coding the two chains of human insulin. The strands were then separately attached to bacterial DNA. Two different hybrid proteins in two different bacteria were synthesized. The two short pieces were cut off, purified, and then joined together to produce *insulin.*

In 1977 Herbert Boyer created *E. coli* capable of synthesizing *somatostatin,* the human growth hormone of the brain hypothalamus. Formerly 500,000 sheep brains were required to produce 5 mg of the hormone. Boyer's bacterium can produce the same quantity of the hormone for the cost of about $2.50. Somatostatin is a small hormone consisting of 14 amino acids coded by a DNA sequence of 42 bases. Itakura and his co-workers chemically synthesized a DNA molecule with the required base sequence for coding somatostatin. A bacterial gene was cut open with a restriction enzyme, and the DNA sequence for the 14 amino acids, followed by a stop signal was inserted. A *hybrid protein* was synthesized from which the *somatostatin* part was cut off chemically and purified.

2. Vaccines. Injecting an animal with an inactivated virus stimulates it into making antibodies against viral proteins. These antibodies protect the animal against infection by the same virus by binding to the virus. Phagocytic cells then remove the virus. Vaccines are manufactured by growing the disease-producing organism in large amounts. This process is often dangerous or impossible. Moreover, there are difficulties in making the vaccine harmless. Recombinant DNA techniques permit the insertion of specific genes into bacteria so that they produce only the proteins against which the antibody response is required.

Although vaccines are available against the disease, there is a slight risk that they can actually cause the disease because they sometimes contain live viruses. Genentech Inc., in collaboration with the U.S. Dpet. of Agriculture scientists, has now produced a safe effective vaccine against the foot and mouth

disease. The virus causing the disease consists of a nucleic acid core covered with a coat of four proteins. One of these proteins, VP3, creates immunity without causing infection, in test animals. The VP3 gene was isolated from the virus and spliced into a plasmid from *E. coli*. The offspring of this bacterium now produced VP3. Vaccines against malaria and hepatitis may also be produced in the near future.

3. **Interferon.** Interferons are virus-induced proteins produced by cells infected with viruses. They appear to be the body's first line of defence against viruses. The interferon response is much quicker than the antibody response. Interferons are *anti-viral* in action. One type of interferon can act against many different viruses, i.e. it is *not virus specific*. It is, however, *species specific*. Interferon from one organism does not give protection against viruses to cells of another organism.

Interferon provides natural defence against such viral diseases as *hepatitis* and *influenza*. It also appears to be effective against certain types of *cancer*, especially cancer of the breast and lymph nodes. Natural interferon is collected from human blood cells and other tissues. It is produced in very small quantities. Each daily injection costs upto $150. If bacterially-produced interferon is found to have no harmful reaction, it may become available for as low as $1 per injection. In 1980 Biogen S.A. produced the first recombinant DNA induced interferon-like human protein. Genentech is offering several types of interferon, one of which is undergoing clinical tests.

Weissmann and his associates have produced interferon by recombinant DNA methods. Messenger RNA for interferon was taken from WBC stimulated by viral infection. Complementary DNA transcribed by the mRNA was converted into double stranded DNA, which was then inserted into vector DNA dn cloned. Some 20,000 clones were examined for the presence of interferon. This was done as folows. Plasmid DNA from the clones was hybridized to mRNA from the WBC. mRNA was then isolated from the DNA-RNA hybrids and injected into frog's eggs to test whether it directed the synthesis of interferon. Once the desired clones were obtained they were tested to see whether they produced interferon. Interferon DNA was inserted into thepenicillinase gene of bacteria, and the product was found to be biologically active.

4. **Enzymes.** The enzyme *urokinase*, which is used to dissolve blood clots, has been produced by genetically engineered microorganisms.

5. **Antibodies.** One of the aims of genetic engineering is the production of *hybridomas*. These are long-lived cells that can produce antibodies for use against disease.

6. **Gene therapy for treating hereditary diseases.** The experiment demonstrates that it may be possible in the future to alter the genetic material in the human egg. This could lead to the elimination of inherited diseases like *haemophilia, Tay-Sachs disease* and *phenylketonuria*. There are, however, still a number of problems to be solved before this is achieved.

The technique could also be used to cure blood diseases like *thalassemia* and *sickle-cell anaemia*,

which result from defects in single genes. Non-defective genes could be transferred into the bone marrow along with the methotrexate-resistance genes. Treatment with methotrexate would then destroy the cells containing defective genes and would permit the non-defective cells to form bone marrow.

2. Industrial applications

The industrial applications of recombinant DNA technology include the synthesis of substances of commercial importance in industry and pharmacy, improvement of existing fermentation processes, and the production of proteins from wastes. Cetus Corp. has used genetic engineering methods to modify microorganisms for the production of industrial chemicals such as *ethylene* oxide (for making plastics) *ethylene glycol* (antifreeze) and *alcohol*. In future it will be possible to produce fuels, plastics and other industrial chemicals out of industrial wastes.

Construction of industrially important bacteria. Bacteria with novel phenotypes can be produced by genetic engineering, sometimes by combining the features of several other bacteria. For example, several genes from different bacteria have been inserted into a single plasmid that has then been placed in a marine bacterium, yielding an organism capable of metabolizing petroleum; this organism has been used to clean up oil spills in the oceans. Furthermore, many biotechnology companies are at work designing bacteria that can synthesize industrially important chemicals. Bacteria have been designed that are able to compost waste more efficiently and to fix nitrogen (to improve the fertility of soil), and an enormous effort is currently being expended to create organisms that can convert biological waste to alcohol. An interesting bacterium is a strain of *Pseudomonas fluorescens*, which lives in association with maize and soybean roots. A lethal gene from *Bacillus thuriengis*, a bacterium pathogenic to the black cutworm, has been engineered into this bacterium. The black cutworm causes extensive crop damage and is usually combatted with noxious insecticides. In preliminary studies inoculation of soil with the engineered *Psn. Fluorescens* resulted in death of the cutworm. This type of genetic engineering will surely have a great impact on world economy and environmental quality.

3. Environmental applications

Generally modified microorganisms could be used for degradation of wastes in sewage, oil spills, etc. Scientists of the General Electric Laboratories of New York have added plasmids to create strains of *Pseudomonas* that can break down a variety of hydrocarbons. This strain has been used to clear up oil spills. It can degrade 60% of the crude oil, while the four parents from which it was derived break down only a few compounds. The discovery was awarded a patent, this being the first patent for a living organism

4. Agricultural applications

It would be possible to prepare clones of genetically manipulated plants and animals of agricultural importance having desirable characteristics. This would increase the nutritive value of plant and animal food. Genetic engineering could lead to the development of plants that would *fix nitrogen* directly from the

atmosphere, rather than from fertilizers which are expensive. Creation of nitrogen fixing bacteria which can live in the roots of crop plants would make fertilization of field unnecessary. Production of such self-fertilizing food crops could bring about a new green revolution. Genetic engineering could create microorganisms which could be used for *biological control* of harmful pathogens, insect pests, etc.

Genetic engineering of plants. Altering the genotypes of plants is an important application of recombinant DNA technology. The bacterium *Agrobacterium tumefaciens* and its plasmid Ti, which produces crown gall tumors in plants. These tumors result from integration of the plasmid DNA into the plant chromosome. It is possible by genetic engineering to introduce genes from one plant into this plasmid and then, by infecting a second plant with the bacterium, transfer the genes of the first plant to the second plant. (Actually genes are first cloned in an *E. coli* plasmid and then recloned in Ti.) Attempts are being made to perform plant breeding in this way. An example is the attempted alteration of the surface structure of the roots of grains such as wheat, by introducing certain genes from legumes (peas, beans), in order to give grains the ability of the legumes to establish root nodules of nitrogen-fixing bacteria. If successful, this would eliminate the need for the addition of nitrogenous fertilizers to grain-growing soils.

The first engineered recombinant plant of commercial value was developed in 1985. An economically important herbicide (weed killer) is glyphosate, which inhibits a particular essential enzyme in many plants. However, most herbicides cannot be applied to fields growing crops because both the crops and the weeds would be killed. (The chlorinated acids that selectively kill dictyledonous plants but not the grasses, such as maize and the cereals, are out of favour because of their persistence in soil, toxicity to animals, and possible carcinogenicity in humans). The target gene of glyphosate is also present in the bacterium *S. typhimurium*. A resistant form of the gene was obtained by mutagenesis and growth of *Salmonella* in the presence of glyphosate; the gene was cloned in *E. coli* and then recloned in *Agrobacterium*. Infection of plants with purified Ti containing the glyphosate-resistance gene has yielded varieties of maize, cotton and tobacco that are resistant to glyphosate. Thus, fields of these crops can be sprayed with glyphosate at any stage of growth of the crop.

5. Fundamental understanding of biological processes

Genetic engineering techniques have been used for acquiring basic knowledge about biological processes like gene structure and expression, chromosome mapping, cell differentiation and the integration of viral genomes. This could lead to a better understanding of the genetics of plants and animals, and ultimately of humans. As compared to the 3,000-4,000 genes in *E. coli* the human cell contains hundreds of thousands of genes. At present the exact location and function of a vast majority of the genes is not known. Human genes could be transplanted into *E. coli* one at a time, and the bacterium could then be made to reproduce on a large scale. This would provide millions of copies of individual genes, and enable a detailed analysis of their structure and function. It will be possible to construct complete DNA maps of eukaryotes by cloning specific genes inserted in *E. coli* or other microorganisms. Many of the genes of *Drosophila* have been isolated and identified by this method.

Determination of the primary structure of DNA, i.e. the sequence of bases, is important for decoding the genetic information stored in genes. For this purpose it is essential to have multiple copies of specific genes. This can be achieved by inserting the genes into bacterial DNA, and then cloning the cell to produce multiple copies.

For some types of research work a specific protein is required for the study of its enzymatic or physical properties. To obtain such a protein from a bacterial culture would require thousands of gallons of the culture, as some proteins are made in very small quantities (e.g. 10 molecules in each bacterial cell). If the gene for the protein is inserted in the lambda phage DNA, the phage would replicate and produce several hundred copies of the protein per cell. Thus there would be a far greater yield of protein per bacterial cell. A gene could also be coupled to a promoter sequence of the host cell, e.g. the *lac* (lactose) promoter of *E. coli*. Transcription of mRNA, and hence synthesis of a particular protein, could now be regulated and started or stopped as required. Under suitable conditions of growth the bacterial cell could be made to produce a specified protein to the extent of 5% of the total bacterial protein.

POTENTIAL BIOHAZARDS OF GENETIC ENGINEERING

1. **Dangers.** Recombinant DNA-research involves potential dangers. Genetic engineering could create dangerous new forms of life, either accidentally or deliberately. A host microorganism may acquire harmful characteristics as a result of insertion of foreign genes. If disease-carrying microorganisms formed as a result of genetic manipulation escaped from laboratories, they could cause a variety of diseases. For example, *Streptococcus*, a bacterium causing rheumatic fever, scarlet fever, strep throat and kidney disease, never acquired penicillin resistance in nature. If a plasmid carrying a gene for penicillin resistance is introduced into *Streptococcus* it would confer penicillin resistance on the bacterium. Penicillin would now become ineffective against the resistant organism. A strain of *E. coli* could be made capable of synthesizing the toxin for diphtheria or botulism. This would convert the normally harmless inhabitant of the human large intestine into a lethal pathogen. Even useful genetically engineered bacteria could become dangerous. Thus if an *E. coli* strain producing a human hormone infects a human being, the large quantities of the hormone released into the intestine could upset the body chemistry.

2. **Escape of chimeric microorganism.** The possibility exists that microorganisms with recombinant DNA might escape from the laboratory and be perpetuated and irreversably fixed in living organisms. If the microbial hosts of recombinant DNA inhabit humans, the likelihood of escape from the laboratory to the natural ecological environment is greater than for microorganisms living on soil and/or plants. Use of microorganisms that occupy special ecological niches like hot springs and salt water would pose less hazard in the event of escape.

   The agencies by which microorganism can escape from the laboratory are *laboratory personnel* (such as scientists, dish washers, media prepares, visitors, etc.) *laboratory facilities* (glassware,

drainage) *spills* and *aerosolization*. Laboratory personnel may not be fully conversant with the precautions to be followed in research on pathogenic microorganisms, or may not be fully conversant with the precautions to be followed in research on pathogenic microorganisms, or may not observe the precautions, Laboratory workers often acquire immunity against microorganisms with which they are working and may thus act as carriers.

3. Pros and cons of genetic engineering research

Currently there is a great scientific controversy going on as to whether recombinant DNA experiments should be permitted or not. Equally well known scientists are found supporting genetic engineering and opposing it. The arguments *against* recombinant DNA experiments are as follows :

1. Escape of genetically modified microorganisms through drainage, laboratory glassware, laboratory personnel, etc. could lead to the origin and spread of new types of diseases.
2. The experiments interfere with the natural evolutionary process and lead to a breakdown of the natural barrier between prokaryotic and eukaryotic organisms.
3. Genetic engineering is unnecessary, and many of its anticipated results would be achieved by other, less dangerous, techniques.
4. There is a danger that genetic engineering techniques will be used for biological warfare. Disease-carrying microorganism could be used against the enemy either in undeclared, silent warefare, or in organised warfare. They could also be employed in terrorist activities.

The arguments *for* recombinant DNA experiments are as follows :

1. There is no genetic barrier between prokaryotes and eukaryotes. Exchange of DNA between the two has taken place continuously in evolution.
2. The dangers of recombinant DNA experiments will decline with growth of experience and accumulation of knowledge.
3. With use of safety precautions which are common in medial research on bacteria and viruses there need be no danger of infections.
4. Bacterial cells produced by genetic engineering have additional traits and would not be able to complete against normal cells. Thus the chaces of survival of escaped organisms would be remote.
5. It is very difficult for microorganisms to become pathogenic. Virulent gene combinations are rejected by host bacterial cells, and the unwanted genes die. Many combinations have been tried in evolution and rejected.

6. All recombinant DNA research work need not be stopped. A temporary ban on certain types of work involving tumour viruses and increasing drug resistance would be enough.
7. If recombinant DNA research is stopped, a promising field with a great potential for solving problems of mankind would be wrecked.

5. Safeguards.

The general safeguards for recombinant DNA research are outlined below.

1. Genes coding for the synthesis of *toxins* or *antibiotics* should not be introduced into bacteria without proper precautions.
2. Genes of animals, animal viruses or tumour viruses should also not be introduced into bacteria without proper precautions.
3. Laboratory facilities should be equipped to reduce the possibility of escape of pathogenic microorganism by using microbial safety cabinets, hoods, negative pressure laboratories, special traps on drains lines and vacuum lines.
4. Use of microorganisms occupying special ecological niches such as hot springs and salt water should be encouraged. If such organisms escape they will not be able to survive.
5. Use of nonconjugative plasmids as plasmid cloning vectors is recommended as such plasmids are unable to promote their own transfer by conjugation.
6. The plasmid cloning vector should be genetically manipulated so that its replication depends upon the host strain, and transmission to other microbes is lethal.
7. Use of microbial host strains that can survive only under laboratory conditions that are not likely to be found in nature (safe laboratory strains) is desirable.

GENE LIBRARIES

Many laboratories utilize recombinant DNA techniques repeatedly as a means of isolating particular genes or DNA segments from a single organism. It is quite time-consuming to go through the complete cloning procedure each time a new DNA segment is needed. Thus, collections have been established of hybrid plasmid-containing bacteria that are of sufficient size that each segment of the cellular DNA is represented once (or, on occasion, twice) in the collection. Such collections are called colony banks or gene libraries. In this section we describe how a gene library is established and how to determine how many clones are needed in order that each sequence will be represented.

One of the first libraries was a collection of *E. coli* colonies containing the ColE1 plasmid in which yeast DNA had been cloned. To form the library, purified ColE1 DNA was cleaved with the EcoRI nuclease.

which makes a single cut in this DNA molecule. The yeast DNA was not cleaved with a restriction enzyme; doing that would certainly have prevented many genes from being in the library as intact units in as much as some genes would be cleaved by EcoRI. Instead, yeast DNA was broken at random by hydrodynamic shear forces. In order to link the ColEI and yeast DNA molecules, poly (dT) tails were added to the 3'-OH termini of the cleaved ColE1 DNA and poly (dA) tails were added to the yeast DNA fragments. The molecules were joined by homopolymer tail joining, and the bacteria were transformed. Cells containing the ColE1 factor are resistant to colicin E1 added to the agar, so plasmid containing cells were readily isolated. The colonies were then transferred to blocks containing small wells filled with storage medium.

The following simple calculation enables one to determine how many colonies are required to make a complete library. Consider a DNA sample containing fragments of such a size that each fragment represents a fraction f of the genomic DNA. The probability P that a particular sequence is present in a collection of n colonies is $P = 1 - (1-f)^N$ or $N = \ln (1 - P) / \ln (1 - f)$.

Let us assume that we want every sequence to be in the library with a probability of 0.99 – that is, $P = 0.99$. If the donor DNA is haploid yeast (whose molecular weight is about 10^{10}) and the average molecular weight of the sheared DNA is 8×10^6, then $f = 8 \times 10^6 / 10^{10} = 0.0008$ and, $N = [\ln (1 - 0.99)] / [\ln (1 - 0.0008)] = 5754$. Thus, if the library contains about 5800 colonies, there is a 99 percent probability that any yeast gene will be present in at least one colony. Furthermore, if a few colonies are selected at random for further study, it is exceedingly likely that their yeast DNA sequences do not overlap. For cells with a large DNA content (such as *Drosophila*) the required number of colonies is about 300,000; this can be reduced roughly n-fold by increasing their size of the constituent fragments by a factor of n.

SUGGESTED QUESTIONS:

1. Restriction enzymes generate three types of termini. What are they?
2. Are the termini of a restriction fragment produced by a particular enzyme always the same or can they be different?
3. Can two different restriction enzymes act at the same site?
4. What is the source of restriction enzymes?
5. What three methods can be used to join fragments with blunt ends?
6. What advantage results from the use of a cosmid?
7. Write notes on
   i) Restriction endonuclease
   ii) Vectors
   iii) Gene Libraries

Ref. Books:

Microbial Genetics – *David Freefelder*

Biochemistry – Zubey.

Botany

Part -1 Paper -I

Module No -8

ALGAE

Contents

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Chapter-1

Classification of Algae

INTRODUCTION

1.1. What are algae?

Modern Phycologists would accept the spirit if not letter of Fritsch's (1935) definition: *'unless purely artificial limits are drawn the designation of an alga must involve all holophytic organisms (as well as their numerous colorless derivatives) that fail to reach the level of differentiation of archegoniate plants'.*

The algae are thus thallophytes (plants lacking roots, stems and leaves) that have chlorophyll a as their primary photosynthetic pigment; with exception of the Charophyceae the algae are distinguished from the bryophytes by their lack of multicellular sex organs contained with sterile jacket of cells and by their lack of retention of sporophyte within the female organ i.e. they do not form embryo. These features encompass a number of plants that are not necessarily closely related for example bluegreen algae (cyanobacteria) and prochlorophytes which are closer in evolution to the bacteria than to the rest of the algae. If we accept five kingdoms of life (Whittaker, 1965) the algae of course dwell into 'MONERA' & 'PROTISTA'.

1.2. A SURVEY OF CLASSIFICATION BY DIFFERENT AUTHORS

Classification of the algae has long been the subject of debate. Harvey (1836) was the first to recognize four major divisions based primarily on colour (brown, red, and green algae, and diatoms) and his scheme has proved to be the basis of most modern classifications of algae into division. While recent treatments recognize more than four divisions, the importance of the major photosynthetic pigments and the accompanying biochemical and structural similarities as a means of segregating algae into divisions has prevailed. There remain, nonetheless, wide differences of opinion among Phycologists as to the details of classification. A continuous stream of new informations on the fine structure and biochemistry of algae is stimulating new approaches to algal classification, and any scheme proposed at the present time is necessarily tentative. Factors which

contribute to difficulties of classification include the evident polyphyletic origin of the algae, the seemingly poor fossil record to most groups, and the tendency to make wide ranging conclusions on the basis of an examination of a relatively small number of species.

Following are some important classification systems. Prior to 1950 the classification systems were based on light microscopic observations, pigmentations, food reserve etc. for example as proposed by Fritsch (1935, 1945), and Smith (1950, 1955). Advent of electron microscope added new dimension to algal structures and therefore later classifications have given more stress to ultrastructure, for example in the schemes of Chapman and Chapman (1973), Round (1977, 1981), Bold and Wynne (1978, 1985) etc. While in the later years endosymbiosis theory has been given due emphasis and therefore classification systems proposed by Van den Hoek *et al.*, (1995), Lee (1999) and Graham and Wilcox (2000) may be considered in this view.

Important Systems:

Fritsch (1935, 1945)

1. Myxophyceae
2. Chlorophyceae
3. Xanthophyceae
4. Chrysophyceae
5. Bacillariophyceae
6. Cryptophyceae
7. Dinophyceae
8. Chloromonadineae
9. Phaeophyceae
10. Euglenineae
11. Rhodophyceae

Smith (1950, 1955)

1. Cyanophyta

   Myxophyceae

2. Chlorophyta
   Chlorophyceae
   Charophyceae
3. Chrysophyta
   Xanthophyceae
   Chrysophyceae
   Bacillariophyceae
   Cryptophyceae
4. Pyrrophyta
   Desmophyceae
   Dinophyceae
5. Phaeophyta
   Phaeophyceae
6. Euglenophyta
   Euglenophyceae
7. Rhodophyta
   Rhodophyceae

Round (1977)

1. Cyanophyta
   Cyanophyceae
2. Chlorophyta
   Bryopsophyceae
   Zygophyceae
   Oedogoniophyceae
   Chlorophyceae
3. Charophyta
   Charophyceae
4. Prasionophyta
   Prasinophyceae
5. Euglenophyta

Euglenophyceae

6. Xanthophyta

Xanthophyceae

7. Chrysophyta

Chrysophyceae

8. Haptophyta

Haptophyceae

9. Bacillariophyta

Centrobacillariophyceae

Pennatobacillariophyceae

10. Dinophyta

Desmophyceae

Dinophyceae

*Eustigmatophyceae

11. Phaeophyta

Phaeophyceae

12. Rhodophyta

Rhodophyceae

13 Cryptophyta

Cryptophyceae

* Division not cited

Bold and Wynne (1985)

1. Division Cyanophyta
2. Division Prochlorophyta
3. Division Chlorophyta
4. Division Charophyta
5. Division Euglenophyta
6. Division Phaeophyta
7. Division Chrysophyta

Class 1. Chrysophyceae

Class 2. Prymnesiophyceae
Class 3. Xanthophyceae
Class 4. Eustigmatophyceae
Class 5. Raphidophyceae
Class 6. Bacillariophyceae

8. Division Pyrrhophyta
9. Division Rhodophyta
Subclass : Bangiophycidae
Subclass : Florideophycidae
10. Division Cryptophyta
11. Division Algae of uncertain affinity

LEE (1999):

Group 1 Prokaryotic algae

Cyanophyta (cyanobacteria): Chlorophyll *a*; phycobiliproteins.

Group 2 Eukaryotic algae with chloroplasts surrounded only by the two membranes of the chloroplast envelope.

Glaucophyta: Algae that represent an intermediate position in the evolution of chloroplasts; photosynthesis is carried out by modified endosymbiotic cyanobacteria.

Rhodophyta (red algae): Chlorophylls *a* and *d*; phycobiliproteins; no flagellated cells; storage product is floridean starch.

Chlorophyta (green algae): Chlorophylls *a* and *b*; storage product, starch, is found inside the chloroplast.

Group 3 Eukaryotic algae with chloroplasts surrounded by one membrane of chloroplast endoplasmic reticulum.

Euglenophyta (euglenoids): Chlorophylls *a* and *b*; one flagellum with a spiraled row of fibrillar hairs; proteinaceous pellicle in strips under the

plasma membrane; storage product is paramylon; characteristics type of cell division.

Dinophyta (dinoflagellates): Mesokaryotic nucleus; chlorophylls *a* and c_1; cell commonly divided into an epicone and a hypocone by a girdle; helical transverse flagellum; thecal plates in vesicles under the plasma membrane.

Group 4 Eukaryotic algae with chloroplasts surrounded by two membranes of chloroplast endoplasmic reticulum.

Cryptophyta (cryptophytes): Nucleomorph present between inner and outer membrane of chloroplast endoplasmic reticulum; starch is formed in grains between inner membrane of chloroplast endoplasmic reticulum and chloroplast envelope; chlorophylls *a* and *c*; phycobiliproteins; periplast is inside plasma membrane.

Heterokontophyta (heterokonts): Anterior tinsel and posterior whiplash flagellum, chlorophyll *a* and *c*, fucoxanthin, storage product usually chrysolaminarin occurring in vesicles in cytoplasm.

Chrysophyceae (golden-brown algae).

Synurophyceae

Dictyochophyceae (silicoflagellates)

Pelagophyceae

Bacillariophyceae (diatoms)

Raphidophyceae (chloromonads)

Xanthophyceae (yellow-green algae)

Eustigmatophyceae

Phaeophyceae (brown algae)

Prymnesiophyta (haptophytes): Two whiplash flagella, haptonema present, chlorophylls *a* and *c*, fucoxanthin, scales common outside cell, storage product usually chrysolaminarin occurring in vesicles in cytoplasm.

1.3. CRITERIA OF ALGAL CLASSIFICATION

(1) Diversity of cellular organization:

Included among the algae are both eukaryotic and prokaryotic types. The cyanobacteria and prochlorophytes are prokaryotes. Prokaryotic algae resemble structurally other Gram negative eubacteria, but differ from other photosynthetic bacteria by having two photosystems with chlorophyll a producing oxygen. The pigments of both cyanobacteria and prochlorophytes are located on internal membranes called thylakoids.

Other members are included under eukaryotic types. A eukaryotic cell is often surrounded by a cell wall composed of polysaccharides and secreted by golgi bodies. The plasma membrane surrounds the remaining part of the cell. Locomotary organs, the flagella propel the cell through the medium by their beating in motile cells. The flagella are enclosed in the plasma membrane and have specific number and orientation of microtubules. The cytoskeleton of the cell is associated with basal bodies and controls the shape of the cell, the position of organelles and movement of materials within the cell. The chloroplasts are generally surrounded by two membranes of the chloroplast envelope. An eyespot (stigma) consisting of several layers of carotenoid granules; may also be part of the chloroplast. The eyespot while involved with sensing the direction of light is not the actual photoreceptor which is either part of the cell membrane or a swelling near the base of a flagellum. The chloroplast may contain a distinct proteinaceous region, called the pyrenoid. The pyrenoid contains the enzyme ribulose 1, 5 bisphosphate carboxylase/oxygenase (McKay and Gibbs, 1991), which is responsible for the incorporation of carbon dioxide into organic compounds in the Calvin cycle. Other cellular structures are mitochondria, golgi bodies, endoplasmic reticulum and ribosomes. Contractile vacuoles, organelles often present near the base of flagella in fresh-water flagellates, regulate the fluid content of the cell by slowly filling the excess liquid and expelling it through pores. Other vesicles (microbodies) and inclusions may be present. In some algal groups, the carbohydrate reserve is stored in cytoplasmic vesicle rather than in the chloroplast.

The nucleus which contains genetic material of cell is surrounded by a double

membrane with pores in it. There are 2 basic types of nuclei in eukaryotic algae. These are: (i) that occur in the Dinophyta and Euglenophyta and (ii) that occurring in the rest of the eukaryotic algae. The Dinophyta & Euglenophyta have following mesocaryotic nuclear characteristics:

(a) Chromosomes condensed throughout the miotic cycle

(b) A persistent nucleolus (endosome), which does not disperse during prophase and which divides pinching into two.

(c) Large nuclei.

(d) Chromosomes that are attached to the nuclear membrane and not to spindle microtubules inside the nucleus.

(e) An intact nuclear membrane during whole mitotic cycle. In the Dinophyta there are few basic proteins (histones) associated with DNA.

(2) Flagella:

The different group of algae show characteristic features of their flagella and associated structures composing the flagellar apparatus. The flagellar apparatus consists of the emergent flagella, the transition region where a flagellum joins its basal body, basal bodies and the cytoskeletal elements radiating from the basal body.

Flagella consist of an axoneme of nine doublet microtubules surrounding two central microtubules, with all the microtubules surrounded by the plasma membrane of the cell. On entering the cell body, two central microtubules end at a dense plate, whereas the nine doublets continue into the cell usually picking up an additional structure that transform them to triplets. Although basal bodies are similar among algae, the transition region shows characteristic features for different groups. Some algal groups have a swelling (paraflagellar body) near the base of one flagellum which functions as photoreceptor allowing the cell to respond to the direction of light. The cytoskeleton of a flagellate cell, composed of structural proteins radiating from basal bodies is divided into three components: connecting fibers between the basal bodies (proximal connecting fibers and distal connecting fibers), root composed of microtubules (called microtubular roots) and fibrous roots (rhizoplast). A contractile protein centrin is

present in connecting fibers and fibrous roots (Salisbury 1989). The types of cytoskeletal components are characteristic of each group and important in determining evolutionary relationship.

The flagellar membrane may have no hairs (mastigonemes) on its surface (whiplash or acronematic or peitgeissel flagellum), or it may have hairs on its surface (tinsel or hairy, or pantonematic or Flimmergeissel). There are two types of flagellar hairs: (i) fibrous solid hairs (ii) tubular hairs composed of a tapering basal region attached to the basal membrane, a microtubular shaft and a few long terminal filaments. In addition to hairs, a number of different scale types are found on the surface of flagella.

Algal motile cells have different arrangement of flagella. These arrangements are also important for designating a characteristic group.

(3) Cell Coverings: Cell Wall - While many flagellates and algal spores and gametes described as naked; the majority of algal cells are covered by one or more bounding layers of relatively inert material (principally carbohydrates), which mayor may not be impregnated with inorganic substances such as calcium carbonate and silica (e.g. diatoms). Many of the walls are comparable with those found in higher plants, although specialized coverings (such as pellicle or theca) may be distinctive for a particular group of algae. Members of different class such as Chrysophyceae, Prymnesiophyceae, Prasinophyceae etc. possess scaly coverings (body scales) deposited on the surface of plasma lemma and are organic, calcite or silica by chemical nature.

In general algal cell walls are made of two components, (i) Fibrillar componant, which forms the skeleton of the-- wall, and (ii) the amorphous component which forms a matrix within which the fibrillar component is embedded..

(i) Fibrillar component

(a) Cellulose (a polymer of 1, 4 linked ß- D - glucose)

(b) Mannan (a polymer of 1, 4 linked ß - D - Mannose)

(c) Xylan

(ii) Amorphous component:

(a) Aliginic acid (a polymer mostly composed of ß - 1, 4 linked D-

mannuronic acid residues with various amounts of L - guluronic acid) e.g. Phaeophyta

(b) Fucoidin (a polymer of α-1, 2, α -1, 3, & α -1, 4 linked residues of L - fucose sulfated at C-4) e.g. Phaeophyta

(c) Agar-agar (galactans made of agarose & agaropectins).

(d) Carrageenan (galactans).

(e) Furcelleran & Funoran.

The cell walls of the Cyanophyta and Prochlorophyta are unique in the sense that they ultrastructurally resemble with gram negative bacteria and are made of peptidoglycans and lipopolysaccharides.

(4) Chloroplast Ultrastructure:

In Rhodophyta and Chlorophyta the chloroplasts are bounded by the double membrane of chloroplast envelope. In other eukaryotic algae, the chloroplast envelope is surrounded by one or two membranes of chloroplast endoplasmic reticulum. In Euglenophyta and Dinophyta there is one membrane of chloroplast E.R. In other divisions of algae there are two membranes of chloroplast E.R. Between chloroplast E.R. and chloroplast envelope is a space that contains tubules, ribosome; and in Cryptophyta, reserve products. The contents of this space probably had a cytoplasmic origin and the arrangement of the membranes of chloroplast E.R. might be the remains of an earlier endosymbiotic event (Lee 1977).

The arrangements of thylakoids in different groups are as follows:

(i) Unstacked: - Cyanophyta, Rhodophyta

(ii) Stacked: -

(a) Two- Cryptophyta, Prochlorophyta

(b) Three- (i) With girdle band- Euglenophyta, Xanthophyta, Bacillariophyta, Phaeophyta, Chrysophyta, Raphidophyta.

(ii) Without girdle band - Dinophyta, Prymnesiophyta, Eustigmatophyta

(c) Two to six - Chlorophyta

In between thylakoids phycobilisomes are present in Cyanophyta & Rhodophyta.

(5) Pigments:

There are three types of photosynthetic pigments occur in algae: chlorophyll, carotenoids and phycobiliproteins, the former two are insoluble in water but are soluble in organic solvents whereas the latter one is soluble in water.

(i) Chlorophyll: It is composed of a porphyrin ring system with a phytol tail. The algae have four types of chlorophyll,a,b,c (c_1 & c_2) and d.

Chlorophyll a - all photosynthetic algae

Chlorophyll b - Euglenophyta, Chlorophyta

Chlorophyll c_1 & c_2 - Raphidophyta, Chrysophyta, Prymneriophyta, Bacillariophyta, Xanthophyta & Phaeophyta

Chlorophyll c_2 only - Dinophyta & cryptophyta

Chlorophyll d – Rhodophyta and in a cyanophyte *Acaryochloris marina.*

(ii) Carotenoids - These are yellow, orange or red pigments that usually occur inside the plastid but may be outside in certain cases.

(a) Carotenes – Oxygen-free hydrocarbons

α -carotene - Rhodophyta, Cryptophyta Chrysophyta, some Chloro- & Dinophyta

ß-carotene - Almost all groups

γ-carotene - some Eugleno - & Chlorophyta

ε-carotene - Chrysophyta, Bacillariophyta, Phaeophyta

(b) Xanthophylls - These are oxygenated derivatives of hydrocarbons. A large variety of xanthopheyll occur in algae, mixture of which gives the algae their characteristic coloration along with other pigments. Fucoxanthin is the principal xanthophyll in the golden brown algae (Chrysophyta, Bacillariophyta, Prymnesiophyta & Phaeophyta). Some xanthophylls are exclusive to their representative groups. e.g.

Oscillaxanthin, Myxoxanthophyll - Cyanophyta

Heteraxanthin- Chrysphyta

Peridinin - Dinophyta

Siphonein, Siphonoxanthin - Siphonaceous marine green algae.

(iii) Phycobiliproteins: These are water soluble blue or red pigments located on

(Cyanophyta & Rhodophyta) or inside (Cryptophyta) thylakoids of algal chloroplast. The prosthetic group (non-protein part) is tightly bound by covalent linkage to its apoprotein (protein part of the molecule). Therefore they are called phycobiliproteins. The prosthetic group (chromophore) is a tetrapyrole (bile pigment). The major 'blue' chromophore occuring in phycocyanin and allophycocyanin is phycocyanobilin and the major 'red' chromophore occuring in phycoerythin is phycoerythrobilin. In addition, in B - and R- phycoerythrin there is the chromophore phycourobilin.

(6) Storage products:

(A) High molecular weight compounds

(i) α-1, 4 linked glucans:

a. Floridian starch - This substance occurs in Rhodophyta and is similar to the amylopectin of higher plants.

b. Myxophycean starch - Found in the Cyanophyta, myxophycean starch has a similar structure to glycogen or amylose fraction of starch.

c. Starch - In the Chlorophyta, the starch is composed of amylose and amylopection. Also occur in Cryptophyta & Dinophyta.

(ii) ß-1, 3 linked glucans:

a. Laminarin - In the Phaeophyta, laminarin consists of a related group of predominantly ß-linked glucans containing 16 to 31 residues.

b. Chrysolaminarin (leucosin) - In the Chrysophyta, Prymnesiophyta and Bacillariophyta.

c. Paramylon - In the Eugenophyta, Xanthophyta & Prymnesiophyta *(Pavlova mesolychnon)*, paramylon occurs as water soluble single membrane bounded inclusions of various shapes and dimensions outside the chloroplast.

(iii) Fructosans - *Acetabularia* (Chorophyta) has an inulin like storage product. Also occurring in Cladophorales.

(B) Low molecular- weight compounds.

(i) Sugars - Chlorophyta & Euglenophyta form sucrose as a reserve product.

(ii) Glycosides - The glycerol glycosides, floridoside and isofloridosides are widely distributed in Rhodophyta.

(iii) Polyols - Mannitol is universally present in phaeophyta. Also present in Prasinophyta and some marine Volvocales e.g. *Dunaliella*

(7) Mitosis & Cell Division:

Mitosis and cell division are associated with cellular reproduction. In a primitive alga nuclear envelope persists, enclosing mitotic spindle (closed spindle) and centrioles serve as organizing centres for the microtubules composing the spindle. In more advanced algae, an open spindle occurs as a result of breakdown of the nuclear envelope other organelles divide along with the nucleus. As mitosis is completed a cell divided at its equator by in-growth of the membrane under the control of actin filaments- this is called furrowing. In green algae the terms phycoplast and phragmoplast are applied. In the Chlorophytes, mitosis occurs without complete break down of the nuclear envelope (closed spindle), the daughter nuclei resulting from mitosis remain closely associated, and the microtubules of spindle are reorganized into a phycoplast in which the microtubules are oriented parallel to the plane of division. In contrast during mitosis in the charophytes, the spindle is open as a result of breakdown of nuclear envelope. At completion of mitosis, daughter nuclei move to opposite ends of the parent cell, and the spindle persists as phragmoplast, with microtubules perpendicular to the plane to the plane of cell division. Cytokinesis occurs by furrowing or cell plate formation.

1.4. MODERN TRENDS IN CLASSIFICATION

Light microscopic studies and existing tools in biochemistry and physiology led to the classificatory schemes till 1950. Advent of electron microscopy added new dimension to the understanding of algal structure and function in the latter years. This and many modern tools helped not only to identify and clarify the systematic and phylogenetic relationship among algae but also contributed significantly to understand the process of evolution leading to the origin and evolution of

different extinct and extant form of organisms. This is due to these data we are now very clearly understanding the basic processes of endosysmbiosis and also the origin of land plants beside many more mysteries waiting to be resolved.

1) Culture Studies: Culturing algae in laboratory is not new or recent tool but searching for new dimensions provoked to open new and novel findings. For example systematics of Cyanobacteria is largely dependent on culture studies. This is because of the fact that many of the forms occurring in nature are actually various life histories stages rather than the organism themselves.

Life histories studies on many red, brown and green algae have helped to solve taxonomic riddles. For example the establishment of relationship between *Porphyra* and *Conchocelis* and similar several other pairs not only solved the debate regarding their status but also prompted to take concrete measures for establishment and promotion of several billion dollar industries involved in commercial exploitation of several sea weeds. Parasitism and hyperparasitism known very common in red algae also lead to understand the nature and principles involved in cell to cell communications also due to culture studies. It is now well known that only in algae several forms have same morphology in gametophyte and sporophyte e.g., *Dictyota, Cladophora, Ectocarpus* etc.

(2) Cytology and Ultra structure: - Concrete steps taken by Godward (1966) to study chromosomes of algae promoted many Phycologists in the world including our country to understand the cytotaxonomic problems, Many interesting facts emerged such as the nature of polyploidy in charophytes and other algae, nature of mesokaryotic nuclei in dinoflagellates and also in englenophytes, nature of endosymbiosis etc. This is due to these studies it is established that centre of origin of Charales was probably Bihar and it adjoining regions. Presence of nucleomorph in some groups led to understand nature of endosymbiosis and in turn the systematic and phylogenetic relationship among the groups.

Nature of *Beggiatoa, Prototheca* and *Plasmodium* (malarial organism) is now clearly known by ultrastructural studies that they had their roots in algae.

Ultrastructure of mitochondria is now considered of taxonomic significance utilized in delineating taxonomic groups. Major breakthrough in algal systematic studies emerged due to works of Stewart and Mattox (1984) and Pickett-Heaps (1975). Findings of Steward and Mattox (1984) on the ultrastructure of flagella and its roots has given new dimension in reclassification of algal groups in green algae and clearly established the relationship between *Coleochaete* and related plants to the origin of land plants. Pickett-Heaps (1975) also supported these findings on the basis of phycoplast and phragmoplast. Graham (1983), therefore, has given a clear insight into origin of land plants on the basis of these and several other data accumulated from various sources.

(3) Biochemical studies: - Many modern and sharp tools involved in these studies have given more clear understanding for various algal groups. For example CCM in algae are various. Rubisco involved in this process are also various. Nature and transfer of plastid genes is interesting aspect in studies on Rubisco. It not only gives an insight into the process of evolution of photosynthesis but also the relationship of various algal groups and genera. For example spectroscopic methods led to know the systematic position of various algal genera occurring as nannoplankton.

Degradation of urea by urease or urea amidolyase, glycolate degradation by glycolate oxidase or glycolate dehydrogonase, nature of cellulose terminal complexes and pyrenoids etc. are various such aspects which have given concrete results.

1.5. MOLECULAR BIOLOGY AS AN AID TO CLASSIFICATION

This is more recent tool applied to understand systematic relation between various organisms initiated by Stackenbradt (1985) and Carl Woese (1990) for bacterial evolution. The basis is that a conservative gene sequence where change occurred only due to mutation may give an insight into the understanding

of the phylogenetic connections. 16SrRNA, 18SrRNA various other such conservative gene segments are now compared to yield fruitful results. For example *Prochloron, Prochlorococcus* and *Prochlorothrix* isolated into a new division prochlorophyta on the basis of pigment composition are now negated and considered "green cyanobacteria" on the basis of molecular phylogeny. Similarly relationship of cyanobacteria to eubacteria particularly to Gram negative bacteria is supported by such data as also as by ultrastructural features. But it is to be clearly noted that molecular data are data as also obtained from other sources and may only be interpreted like that. It may add to our understanding. It is not superior to other data neither be treated inferior.

Chapter –2

SALIENT FEATURES OF DIFFERENT CLASSES OF ALGAE AND THEIR PHYLOGENY

I. CYANOPHYCEAE:

i. Prokaryotic cellular organization.

ii. Cell wall similar to gram negative bacteria.

iii. Presence of chlorophyll a. Some cyanobacteria previously classified as prochlorophytes have chlorophyll b also.

iv. Thylakoids unstacked and equidistant having phycobilisomes in-between.

v. Presence of echinenone (4-keto-β-carotene) and myxoxanthophyll as carotenoid pigments. β-carotene occurring in higher proportion.

vi. Presence of heterocyst.

vii. Absence of sexual reproduction by production of gametes rather genetic recombination occurs like bacteria.

II. RHODOPHYCEAE:

i. Chiefly marine, a few freshwater.

ii. Thallus mostly filamentous, uni-or multiaxial in organization.

iii. Flagellate stage is completely lacking neither in vegetative nor in reproductive form.

iv. Chloroplast commonly discoid, without chloroplast endoplasmic reticulum; thylakoids unstacked with phycobilisomes.

v. Photosynthetic pigments chlorophyll a, α & β-carotene, lutein, zeaxanthin, antheraxanthin, violaxanthin, phycocyanin and large amounts of phycoerythin.

vi. Storage product floridean starch formed in the cytoplasm, outside the chloroplast.

vii. Mitosis closed, centrioles lacking, polar rings are present instead.

viii. Cleavage incomplete in most algae, presence of pit connection & pit plugs in most.

ix. Male gametes nonflagellate called spermatia produced in spermatangium & female sex organ carpogonia generally with a projection called trichogyne; gametic fusion always oogamous.

x. Post fertilization changes complicated.

xi. Life cycle iso- or heteromorphic, generally extended and triphasic.

III. PRASINOPHYCEAE (MICROMONADOPHYCEAE):

i. Primarily marine.

ii. Most are unicellular flagellates.

iii. Vegetative cells are covered with delicate organic scales, composed largely of carbohydrates. Scales are also present on flagella.

iv. Flagella typically emerge from an apical depression or pit commonly covered with scales.

v. Basal body orientation variable, with variable microtubular roots (cruciate roots, rhizoplasts, some with MLS); often very long and parallel.

vi. Ejectile structures extrusomes may occur.

vii. Generally have a single plastid, usually with one starch-sheathed pyrenoid.

viii. An eyespot may present, but always within the plastid.

ix. Photosynthetic pigments chlorophyll a & b usually have distinctive accessory pigment, prasinoxanthin.

x. Mitosis variable, mitotic spindle usually persistent.

xi. Microtubular organization phycoplast or phragmoplast

xii. Life cycle haplontic [haplobiontic, haploid type (H, h)]

xiii. Sexual reproduction uncommon (& poorly known), isogamous.

xiv. A spherical cyst (phycoma stage) may occur in many forms.

IV. CHLOROPHYCEAE:

i. Thallus unicellular to unbranched and branched filamentous forms; multinucleate coenocytic forms also occur. All forms produce flagellate reproductive units.
ii. Plastids with two chloroplast envelop.
iii. Motile cells with radial or near radial external symmetry.
iv. Flagella attached at anterior end of cell.
v. Flagella roots four (X-2-X-2) cruciate roots with no scales, rhizoplast may occur, no multilayered structure (MLS).
vi. Eyespots common.
vii. Mitosis closed with non-persistent spindle.
viii. Phycoplast produces new cross wall in cell division; cleavage may occur by furrowing.
ix. Glycolate break down by glycolate dehydrogenase.
x. Urea breakdown urea amidolyase.
xi. Zygotic meiosis.
xii. Predominantly freshwater.

V. ULVOPHYCEAE:

i. Thallus multicellular filaments, parenchymatous sheets, or a single, large coenocyte.
ii. Mostly marine.
iii. Flagellate stages are characterized by basal bodies in a counter clockwise orientation and cruciately arranged microtubular roots, rhizoplast present.
iv. A persistent mitotic spindle occurs during mitosis but neither a phycoplast nor phragmoplast is present during cell division. Furrowing at cytokinesis.
v. Telophase nuclei typically exhibit an elongated dumb-bell shape and daughter nuclei tend to remain well separated after mitosis.
vi. Photorespiratory enzyme glycolate dehydrogenase.

vii. Motile cells commonly covered by scales.
viii. Urea degradation enzyme urease.
ix. Life cycles are either diplohaplontic diplobiontic with sporic meiosis (D, h+d) or diplontic [Haplobiontic, diploid type with gametic meiosis (H, d)]

VI. CHAROPHYCEAE:

i. Thallus unicells to unbranched (microscopic), and highly branched macroscopic forms.
ii. Motile cells asymmetrical.
iii. Flagella two, attached laterally.
iv. Flagellar roots asymmetric constituting of a broad band of microtubules and a second smaller microtubular root.
v. Multilayered structure (MLS) may be present but rhizoplast rarely occur.
vi. Body and flagellar scales usually occur.
vii. Mitosis open, persistent spindle.
viii. Phragmoplast commonly produces new cross walls after cell division
ix. Eyespots rarely occur.
x. Glycolate broken down by glycolate oxidase.
xi. Catalase in peroxisomes.
xii. Cellulose terminal complex in rosettes.
xiii. Zygotic meiosis.
xiv. Predominantly freshwater.

VII. EUGLENOPHYCEAE:

i. Unicellular flagellates may or may not have plastids.
ii. Mostly freshwater; prefer increased levels of dissolved organics particularly vitamins B_1 and B_{12}.
iii. Exhibit metaboly, a form of motility that does not involve flagellar action but achieved due to their unusual cell surface or pellicle.

iv. Vegetative cells are naked having plasmalemma as outermost layer and a pellicle inside which gives a spiral look to the cells. The pellicle is composed of abutting strips of protein which wind helically around the cell from one end to the other.

v. Only about one third of the known genera possess green-pigmented chloroplast. Most others either lack plastids or have colourless plastids. Heterotrophic forms are therefore abundant. A number of forms are phagotrophic that have cytostome – a special apparatus for capturing and ingesting prey.

vi. Flagellate members have two emergent flagella arising from the bottom of a flask shaped invagination called ampulla. Each flagellum has a paraxonemal rod (paraxial rod) that runs the length of the flagellum and helically arranged unilateral array of delicate hairs with a felt like covering of shorter hairs.

vii. Chloroplasts are usually discoid or plate like with a central pyrenoid. Each chloroplast is surrounded by two chloroplast envelope and one chloroplast endoplasmic reticulum not connected to nucleus. Thylakoids are grouped in three. Photosynthetic pigments are chlorophyll *a* & *b* & commonly β carotene, neoxanthin & diadinoxanthin.

viii. The storage product is paramylon, a β-1, 3-linked glucan that lies as granules in the cytoplasm.

ix. The nucleus is mesokaryotic, while mitosis is closed.

x. At the anterior end of the cell there lies a large contractile vacuole.

xi. Sexual reproduction does not occur in euglenoids with regularity, if at all.

VIII. Dinophyceae:

i. Mostly marine

ii. Majority are unicellular flagellates, only a few are coccoid or filamentous.

iii. Exhibit an amazing diversity of nutritional types, including autotrophs, mixotrophs, osmotrophs, phagotrophs and parasites.

iv. Outer most boundary of the cell is cell membrane and cell covering components lie beneath the cell membrane in flattened thecal vesicles. The entire array is known as amphiesma. Thecal vesicles may most often contain thecal plates composed of cellulose.

v. The flagellate cells possess two dissimilar flagella arising on the ventral side of the cell. The former is transverse flagellum with one row of mastigonemes and lying in a transverse furrow while the latter longitudinal flagellum with two rows of mastigonemes lying in the longitudinal groove directed posteriorly.

vi. Chloroplasts with one chloroplast endoplasmic reticulum besides its normal two envelops, thylakoids united in stacks of three.

vii. Photosynthetic pigments are chlorophyll a, c_2, β-carotene and unique accessory xanthophylls peridinin.

viii. Nuclei mesokaryotic and mitosis closed.

ix. Reserve food starch and lipids.

x. Sexual reproduction iso- or anisogamous and life cycle haplobiontic haploid (haplontic) type with only the zygote nucleus being diploid.

IX. Cryptophyceae:

I. As literally means these are the most inconspicuous.

II. Relatively small 3-50μm in length, members of phytoplankton, abundant in cold or deep waters.

III. They are characterized by a dorsiventral cell body flattened in one plane.

IV. Contains chlorophyll *a* and c_2 and phycobiliproteins occurring inside the thylakoids of the chloroplast (rather than outside the thylakoids in form of phycobilisomes as in cyanophytes and rhodophytes).

V. A few are however heterotrophic and lack chloroplast.

X. Chrysophyceae:

i. Mostly freshwater, occurring in soft waters.

ii. Mostly unicellular, solitary or colonial, motile.

iii. Flagella inserted at the apex perpendicular to each other; anterior pleuronematic and lateral smooth.

iv. A distinct photoreceptor as a swelling at the base of smooth flagellum.

v. Photosynthetic pigments chlorophyll *a*, c_1 & c_2 & fucoxanthin as main carotenoid pigment.

vi. Chloroplast parietal, few in number; golden brown due to fucoxanthin.

vii. Chloroplast with two chloroplast endoplasmic reticulum, thylakoids in stacks of three per band, transition helix present.

viii. Reserve food chrysolaminarin.

ix. Mitosis is open.

x. Most members of the class produce statospores.

xi. In sexually reproducing species reproduction is iso- or physiologically anisogamous.

xii. Life cycle haplobiontic, haploid type (H, h).

XI. Bacillariophyceae:

i. Widespread in both marine and freshwater habitats.
ii. Unicellular & eukaryotic, may form colony.
iii. Each cell is encased by unique cell wall impregnated with silica and two parted like Petri dishes or oval Tiffin boxes with an overlapping lid, termed frustules.
iv. Chloroplast many, discoid (in centric forms) to just two, large and plate-like (in pennate forms).
v. Chloroplasts with two chloroplast endoplasmic reticulum and thylakoids grouped into stacks of three.
vi. Photosynthetic pigments are chlorophyll *a* & c_2 (sometimes also c_1 or c_3) together with fucoxanthin as major carotenoid pigment.
vii. Storage product is chrysolaminarin, which is located in vesicles in the cytoplasm.
viii. Only flagellate cells are the male gametes in centric diatoms. It has a single, apically inserted pleuronematic flagellum that lacks two central microtubules of axoneme. Also transition zone of flagellum lacks a transition helix.
ix. Mitosis is open.
x. Cell division is unusual in that cell size is diminished gradually for the one counterpart. It is re-established by formation of unique spores called auxospores or growth spores but these spores are formed always as a product of sexual reproduction.
xi. Sexual reproduction isogamous or oogamous
xii. Sexually reproducing species have a haplobiontic, diploid (H, d) type of lifecycle as the vegetative cells of diatoms are always diploid and meiosis is gametic.

XII. Xanthophyceae:

i. Primarily freshwater, only a few are marine (20 species of *Vaucheria*).

ii. Unicellular or colonial, coccoid; filamentous forms are few; some a siphonaceous, coenocytic.

iii. Chloroplast many, discoid.

iv. Chloroplast with two chloroplast endoplasmic reticulum & thylakoids grouped in stacks of three..

v. Photosynthetic pigments chlorophyll a, small amounts of chlorophyll c_1 & c_2 with accessory pigments β-carotene, diatoxanthin & diadinoxanthin. Fucoxanthin conspicuously absent.

vi. Cell wall generally two parted & composed primarily of cellulose.

vii. Reserve food a β-1, 3 linked glucan similar to paramylon, never starch.

viii. Zooids with flagella inserted at the apex & not laterally.

ix. Heterokont, with forwardly directed tinsel & posterior whiplash flagella.

x. A typical photoreceptor present at the base of smooth flagellum as paraflagellar body and eye spot nearby within the chloroplast.

xi. Presence of transition helix in transition region of each flagellum.

xii. Sexual reproduction reported in few members, isogamous or oogamous.

XIII. Phaeophyceae:

i. Mostly marine, only a few freshwater.

ii. All species are multicellular; the plants may exhibit complex morphology with highly differentiated tissues.

iii. Flagella laterally inserted heterokont with one anterior longer tinsel and another posterior shorter, whiplash; flagellate cells are always reproductive; no free living flagellates.

iv. Flagellate cells with typical heterokont photoreceptor at the base of smooth flagellum and an adjoining eyespot inside the cell within the chloroplast.

v. Transition region of flagellum lacks a transition helix.

vi. Chloroplast many discoid.

vii. Chloroplast with two membranes of chloroplast endoplasmic reticulum; thylakoids grouped into stacks of three; girdle lamella present.

viii. Photosynthetic pigments chlorophyll a, c_1, c_2 & fucoxanthin. Additional xanthophylls violaxanthin, antheraxanthin, neoxanthin diadionoxanthin & diatoxanthin may be present; β-carotene present.

ix. Mitosis semi-closed.

x. Cell wall composed of felt like network of cellulose microfibrils, stiffened by calcium alginate & amorphous fucoidan & mucilaginous alginates.

xi. Sexual reproduction iso-, aniso-, to oogamous.

xii. Gametes are produced in plurilocular gametangia, while meiospores in unilocular sporangia.

xiii. Life cycle diplobiontic (D^l,h+d or D^h, h+d) or haplobiontic diploid (H,d) type. In later case meiosis is gametic.

2.2. Phylogeny of Algae

Starting from the earliest prokaryotes and proceeding to the present time, the process of algal evolution spans most of the period of known life on earth, and embraces some of the main events in botanical evolution the emergence of photoautotrophic prokaryotes, the origin of eukaryotes, mitosis, syngamy and meiosis, alternation of generations, and adaptations to terrestrial life.

The origins of the prokaryote lie remarkably in time to the beginning of the geological record of *ca.* 3.8 billion years ago (bya). The first autotrophic organisms, the cyanophytes, made their appearance about half way through the Archaean (3.8 – 2.6 bya), the earlier part of the Precambrian. In the latter part of the Precambrian, the Proterozoic (2.6 – 0.5 bya), they were ubiquitous, and representatives of most present-day cyanophycean orders have been recorded from the fossil record of this period often referred to as the age of cyanobacteria.

The development of the nucleated cell type took place probably about 1.4
Meiosis and eukaryotic sexuality originated at least 0.8 bya, but may have
into existence well before then, possibly 1.33 bya. The subsequent
opment of life histories with an alternation between diploid and haploid
ations in a meiosis-syngamy cycle evolved in four major patterns; there is
ar record of the precise time of initiation of each of these patterns, although
equence in which they developed can be deducted from knowledge of life
es in modern plants. Alternation of generations was one of the adaptations
tial for a terrestrial existence that eventually played a significant role in the
lion of the land plants (embryophytes). It has been widely accepted that it
mong the ancestors of the green algae that this major advance took place,
bly somewhere between 0.7 and 0.4 bya.

ry endocytobiosis

It is a great concern that how oxygenic photosynthesis evolved in the
t earth. There is no doubt that cyanophytes (cyanobacteria) were the
st oxygen evolving photoautotrophs that had their ancestry in eubacteria.
phytes utilized H_2O to evolve oxygen and in this way created an
nment. Gradually the oxygen level in the atmosphere increased and the
shield was created. Origin of eukaryotic algae also had a root in
bacteria. The integration of a cyanobacterium into heterotrophic eukaryote
h endocytobiosis and its conversion to a reduced organelle led to primary
ls surrounded by two membranes. Such organelles are found in three
lineages: (1) the chlorophyll a / b (green algae and land plants), (2) the red
and (3) the glaucophytes, a small group of unicellular algae with several
t characteristics. Phylogenetic analysis of symbiont (plastid) and host
es indicated that irrespective of the diversity, primary plastids evolved
hyletically (Cavalier-Smith 2000).

The origins of primary plastids in green algae through the endocytobosis
nobacteria essentially lead to two basic modifications. These are: synthesis
orophyll b as secondary pigment and loss of phycobiliproteins. The

hypothesis; that the photosynthetic ancestor of green lineage was a prochlorophyte that possessed chlorophylls a and b and lacked phycobiliproteins, has lost its favour as established by molecular phylogeny data. The green lineage played a major role in oceanic food webs and the carbon cycle from about 2.2 billion years ago until the end-Permian extinction, approximately 250 million years ago (mya). It was this similarity to the pigments of plants that led to the inference that the ancestors of land plants (i.e., embryophytes) would be among the green algae, and is clear that phylogenetically plants are a group of green algae adapted to life on land.

The plastids of the red algae (Rhodophyta) constitute the third primary plastid lineage. Like the green algae, the red algae are an ancient group in fossil record, and some of the oldest fossils interpreted as being of eukaryotic origin are often referred to the red algae, although clearly these organisms were very different from an extant alga. Like those of green algae, the plastids of red algae are surrounded by two membranes. However, they are pigmented with chlorophyll a and phycobiliproteins, which are organised into phycobilisomes.

Glaucophyte lineage occupies a key position in the evolution of plastids. Unlike other plastids, the plastids of glaucophytes retain the remnant of Gram-negative bacterial cell wall type found in cyanobacteria, with a thin peptidoglycan cell wall and cyanobacterium like pigmentation. In fact these ancient plastids are morphologically so similar to cyanobacteria that the term 'cyanelle' is applied to them instead of plastids.

Secondary endocytobiosis:

Major groups of photosynthetic algae do not share the plastid morphology found in primary evolved plastids. The most striking difference is a plastid surrounded by three or four membranes instead of two. In the last decades of the twentieth century it was speculated that these multimembranous plastids evolved by the engulfment of a phototrophic eukaryote by a heterotrophic host cell. This was found to be correct and was called secondary endosymbiosis or endocytobiosis. Today, it is generally accepted that the stramenopiles,

haptophytes, cryptophytes, chlorarachniophytes, and the apicomplexa, as well as the phototrophic euglenophytes, all contain plastids surrounded by three to four membranes, evolved by secondary endosymbiosis. However, the origin of peridinin – containing dinoflagellates seems to be still a matter of debate and is either explained by secondary or tertiary endosymbiosis.

Phylogenetic analyses of plastid and nuclear-encoded plastid genes demonstrated that the phototrophic euglenophytes and chlorarachniophytes were evolved by the engulfment and reduction of a green alga to a secondary plastid.

A number of algal groups have secondary plastids derived from those of red algae, including several with distinctive pigmentation. The cryptophytes were the first group in which secondary plastids were recognized on the basis of their complex four membrane structure. Like red algae, they have chlorophyll a and phycobiliproteins, but these are distributed in the intra-thylakoidal space rather than in phycobilisomes found in red algae, glaucophytes and cyanophytes. In addition cryptophytes possess a second type of chlorophyll, chlorophyll c, which is found in the remaining red lineage plastids.

These groups, which include the Heterokontophytes (including Phaeophyceae, Bacillariophyceae, Cryptophyceae and related group), Haptophytes (Prymnesiophytes), and probably those dinoflagellates pigmented with peridinin, have chlorophylls a and c, along with a variety of carotenoids, for pigmentation. Stacked thylakoids are found in those lineages (including the cryptophytes) that lack phycobilisomes. The derivation of chlorophyll c containing plastids from the red lineage is still somewhat conjectural, but recent analyses of both gene sequences and gene content are consistent with this concluding.

A few groups of dinoflagellates have plastids now recognized to be derived from serial secondary endosymbiosis or tertiary endosymbiosis (the uptake of the secondary plastid-containing endosymbiont).

Chapter-3
3.1.Economic Importance of Algae

3.1.1. Industrial uses:

A. **Fertilizer / soil amendments:** Twin ability of nitrogen fixation and photosynthesis prompted cyanobacteria on the barren lands. They add moisture to the soil, release growth promoting substances, solubilise phosphate and potash in the soil etc. These properties are now well exploited as potential biofertilizer in many parts of the world. For soil amelioration and reclamation of usar lands cyanobacteria have been utilized with promising results. Miscellaneous species of Kelps (Brown algae), e.g. *Laminaria, Macrocystis* etc are used as fertilizers in coastal countries.

B. **Diatoms in the form of Diatomaceous earth (diatomite):** Also called diatomite or kieselguhr actually is frustules of fossil diatoms used for various purposes. It is used in industrial filtration processes, sugar refining and brewing industries. For the removal of waste mycelium in the production of antibiotics it is used as filter and is also used as an absorbent for nitro-glycerine in the manufacture of dynamite. Diatomite is used as a cleaning agent in soap, toothpaste and metal polish industries. The sprinkling of diatomaceous earth on the floor and walls reduces the chances of explosion in coral mines.

C. **Sewage treatment to remove inorganic nutrients and toxins:** In waste water treatment plants microalgae are utilized for rapid sewage disposal. Sewage oxidation ponds have been created for bringing about its complete oxidation into mineral components. Such ponds support luxuriant growth of unicellular algae, e.g., *Chlorella, Chlamydomonas, Scenedesmus,* and *Euglena*. These help in the bacterial decomposition of sewage by providing oxygen; in addition, they recover the mineral nutrients from sewage which would otherwise have been lost in the effluent.

D. **Source of chemicals:** *Dunaliella*, a green flagellate, can manufacture 50 times the β-carotene produce by *Spirulina*. This and other microalgae are often cultivated in open-air commercial ponds that may several thousand square meters in surface area, but less than a meter deep. *Phaeodactylum tricornutum* is harvested for the extraction of eicosapentaenoic acid, a long-chain polyunsaturated fatty acid used as a human food supplement. *Dunaliella* is well exploited for glycerol in saline lakes.

3.1.2. Phycocolloids: Mucilaginous material extracted from the walls of some brown and red algae are called phycocolloid. These are hydrophilic in nature and used as a thickener and gel in commercial products. Three principal types of phycocolloids are obtained from algae. These are:

A. **Alginic Acid (Alginate):** It is a complex polysaccharide composed of β-1, 4-linked mannuronic acid units and α-1, 4-linked L-guluronic acid units in varying proportions. Alginic acid is present in the intercellular spaces and cell walls of the brown algae. It also occurs in the cell walls of some red algae. (i) About half of the alginate produced is used for making ice cream and other dairy products, the rest is used in other products, including shaving cream, rubber, or paint. (ii) In textiles, alginates are used to thicken fiber-reactive dye pastes, which facilitate sharpeness in printed lines and conserves dyes. (iii) Dentists use alginates to make dental impressions of teeth. Sources: Kelps: *Macrocystis*, *Laminaria*, Other: *Ascophyllum*, *Fucus*, and *Sargassum*.

B. **Carrageenan (carrageenin, carragheen):** It is phycocolloid derived from certain red algae. It occurs as amorphous content in cell wall. Similar to agar it is also a galactan but due to higher ash content it requires higher concentrations to form gels. carrageenan is used for stabilizing chocolate, milk, egg nog, ice cream, sherbets, instant puddings, frostings, creamed soups, etc. Sources: *Gigartina stellata, Chondrus crispus, Eucheuma.*

C. **Agar-agar:** Agar is a Malay word for the gelling substance extracted from *Eucheuma* (red algae) which is ironically now known to be a carrageenan. Agar is a galactan and constitutes the gelatinous fraction of the cell wall of certain red algae (e.g. *Gelidium, Pterocladia, Gracilaria etc)* consisting of a sulphated complex polysaccharides composed mainly of β-1,3 linked D-galactose and 1,4-linked anhydro-L-galactose. Agar is used as solidifying agent in various culture media. It remains as stiff gel in 1-2% aqueous solution at normal temperature but are liquefied when hot. Pure agarose is now used as a gel in electrophoretic and chromatographic studies.

3.1.3. Soil Reclamation: It was Prof. R. N. Singh of Banaras Hindu University who first established that BGA could be utilized in reclamation of 'Usar' or barren lands. In our country over 10 million hectare land, mainly in Indo-Gangetic plains, black cotton soils, Indian coast line, is estimated to be affected by salts and is inhospitable to crops. Although chemical amendments, like application of gypsum, followed by leaching with good quality irrigation water are being used, they have some ecological limitations. Certain cyanobacteria have been found not only to grow in such inhospitable ecosystems (Kaushik, 1991), but also improve the physico-chemical

properties of the soil by enriching them with carbon, nitrogen and available phosphorus (Kaushik and Subhashini, 1985). Considerable amount of sodium has been found to be scavenged by these cyanobacteria. Cyanobacteria also reduce sodium ion content of the soil by making calcium ions available through solubilisation of calcium carbonate nodules, possibly by releasing various organic acids, like oxalic, oxaloacetic-, lactic-and succinic-acids. A remarkable improvement in the reduction of soil pH (towards neutrality), exchangeable sodium and soil conductivity has also been shown by. The protection against alkaline environment is also provided by the synthesis of certain fatty acids and sucrose and osmotic stress induced proteins.

3.1.4. Single cell protein: Health foods based on microalgae like *Spirulina, Scenedesmus* and *Chlorella* is now popular in global markets. The chief merits of these foods are their contents particularly proteins present in a range of 50-70% and popularly known as Single Cell Protein (SCP). *Spirulina* has traditionally been harvested from African lakes and consumed in sauces at the rate of some 9-13 gms per meal. The protein level of *Spirulina* can be as high as nuts, grains and soybeans, ranging from 50-70% of algal dry weight, and shows promise for use as a protein supplement for malnourished populations. Because the cell walls are composed on mucopolysaccharides rather than cellulose, *Spirulina* is more digestible than some other micro-algae. *Spirulina* is also naturally high in B-vitamins and essential unsaturated fatty acids as well as high levels of β-carotene, which is converted to Vitamin-A during digestion.

3.1.5 Use in Space research: Algae like *Chlorella* have played a significant role in space research for several decades and will have future applications in spacecraft life- support systems, colonization of the Moon and Mars, and eventually in terraformation of other planetary bodies. Experiments

designed to study the growth of algae in the zero-gravity conditions of space have flown abroad rockets, satellites, the space shuttle, and the Salyut and Mir space stations.

3.1.6. Use in Fisheries: Microalgae feeds are currently used in relatively small amounts in aquaculture, mainly for the production of larvae and juvenile shell- and finfish, as well as for raising the zooplankton required for feeding of juvenile animals. The blue-green alga *Spirulina* is used in substantial amounts (over 100 t y^{-1}) as a fish and shrimp feed. Another potential large-scale application of microalgae is the cultivation of *Haematococcus* for the production of the carotenoid astaxanthin, which gives salmon flesh its reddish color. In the long-term microalgae biomass high in lipids (omega-3 fatty acids) may be developed as substitutes for fish oil-based aquaculture feeds. Diatoms are preferred food for fresh water fishes due to its oil contents.

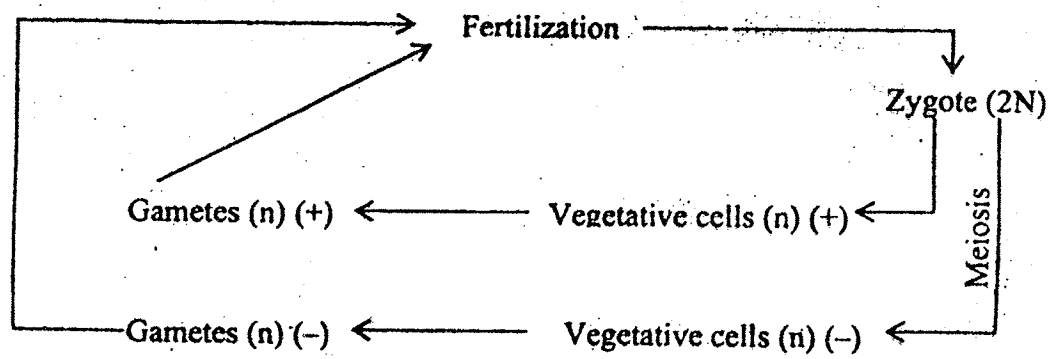
Chaper-4

4.1. Life Cycle Patterns in Algae

Algae exhibit an amazing diversity of life histories. Three principal types are illustrated below. The primary differences between them include the point where meiosis occurs and the type of cells it produces, and whether or not there is more than one free-living stage present in the life cycle.

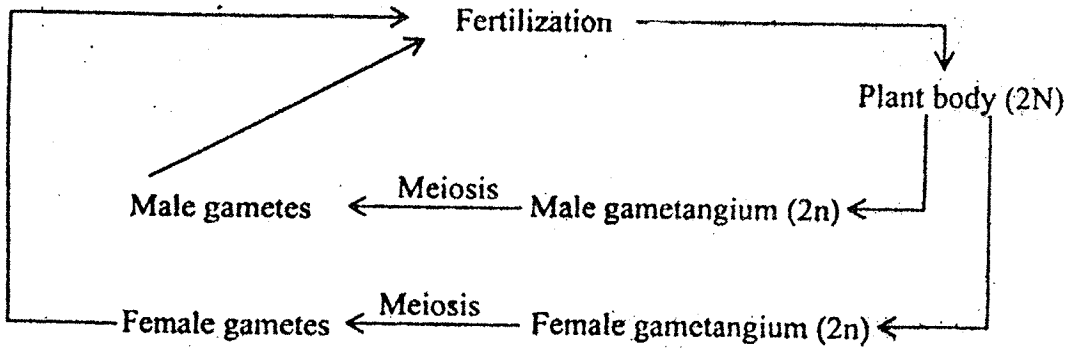
I. Haplontic or Zygotic life cycle:-

This cycle is characterised by a single predominant vegetative phase, with meiosis taking place upon germination of zygote. *Chlamydomonas* exhibits this type of life cycle.



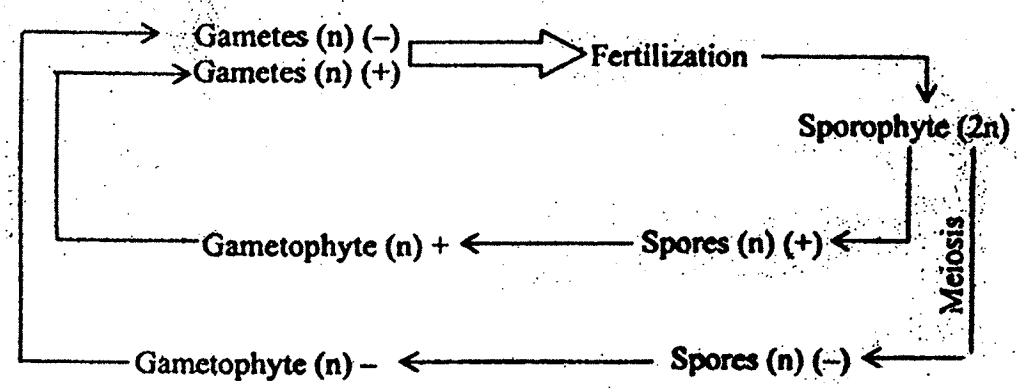
II. Diplontic or Gametic Life Cycle:-

This life cycle has a single predominant vegetative diploid phase, and meiosis gives rise to haploid gametes. Diatoms and *Fucus* (Phaeophyceae) exhibit this type of life cycle.



III. Diplohaplontic or Sporic Life cycle:-

These cycles present an alternation of generation between two different phases consisting in a haploid gametophyte and a diploid sporophyte. The gametophyte produces gametes by mitosis; the sporophyte produces spores through meiosis. Alternation of generations in the algae can be isomorphic, in which the two phases are morphologically identical as in *Ulva, Ectocarpus, Cladophora* etc. or heteromorphic, with the predominance of the sporophyte as in *Laminaria* (Phaeophyceae) or with the predominance of the gametophyte as in *Porphyra* (Rhodophyceae).

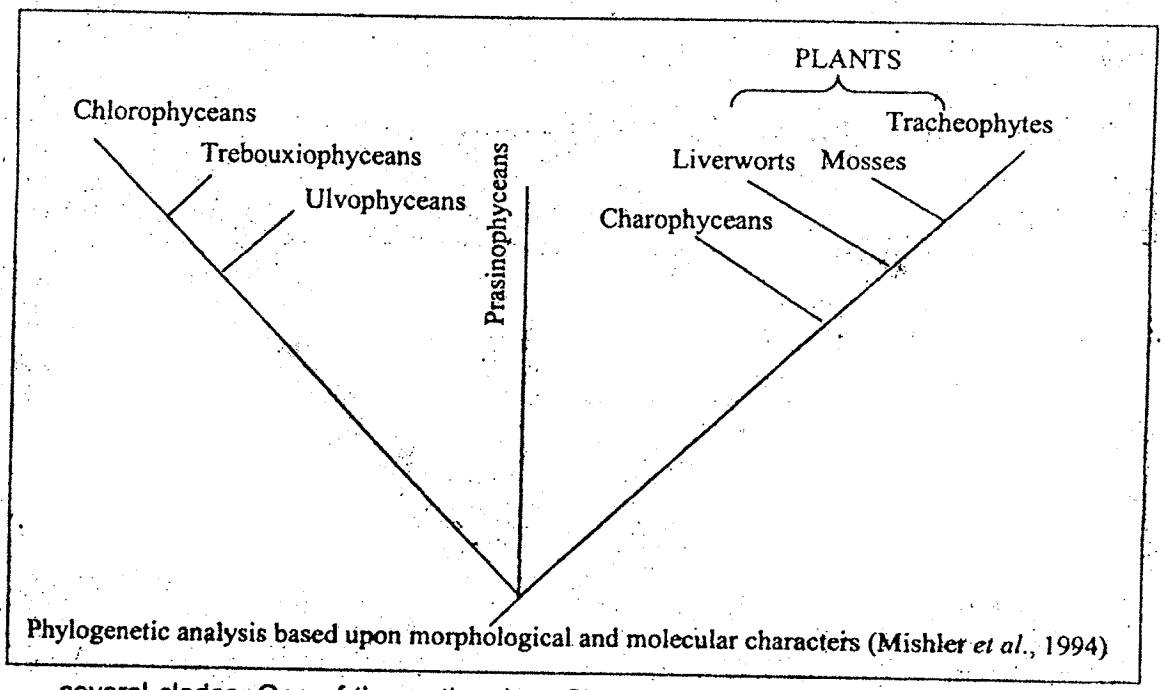


Higher red algae exhibit extended life cycle. The haploid gametophyte may be succeeded by a diploid carposporophyte, and a diploid tetrasporophyte in major cases. Several other variations are noted in Rhodophyceae and certain Phaeophyceae.

Chapter-5

5.1. Evoluionary trends in Chlorophyceae

The traditional concept of Fritsch (1935) to group the Chlorophyceae into 9 orders has been changed from time to time. New tools adopted in deciphering plant relationships have explored new dimensions and hence the ideas. Ultrastructural, biochemical, and molecular sequence evidence suggests that there are two major evolutionary lineages of green algae that contain multicellular forms (multicellular lineages) both of which include



Phylogenetic analysis based upon morphological and molecular characters (Mishler *et al.*, 1994)

several clades. One of these, the class Charophyceae or the charophyceans includes not only stoneworts but zygnematalean algae such as *Spirogyra* and related desmids, as well as several other taxa including *Chlorokybus, Klebsormidium, Chaetosphaeridium* and *Coleochaete* (Mattox and Stewart 1984; Graham 1996; Graham and Wilcox 2000). Charophyceans are characterized my multilayered structure (MLSs), open mitosis, a persistent mitotic spindle, Cu / Zn superoxide dismutase, class I aldolases, and glycolate oxidase containing peroxisomes that structurally resemble those of plants. Further, their SSUrDNA, rbcL, and other gene sequences reveal close relationship to embryophytes (bryophytes + tracheophytes). The fact that many conservative ultrastructural, biochemical, and molecular features of

charophyceans are shared with land plants, strongly suggests that embryophytes arose from charophycean ancestors.

The second multicellular green algal lineage includes the Ulvophyceans (Class: - Ulvophyceae), trebouxiophyceans (Class: - Ulvophyceae), trebouxiophyceans (Class: - Trebouxiophyceae), and the chlorophyceans (Class: - Chlorophyceae). Ulvophyceans primarily occupy marine waters and include *Ulva*, *Codium*, *Enteromorpha*, *Cloadophora*, *Halimeda*, *Caulerpa* and *Acetabularia*. Trebouxiophyceans are freshwater and terrestrial algae that include such familiar forms as *Chlorella*, and the common lichen phycobiont *Trebouxia*, for which the group is named (Friedle 1995). Chlorophyceans include the familiar *Chlamydomonas* and *Volvox*, as well as many other primarily freshwater green algae.

These two multicellular lineages – the charophyceans (C-clade) and the Ulvophyceans + trebouxiophyceans + Chlorophyceans (the UTC clade) – are thought to have arise from distinct types of unicellular flagellates related to the modern prasinophyceans, a polyphyletic group. This group include *Oltmannsiellopsis viridis* (a well-less, scaly flagellate), *Mesostigma* (also a wall-less scaly flagellate) etc. *Mesostigma* has been shown by SSUrDNA sequence studies to be an early divergent member of the Charophyceae and therefore suggested to include with Charophyceae (Graman and Wilcox, 2000). *Tetraselmis* formerly included under Volvocales has been shifted to Prasinophyceae.

5.2. Green algae and origin of land plants

The earlier belief that *Fritschiella tuberosa* a terrestrial member of Chaetophorales may represent a close relative to land plants has been rejected on the ground of ultrastructural, biochemical and molecular evidences. It is now strongly believed and evidenced that charophycean green algae actually represent the lineage that is ancestral to the land plants (embryophytes – bryophytes + tracheophytes). The charophyceans possess plant like features such as MLSs (Multilayered structures) at the flagellar base, open mitosis, persistant spindles or phragmoplasts, glycolate oxidase, plant like peroxisomes, and many other biochemical features. Additional evidences are: - (i) silence introns, (ii) location of the tuf A gene in the nucleus

rather than in the chloroplast, and (iii) presence of lignin and sporophyllenin in the walls (Delwiche *et al.*, 1989; Graham 1993).

Genomic architectural changes suggests relative divergence times for the derived charophyceans (those having sexual reproduction): Zygnematales, Charales, and Coleochaetales, and indicate which are most closely related to embryophytes

It is now established that *Coleochaete* among the charophyceans approaches close to land plants. It share following characteristics shared with land plants :- (i) division with a cell plate, (ii) oogamy, (iii) retention of the egg and the zygote on the gametophyte, (iv) formation of a covering of cells around the zygote, and (v) formation of more than four spores from each zygote. Some species of *Coleochaete* also show localized growth, parenchymatous construction, and multicellular antheridia. If land plants arose from an algal ancestor similar to *Coleochaete*, with zygotic meiosis and a haploid vegetative phase, the following events probably occurred after land plants diverged from the algae:

(1) a change in the time of meiosis to produce an alternation of generations,
(2) retention of the zygote and subsequent development of the sporophyte on the gametophyte, and
(3) establishment of nutritional and developmental relationships between the young sporophyte and gametophyte (Graham 1984).

Self - assessment questions :

1. Make a survey of classification of Algae proposed by different authors and point out the basis of such classifications.
2. Give an account of the classification of Algae proposed by Lee (1999) and mention the salient features of each division.
3. Discuss briefly the citreria of Algal classification with special emphasis on pigments and storage products.
4. Comment on the modern trends in Algal classification.
5. Mention the salient features of the following classes of Algae :

(i) Cyanophyceae (ii) Chlorophyceae (iii) Bacillariophyceae (iv) Phaeophyceae (v) Rhodophyceae.

6. Discuss your idea about phylogeny of Algae.
7. Mention various economic importance of Algae.
8. Write notes on :

(a) Industrial uses of Algae.

(b) Algae and soil reclamation.

9. Describe schematically different life-cycle patterns in Algae citing suitable examples.
10. Comment on the evolutionary trends in Chlorophyceae.

Suggested readings

1. H. D. Kumar (1999): Introductory Phycology. Affiliated East-West Press, New Delhi.
2. Philip Sze (1998): A Biology of Algae.3rd ed. WCB/McGraw-Hill.
3. C. van den Hoek, D. G. Mann, & H. M. Jahns (1995): Algae An introduction to Phycology. Cambridge.
4. R. E. Lee (2008): Phycology. Cambridge.
5. L. Barsanti & P. Gualtieri (2006): Algae: Anatomy, Biochemistry and Biotechnology. Taylor & Francis.
6. L. E. Graham & L. W. Wilcox (2000): Algae. Prentice Hall.

M. Sc. in Botany
Part – I, Paper – I, 2nd Half
MODULE – 9

MYCOLOGY
Marks – 10

Introduction:

Fungi are eukaryotic, spore bearing achlorophyllous organisms that generally reproduce sexually and asexually and whose usually filamentous, branched somatic structures are typically surrounded by cell walls containing chitin and cellulose or both together with other complex organic molecules (Alexopoulos & Mims, 1979).

The branch of botany which deals with fungi is called *Mycology* (Gr. *Mykes* means mushroom or fungus; *logos* means discourse). According to Alexopoulos and Mims (1979) the Italian botanist Pier' Antonio Micheli (1729), deserves the honour of being called the '*founder of mycology*' because of his researches on fungi which he published in *Nova Plantarum Genera*.

The systematic study of fungi is only 300 years approx. old, but manifestations of this group of organisms have been known for thousands of years. Ancient peoples were well aware of biological fermentation while Romans attributed the appearance of mushrooms and truffles to lightning hurled by Jupiter to the earth. This mushrooms also play a role in religion and mythology of Indians of Mexico, Guatemalan Indian Tribes etc. where use of hallucinogenic mushrooms are common. Even in today's science conscious world few people realize how intimately our lives are linked with those of fungi. We are familiar with those which frequently grow on jams, jellies, pickles, bread etc. while farmers are familiar with fungi which cause diseases to the crop plants or spoil their stored fruits and vegetables. A layman, foresters are familiar with large polypores that form bracket and shelf on the trees and the puff balls, toad stools, mushrooms that grow in gardens and forests. Urban people frequently suffer from fungal diseases like Coccidioidomycosis, Aspergillosis, ring worm, athlete's foot or simple fungal allergies.

We are familiar with antibiotics like penicillin, griseofulvin etc.; cheese, soya sauce etc : vitamins like riboflavin and so many drugs which are the direct or indirect products of fungal activity. Fungi are also used today for industrial manufacture of organic acids, enzymes, steroids transformations, feed supplement etc. Similarly, yeasts are used in baking and alcohol production and have played an important role in nutrition of human race.

Fungi are able to break down complex organic substances of all most every kind which is an essential activity involved in recycling of various elements in the cycle of life. Soil fertility is closely linked with fungal activity. Roots of most green plants are infected with mycorrhizal fungi and absorption of minerals and water are thus enhanced. This ability allow such plants to grow in infertile natural soil.

Another interest of man in fungi is their use as food. Mushrooms like *Agaricus. Calocybe, Auricularia* etc. are being cultivated widely in India and abroad, as food supplement. Besides these beneficial aspects they also cause destruction of wooden materials, textiles, leather goods, electrical insulations, lens of camera, microscope, telescope and other instruments particularly in the humid climates. Apart from these applied aspects, they are also used as tools for study of fundamental biological processes by geneticists, cytologists, physiologists, biochemists, microbiologists, biotechnologists mainly.

Thus, in brief fungi as a group have what it takes to survive. They are progressive and evolve rapidly and capable of rapidly adopting to every condition of life. Fungi were around long before man appeared and many of them are sure to be around in the future.

CLASSIFICATION OF FUNGI

The classification of the fungi is a very difficult task since it represents various problems which originate from the differences of opinion of different workers. Such differences are due to the differences in the approach, interpretation of collected data etc. The history of classification of fungi can be traced back from the time of herbalists Bauhin, who listed about 100 species of fungi in his "*Pinax Theatri Botanici*" published in 1623. After that a number of classifications have been proposed of which most

important and famous are: Saccardo (1882), Gwynne-Vaughan and Barnes (1926), Clements and Shears (1931), Bassey (1950), Martin (1961), Alexopoulos (1962), Gaumann (1966), Kreisel (1969), Webster (1970), Ainsworth (1973), Alexopoulos and Mims (1979), Moore (1980), von Arx (1981), Hawksworth *et al.* (1983), Margulis and Schwartz (1988)... etc. From the above account it is clear that the classification of fungi is still in a state of flux and as such a stable as well as ideal system of classification is yet to be proposed.

The classification of fungi is mostly based on the following features-

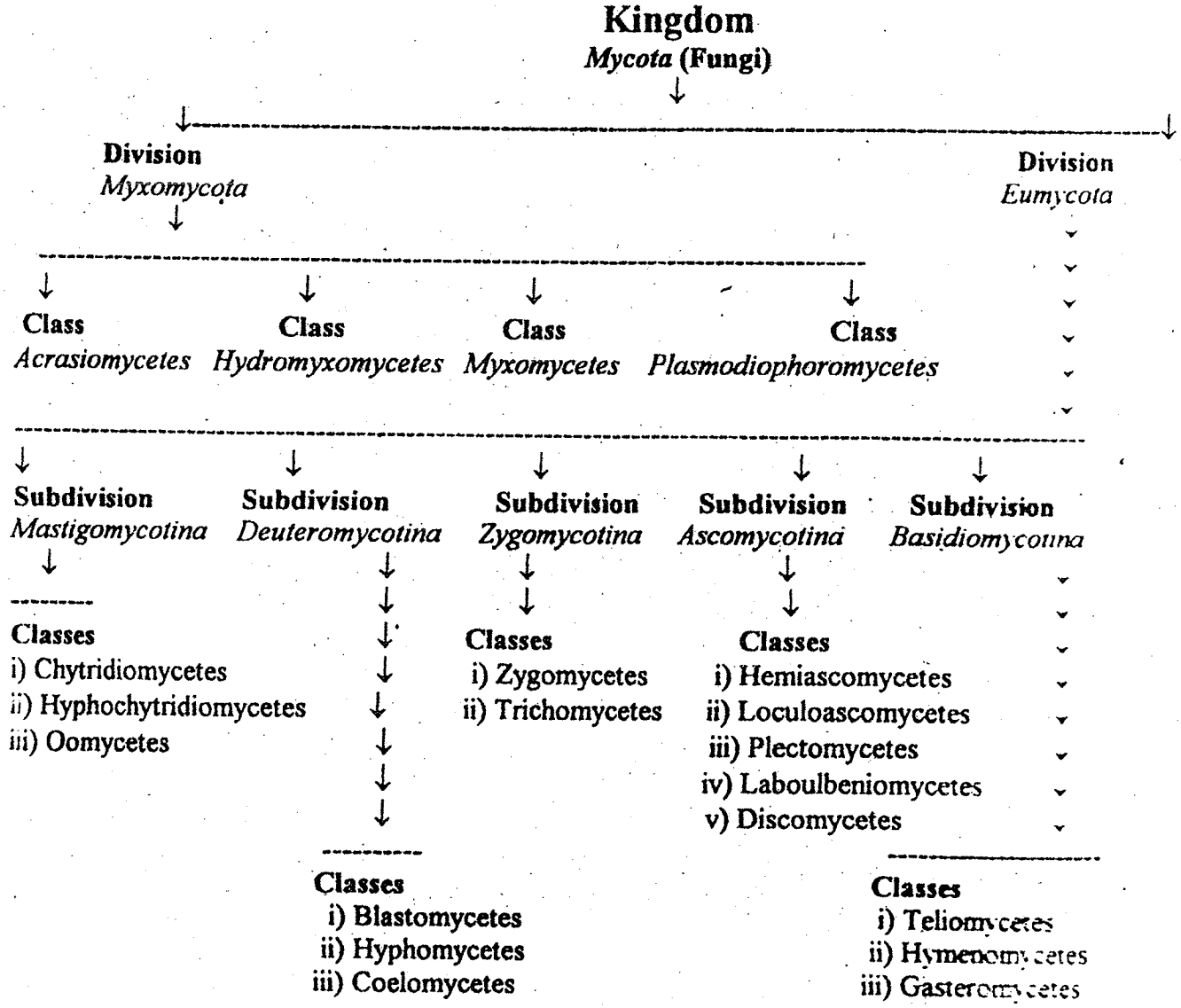
i) Nature of Somatic phase

ii) Types of asexual spores

iii) Nature of sporangia

iv) Life cycle pattern (Asexual; sexual cycle which includes Haplontic, Haplontic Cycle with restricted dikaryon, Haplontic-dikaryotic Cycle, Diplontic, Haplodiplontic, Dikaryotic Cycle) etc.

Recently, a great deal of information about the fungi has been derived from biochemistry, genetics and molecular biology and as such the system is to be modified frequently to reflect more complete understanding of fungi including their origin, relationship, evolution etc. In this resume a more natural, widely accepted system of fungal classification proposed by Ainsworth, G.C. (1973) is cited-



Kingdom Mycota (Fungi)

Key to Division of fungi

Plasmodium or pseudoplasmodium present	..... Myxomycota
Plasmodium or pseudoplasmodium absent, assimilative phase typically filamentous	..... Eumycota

Key to Classes of Myxomycota

Assimilative phase free-living amoebae which unite as a pseudoplasmodium before reproduction ..... Acrasiomycetes

Assimilative phase a plasmodium, plasmodium forming a network ('net plasmodium') ..... Hydromyxoycetes

Plasmodium not forming a network, plasmodium saprobic, free-living, plasmodium parasitic within cells of the host plant ..... Plasmodiophoromycetes

Key to subdivisions of Eumycota

Motile cells (Zoospores) present, perfect state spores typically oospores ..... Mastigomycotina

Motile cells absent, perfect state absent ..... Deuteromycotina

Perfect state present, perfect state spores zygospores ..... Zygomycotina

Zygospores absent, perfect-state spores ascospores ..... Ascomycotina

Perfect state spores basidiospores ..... Basidiomycotina

Key to classes of Mastigomycotina

Zoospores posteriorly uniflagellate (flagella whiplash type) ..... Chytridiomycetes

Zoospores not posteriorly uniflagellate but zoospores anteriorly uniflagellate (flagella tinsel-type) ..... Hyphochytridiomycetes

Zoospores biflagellate (posterior flagellum whiplash-type; anterior flagellum tinsel-type); cell wall cellulosic ..... Oomycetes

Key to classes of Zygomycotina

Saprobic or if parasitic or predacious, having mycelium immersed in host-tissue ..... Zygomycetes

Associated with arthropods and attached to the cuticle or digestive tract by a holdfast and not immersed in

the host tissue ..... Trichomycetes

Key to classes of Ascomycotina

Ascocarps and ascogenous hyphae absent, thallus mycelial or yeast like ..... Hemiascomycetes

Ascocarps and ascogenous hyphae present; thallus mycelial, asci bitunicate, ascocarp an ascostroma ..... Loculoascomycetes

Asci typically unitunicate, if bitunicate, ascocarp an apothecium; asci evanescent, scattered within the astomous ascocarp which is typically a cleistothecium; ascospores aseptate ..... Plectomycetes

Asci regularly arranged within the ascocarps as a basal or peripheral layer; exoparasites of arthropods, thallus reduced; ascocarp a perithecium; asci inoperculate ..... Laboulbeniomycetes

Not exoparasites of arthropods, ascocarp typically a perithecium which is usually ostiolate (if astomous, asci not evanescent); asci inoperculate with an apical pore or slit ..... Pyrenomycetes

Ascocarp an apothecium or a modified apothecium, frequently macrocarpic, epigean or hypogean, asci inoperculate or operculate ..... Discomycetes

Key to classes of Basidiomycotina

Basidiocarp lacking and replaced by teliospores or chlamydospores (encysted probasidia) grouped in sori or scattered within the host-tissue; parasitic on vascular plants ..... Teliomycetes

Basidiocarp usually well-developed; basidia typically organized as a hymenium; saprobic or rarely parasitic, basidiocarp typically gymnocarpous and semiangiocarpous; basidia phragmobasidia (phragmobasidiomycetidae) or holobasidia (Holobasidiomycetidae); basidiospores

ballistospores ..... Hymenomycetes

Basidiocarp typically angiocarpous; basidia holobasidia, basidiospores not ballistospores ..... Gasteromycetes

Key to classes of Deuteromycotina

Budding (yeast or yeast like) cells with or without pseudomycelium characteristics; true mycelium lacking pr not well developed ..... Blastomycetes

Mycelium well developed, assimilative budding cells absent; mycelium sterile or bearing spores directly or on special branches (sporophores) which may be variously aggregated but not in pycnidia or acervuli ..... Hyphomycetes

Spores in pycnidia or acervuli ..... Coelomycetes

The grouping or categories used in the above classification are given below along with the recommendations of the committee on the International Rules of Botanical Nomenclature:

Division should end in ..... *mycota*

Subdivision should end in .....*mycotina*

Class should end in .....*mycetes*

Subclasses should end in ..... *mycetidae*

Orders should end in .....*ales*

Family should end in ..... *aceae*

The following table provides an outline idea of other systems of classification of Fungi according to Hawksworth *et al.* (1983), von Arx (1981), Moore (1980), Alexopoulos and Mims (1979) and Bessey (1950)

	Hawksworth *et al.* (1983)	von Arx (1981)	Moore (1980)	Alexopoulos and Mims (1979)	Bessey (1950)
Kingdom	FUNGI	Kingdom MYCOTA	Kingdom FUNGI	Kingdom MYCETAE (FUNGI)	
Division	Myxomycota	Myxomycota	FUNGI INFERIOR	Gymnomycota	Mycetozoa
Classes	Protosteliomycetes Ceratiomyxomycetes Dictyosteliomycetes Acrasiomycetes Myxomycetes Plasmodiophore- mycetes Labyrinthulomycetes	Myxomycetes Acrasiomycetes Plasmodiopho-phoromycetes Labyrinthulo-mycetes	Moore (1971) included Oomycota, Hyphochytri-diomycota and Zygomycota in 'PHYCOMY-COTERA'	Acrasiogymnomycotina Acrasiomycetes Plasmodiogymnomy-cotina Protostelimycetes Myxomycetes Ceratiomyxomycetidae Myxogastromycetidae Stemonitomycetidae	
Division	Eumycota	Oomycota			Phycomyce-Teae
Sub-Division	Mastigomycotina	Oomycetes Hyphochytri-diomycetes		Mastigomycota Haplomastigomycotina	Carpomyce-teae
Class	Chytridiomycetes Hyphochytridio-mycetes Oomycetes	Chytridiomy- cota Chytridiomy-cetes		Chytridiomycetes Hyphochytridiomycetes Plasmodiophoromycetes Diplomastigomycotina Oomycetes	
Sub-Division	Zygomycotina	Eu-Mycota		Amastigomycota	
Class	Zygomycetes Trichomycetes	Zygomycetes Endomycetes Ustomycetes Ascomycetes	FUNGI SUPERIOR (Moore 1971) included Hemiascomy-cetes and Euascomycetes	Zygomycotina Zygomycetes Trichomycetes Ascomycotina Ascomycetes Hemiascomycetidae Plectomycetidae Hymenoascomyce-tidae Pyrenomycetes Discomycetes Laboulbeniomycetidae Loculoascomycetidae	Ascomyce-teae The Pyreno-mycetes
Sub-Division	Ascomycotina (No classes recognised)				

The following table provides an outline idea of other systems of classification of Fungi according to Hawksworth *et al.* (1983), von Arx (1981), Moore (1980), Alexopoulos and Mims (1979) and Bessey (1950) (Contd...)

	Kawksworth *et al.* (1983)	von Arx (1981)	Moore (1980)	Alexopoulos and Mims (1979)	Bessey (1950)
Sub-Division	Basidiomycotina		BASIDIOMY-COTERA	Basidiomycotina	Basidiomyce-tae
Class	Urediniomycetes Ustilaginomycetes Hymenomycetes Gasteromycetes		Ustomycota Ustomycetes Sporidiomycetes Homobasidiomycia Hymenomycetes Gasteromycetes Heterobasidio-mycia Holobasidiomy-cetes Phragmobasidio-mycetes	Basidiomycetes Teliomycetidae Phragmobasidiomy-cetidae Holobasidiomycetidae Hymenomycetes Gasteromycetes	Teliosporeae Heterobasi-diae Eubasidiae Hymenomy-ceteae Gasteromyce-Teae
Sub-Division	Deuteromycotina		DEUTEROMY-COTERA	Deuteromycotina	Fungi Imperfecti
Class	Hyphomycetes Coelomycetes		Blastomycota Ascoblastomycetes Basidioblastomy-cetes Deuteromycota	Blastomycetidae Hyphomycetidae Coelomycetidae	Sphaeropsi-dales Melanconiales Moniliales Mycelia Sterilia

GENERAL CHARACTERISTICS OF DIVISIONS AND SUBDIVISIONS

Division: Myxomycota

The organisms included in the division **Myxomycota** are called **slime molds** which are characterized by the following characters-

a. The somatic phase may be a plasmodium or pseudoplasmodium.
b. A plasmodium is a mass of naked, multinucleate protoplasm moving by amoeboid movement and usually feeding by ingesting particulate matter (Phagotropic nutrition).
c. A pseudoplasmodium is an aggregation of separate amoeboid cells.
d. The somatic phase is always free living, diploid and halocarpic.
e. Spores are differentiated by meiosis.
f. Spores are enveloped by a rigid cell wall similar to true fungi.

Division: Eumycota

Fungi except slime molds are included in this division. It represents a heterogenous group and frequently called **true fungi**, are characterized by the following characters-

a. The somatic phase is typically filamentous (except a few unicellular forms like *Synchytrium*, *Saccharomyces* etc.) structure called mycelium.
b. The mycelium composed of thin, filamentous, branched structure called hyphae. Thus, structural unit of mycelium or fungal somatic phase is typically hyphae not a cell like higher plants.
c. Hyphal wall is usually composed of fungal cellulose, called Chitin ($C_{22}H_{54}N_4O_{21}$) or a mixture of cellulose and chitin (example Hyphochytridiomycetes) or rarely cellulose.
d. Nucleus is typically bounded by a nuclear membrane.
e. Excepting a few (e.g. *Synchytrium*) all most all are eucarpic.
f. The septa between the cells when present have each a central pore.
g. Reproduction involve both asexual and sexual methods of which former involve sporulation which takes place by means of mitospores, while sexual reproduction always involve union of nuclei or sex cells or sex organs.

h. From the lower to higher fungi there is a gradual and progressive simplification and ultimate elimination of the sexual apparatus/organs.

Subdivision: Mastigomycotina

The mastigomycotina are commonly known as Zoosporic fungi which includes three distinct groups of organisms: the Chytridiomycetes, Hyphochytridiomycetes and Oomycetes. These have been brought together in this subdivision on the basis of their following characteristics-

a. Assimilatory phase represented by either unicellular or filamentous, coenocytic mycelium. The septa when present usually remain suppressed in the actively growing stage.
b. Cell wall composed of either a mixture of chitin-glucan (Chytridiomycetes) or cellulose-chitin (Hyphochytridiomycetes) or cellulose-glucan (Oomycetes).
c. Asexual reproduction takes place either by the conversion of the entire contents of the thallus (in holocarpic one) or through development of elongated sac like sporangium (in eucarpic genera).
d. The spore producing units are usually not grouped into a complex frutification.
e. Three kinds of zoospores occur- i) with a single posterior flagellum of the whiplash type, ii) with a single anterior flagellum of the tinsel type and iii) biflagellate zoospore with apically or laterally attached flagella- one of tinsel and another of whiplash type.
f. Sexual reproduction when present is either isogamous or heterogamous.
g. Karyogamy generally follows plasmogamy almost immediately.

Subdivision: Zygomycotina

The following are the important distinguishing characters of this subdivision-

a. They are mostly terrestrial, have a well developed filamentous thallus called mycelium.
b. Most of the members are saprobes or facultative or weak parasites of plants or specialized parasites of arthropods and obligate parasites of fungi.
c. The mycelial organization is coenocytic.
d. Cell wall contains chitin.

e. The hyphae contain the typical cellular organelles that other fungi contain except that centrioles and typical golgi dictyosomes are lacking.

f. Asexual reproduction takes place by non-mortile aplanospores. The spores are contained in sporangia which may be violently dispersed; but more usually spores are passively dispersed by wind, rain or animals.

g. Sexual reproduction is by gametangial copulation which is typically isogamous, and results in the formation of a Zygospores.

h. Zygospore germinates to produce a short hyphae, the promycelium which bears a terminal sporangium.

i. There is no dikaryophase in the life cycle.

Subdivision: Ascomycotina

It is a large group comprising fungi which are more complex in structure than the members of Mastigomycotina and Zygomycotina. Diagnostic features of this subdivision are-

a. Vegetative body is typically thallus consists of well-developed profusely branched mycelium with regular septation of the hyphae except yeast and yeast like unicellular members.

b. The cell wall contains a microfibrillar network of Chitin and in addition various other components like amino-sugar, mannose, protein and glucose etc. are also present.

c. The septation is incomplete because the septa dividing the hyphae into cells have each a minute, central, simple pore. Each segment often contain several nuclei.

d. Three kinds of nuclei may exist within the hyphae of Ascomycotina. Differences between these nuclei may arise by mutation or by anastomosis of hyphae of different genotypes followed by nuclear migration.

e. Asexual reproduction takes place by means of non-motile, exogenous conidia, which borne terminally on special reproductive hyphae called the Conidiophore.

f. Aggregation of conidiophores are of various types such as the Synnemata, Sporodochia, Pycnidia, Acervuli.

g. Perfect state spores are known as Ascospores which borne endogenously within a small, thin walled, sac-like spore producing unit called asci (sing. ascus).
h. Each ascus bears a definite number of ascospores which is usually eight and disperse explosively.
i. The asci, in most of the species are grouped into a definite complex fruit bodies called ascocarps. These are multicellular bodies enveloped in a sheath of sterile hyphae.
j. Presence of dikaryophase is another important characteristics. However, this is of short lived and parasitic on the haplophase.

Subdivision: Basidiomycotina

Many of the familiar larger fleshy fungi are members of this group, includes the toad stools, bracket fungi, puff-balls, stink horns, bird's nest fungi etc. Following are the important characteristic features-

a. The assimilative phase of Basidiomycotina are typically septate mycelium except some yeast or yeast-like state. There are three kinds of mycelia- **i) Primary mycelium:** produced by the germination of basidiospores and contain only one type of haploid nucleus in each cell (i.e. monokaryotic), is usually of short duration and of limited extent. **ii) Secondary mycelium:** Sooner or later, the primary mycelium undergo dikaryotization and results in the formation of a secondary or dikaryotic mycelium where each cell contains two haploid nuclei (n+n) of two genetic make up. This is long lived and plays a prominent role in the life-cycle. **iii) Tertiary mycelium:** The secondary mycelium does not give rise directly to basidiocarps but it undergoes certain morphogenetic changes to give rise to a tertiary mycelium which actually takes part in construction of basidiocarps.
b. The septal pores are complex in organization except in rust and smut fungi , called dolipores. The ends of the barrel-shaped dolipore remain guarded by cap-like covers called parenthesome.
c. Asexual reproduction plays an insignificant role in the life cycle. The subclass Homobasidiomycetidae never form any asexual spores while Heterobasidiomycetidae form them in the dikaryotic mycelium e.g. Uredospores and Aeciospores in the rust fungi.
d. The motile cells are absent in the life cycle as in Ascomycotina.

e. Dikaryophase is of regular occurrence and each dikaryotic hyphae usually bear clamp connections.

f. Sex organs are completely lacking in this group but the sexual reproduction takes place by plasmogamy and karyogamy only. Karyogamy is immediately followed by meiosis.

g. Basidium is the characteristic reproductive organ in which both karyogamy and meiosis takes place.

h. Each basidium bears usually four exogenous basidiospores but their number may vary from one to many depending on the species. The projection which bear basidiospore in termed Sterigmata.

i. The basidia along with basidiospores are present in the definite fructifications called basidiocarps. In the Heterobasidiomycetidae teleutospores or brand spores germinate to produce basidia bearing basidiospores.

j. The basidiospore germinates to produce the primary mycelium.

Subdivision: Deuteromycotina

This is the second largest subdivision of the fungi which represent an artificial assemblage of fungi reproducing by spores (which are formed without nuclear fusion followed by meiosis). This artificial assemblage embraces not only the imperfect, asexual or conidial stage of Ascomycotina and Basidiomycotina but also those asexual fungi with which no perfect states have been correlated so far. Thus, in other words, it is an assemblage of fungi, whose perfect or sexual states are either unknown or may possibly be entirely lacking and that is why sometimes referred to as **"fungi imperfecti"**. The Deuteromycotina has been called **waste basket** assemblage of organisms to accommodate imperfect fungi for the sake of convenience until their taxonomic place is determined.

The designation of the taxa is often preceded by the prefix **"form"** before the various taxa e.g. Form-class, Form-Subclass, Form-order, Form-family, Form-genus and Form-species, the implication being that the names are proposed without a knowledge of the sexual states of the taxa they represent. However, in practice, the prefix **"form"** is understood and usually omitted.

It is to be noted that whenever the perfect state of the imperfect fungus is known it is transferred to its proper systematic position among the fungal subdivisions the applied name then follow the priority of sexual state. For example, after the discovery of sexual reproduction in *Penicillium*, it was shifted to Ascomycotina. The sexual stage of this fungus was named *Talaromyces*. Thus, these two stages (conidial stages belongs to Deuteromycotina is named *Penicillium* while sexual stage belongs to Ascomycotina named *Talaromyces*) belong to two different fungi.

Following are the important distinguishing features-

a. With exception the Blastomycetes, they typically produce well developed, septate, branched hyphae where the septum is usually perforated.
b. Cell wall composed of chitin-glucan etc. like the members of Ascomycotina.
c. The cells are usually multinucleate.
d. Asexual reproduction takes place by means of exogenously formed asexual spores.
e. Conidiophores may be either simple or branched; and may occur singly or in groups. The aggregation of conidiophores usually lead to the formation of various asexual fructifications like i) **Synnema or Coremium:** where the conidiophores arise very close to each other and are often united along a greater part of their length to form a dense fascicles. It is common in the members of family Stilbellaceae e.g. *Trichurus*, *Graphium* etc. **ii) Sporodochium:** is a hemispherical or barrel-shaped asexual fruiting body as in *Fusarium* sp. consists of two parts- the lower or basal cushioned stroma-like mass of hyphae and the upper part, from the exposed surface of which arise conidiophores bearing conidia at their tips. **iii) Pycnidium:** may be a flsk-shaped or globbose hollow frutification, lined inside wih conidiogenus cells, superficially resemble perithecium of some of the members of Ascomycotina (Pyrenomycetes). **iv) Acervulus:** is a saucer-shaped structure comprising a stromatic mass of hyphae and a fertile layer of conidiophore, which are very short. Intermingled with the conidiophores are sometimes dark, long, stiff bristles called setae. Found in *Colletotrichum* sp.

MODEL QUESTIONS

Short answer type (5 marks)

1. Give an outline scheme of classification of fungi proposed by Ainsworth (1973).
2. Give an outline of classification of fungi proposed by Hawksworth *et al.* (1983).
3. Characterize divisions Myxomycota and Eumycota.
4. Write the diagnostic characters of Mastigomycotina and Zygomycotina.
5. Give the salient features of Ascomycotina and Basidiomycotina.
6. Enumerate the features of "Fungi imperfecti".
7. Compare Mastigomycotina, Ascomycotina and Basidiomycotina on the basis of their diagnostic characters.

Objective type (1 or 2 marks)

1. Define fungi.
2. What do you mean by primary mycelium ?
3. Why members of Deuteromycotina are called fungi imperfecti ?
4. What is pycnidium ?
5. Name one acervulus producing fungi.
6. What is holocarpic fungi ?
7. Where dolipore septum is present ?

SEXUALITY IN FUNGI

The primary source of genetic variation in fungi is mutation but further genetic variation can occur as a result of sexual reproduction. In this group of organisms sexual reproduction involves the union of gametes or gametangia which brings together their nuclei to form a dikaryon and ultimately a zygote. However, in some fungi this process can be accomplished by hyphal fusion alone. Existence of mechanisms that regulate sexuality in fungi is an unique attribute which determine whether or not a particular fungus is able to mate with a compatible partner. The concept of **homothallism** and **heterothallism** explain fundamental differences in sexuality in the fungi leading to inbreeding and ourbreeding respectively. Inbreeding involves sexual reproduction between individuals that are more closely related than those of a random sample of the naturally occurring population, while out breeding occurs between individuals that are less closely related. Thus, inbreeding creates a homogeneous population, while outbreeding encourages greater genetic heterogenecity providing the necessary variability for evolution.

Homothallism:

It is usually considered as the condition where the sexes of a species are not segregated in different thalli and as such sexual reproduction takes place in a single thallus. Therefore, homothallic fungi are always self sterile and self compatible that is two mycelia developing from a single spore are capable of interacting and forming diploid structures after syngamy. The fungi which exhibit homothallism are termed homothallic fungi. The majority of all fungal species are homothallic but predominates among the zoosporic fungi, ascomycetes and in a small number of basidiomycetes. It is believed that in all cases of homothallism, the single nuclear type contains all genetic requirements for full sexual expression because karyogamy and meiosis can occur without involving a second matting partner. Examples of reproduction in different homothallic fungi are given below.

i) *Primary homothallism* in which a single uninucleate spore germinates to form a hyphae which soon becomes multinucleate or binucleate segments. There are no genetic distinction between the

two nuclei present in each cell or even in the mycelium and this mycelium is capable of producing fertile fruiting bodies as in *Coprinus sterquilinus*.

Several fungi other than the above mentioned form two kinds of gametangia, antheridia and oogonia on the same thallus even on the same hyphal branch is common among the members of Saprolegniales. Similar type of response also observed in the members of ascomycetes like *Pyronema domesticum* where male and female sex organs borne by a single stalk cell, self fertilization can occur in these hermaphroditic fungi if plasmogamy occurs between the neighbouring gametangia. The nuclei involved in karyogamy are sister nuclei, derived mitotically from the original one present in the spore that gave rise to the thallus.

ii) *Secondary homothallism*: Where a spore contains two different types of nuclei i.e. heterokaryotic, which on germination produces a dikaryotic mycelium capable of producing the fruit bodies. Such a condition exists in *Coprinus ephemerus* f. *bisporus*. In some cases, however, the non-clamped mycelia produced by the germination of the basidiospores do not lead to the formation of a basidiocarp but on pairing in certain combinations these mycelia from basidiocarps similar to those found in *Neurospora tetrasperma* (4-spored Ascomycetes).

In certain members of Ascomycotina for example *Neurospora tetrasperma*, *Podospora anserine*, the ascospores are binucleate and commonly contain nuclei of both matting types. Such spores on germination would give rise to fully fertile mycelia and it would appear that the fungus is homothallic. However, occasionally uninucleate ascospores are formed which on germination results into sterile mycelium but ascocarps are only formed in 50% of mattings between such mycelia. Since, basically these fungi are heterothallic in their matting behaviour, the term secondary homothallism is used to describe the behaviour of their binucleate ascospores.

iii) *Unclassified homothallism:* In *Agaricus campestris* the mycelium produced by the germination of a single basidiospore produces the basidiocarps, making the fungus homothalloic. Karyogamy takes place in the basidium, followed by two nuclear divisions, presumably meiotic. Clamp connections have not been observed here.

In *Armillariella mellea* most of the cells of the mycelium are monokaryotic and there is no evident of clamp conections in the mycelium. The fruit body primordial arise from monokaryotic rhizomorphs, but the cells making up the gill tissue are dikaryotic and these dikaryotic hyphae are however, associated with clamp connections, whilst the monokaryotic cells formed, in the remaining cells of the stem and cap, have no clamp. It has been demonstrated that the nuclei of monokaryotic cells are diploid whilst those of dikaryotic cells are haploid. It is presumed that, during the formation of gill initials, the diploid nuclei undergoes haploidization by an unknown mechanism.

From the above accounts it would seem that genetic variation is not likely to occur in homothallic fungi because karyogamy occurs between sister nuclei. However, mutations in one sister nucleus (but not the others) followed by karyogamy may lead to development of some genetic difference in them. This genetic differences, however, do not alter the matting behaviour. As a result of these potential mutations and recombination, genetic variation can occur through sexual reproduction in homothallic fungi although it is extremely slow, but there is the advantage that risks are minimized as there will not be great deviations from the parental types (Raper; 1959, 66a).

Heterothallism:

Unlike homothallism, heterothallism represent the condition where the sexes of a species are segregated in two different thalli and thus, for sexual reproduction to occur two different thalli are required. In other words, heterothallic species consists of sexually self sterile and self incompatible thallus. Thus, self sterile individuals do not carry all genetic requirements for sexual development in a single nucleus.

It was A.F. Blackeslee (1904) who for the first time demonstrated that in many mucoraceous species, two strains or races can be distinguished, which when grown apart, formed only sporangia, but formed zygospores when grown together. Blackeslee called these fungi, which required the interaction of two different thalli for zygospore formation, heretothallic and the phenomenon heterothallism. The two strains of such a heterothallic species which were morphologically similar

and indistinguishable into male and female but of positively of different sex were designated as '+' and '–' matting types.

Whitehouse (1949) however, distinguished two major types of heterothallism i.e. *morphological heterothallism* where two interacting thalli produce morphologically dissimilar male and female sex organs (found in *Achylya bisexualis, A. ambisexualis, Dictyuchus monospora* etc.) and *physiological heterothallism* where the interacting thalli differ in matting types or incompatibility and is entirely independent of morphological differences between male and female. Such fungi include many members of Mucorales such as species of *Mucor, Rhizopus*; several ascomycetes including species of *Neurospora, Ascobolus stercorarius*; many rust fungi including *Puccinia graminis* etc. Thus, on one hand heterothallism can be controlled by sexual dimorphism in few fungi while in others by genetic incompatibility factors. This physiological heterothallism or incompatibility systems differ in the number of genetic loci involved and also in the number of alleles than can occur at each locus and on that basis following types of heterothallism may be recognized-

a) Bipolar Heterothallism:

i) Bipolar two-factor (allele) heterothallism: This type of sexual incompatibility is controlled by two alleles [A_1 and A_2 or A and a or (+) or (–)] of a single genetic locus generally termed 'A'. In species like *Coprinus comatus* and *Piptoporus betulinus* when mycelia obtained from single spore from any one fruit body are matted together, dikaryons are formed in 50% of the crosses. This phenomenon is explained on the basis of a single gene or factor with two alleles. Since only one factor is involved, the genetical basis is termed as **unifactorial.** Segregation of the two alleles at meiosis ensures that a single spore carries only one allele. Dikaryons are only formed between monokaryons carrying different alleles at the locus for incompatibility. The members of Uredinales (*Puccinia graminis*) have matting systems of this type. The heterothallic ascomycetes like *Neurospora, Ascobolus stercorarius* also exhibit this type of compatibility where a pair of genic alleles A_1 A_2 that segregate at meiosis just before ascospore formation and as a result 50% of the ascospores normally carry gene A_1 and the other half gene A_2.

ii) Bipolar multiple-factor (allele) heterothallism: This type is characterized by a single genetic locus called 'A' locus, that controls compatibility. A number of factors (alleles) of the 'A' locus exist which are designated A_1, A_2, A_3, A_4 ..... A_n. Fertile dikaryons formation is thus, only possible when the homokaryotic mycelia carry different factors. This means that a mycelium carrying the A_1 factor can not mate with a mycelium also carrying A_1 but it will mate with a mycelium containing any other of the remaining factors. The newly formed dikaryon would then contain both factors and would segregate out only at meiosis. This type of heterothallism is present in most smut fungi and other members of Basidiomycotina.

	A_1	A_2	A_3	A_4
A_1	–	+	+	+
A_2	+	–	+	+
A_2	+	+	–	+
A_4	+	+	+	–

+ = Compatible Cross

– = Incompatible Cross

Mating reactions of a bipolar fungus

b) Tetra polar heterothallism:

Compatibility is governed by two genus with two factors (alleles) at each locus. Because two separate factors are involved the genetic basis is said to be **bifactorial**. Two genes are usually designated A and B and their two alleles as A_1, A_2 and B_1, B_2 respectively. When monosporus mycelia derived from a single fruit body are inter crossed fertile dikaryons result in only ¼ th of the mattings.

	A_1B_1	A_2B_2	A_3B_3	A_4B_4
A_1B_1	–	FL	B	+
A_2B_2	FL	–	+	B
A_3B_3	B	+	–	FL
A_4B_4	+	B	FL	–

+ = Compatible
– = Incompatible
FL = Flat
B = Barrage

Mating reactions of a tetrapolar fungus

iv) *Tetrapolar two factor (alleles) heterothallism*: This type occurs only in some members of Basidiomycotina (e.g. *Heterobasidion annosus*). In this type the monokaryotic hyphae (A_1B_1) derived from uninucleate basidiospore can grow indefinitely but fusuions occur between cells of the same species if they encounter each other during growth but subsequent development takes place only when the mates are compatible (A_1B_1 + A_2B_2). If the strains are compatible the invading nuclei migrate through the recipient hyphae and pair to form stable heterokaryon which will ultimately produce basiodiocarps.

v) *Tetrapolar multiple-factor (allele) heterothallism*: Majority of the members of Basidiomycotina (*Coprinus firmaterius*) and the smut fungi *Ustilago maydis* exhibit this type of compatibility. As in the above type this compatibility is controlled by A and B genes each having two sub-loci K and 2 and each sub-loci with a number of alleles. Thus, compatibility occurs when alleles of both A and B are different. If one or other alleles are common hyphal fusion is possible but not true dikaryon formation. According to Novotony *et al.* (1991) in *Schizophyllum commune* at least $32A_K$ and $9A_2$ alleles and $9B_K$ and $9B_2$ alleles are there and as such 288 (32 x 9) alleles of A and 81 (9 x 9) alleles of B regulates the chances of 23328 (288 x 81) new progenies formation by the compatible crosses where all the alleles of A and B factors are involved.

c) Octopolar Heterothallism:

A trifactorial matting system was reported in 1973 by Jurand and Kemp in *Psathyrella coprobia* a member of Basidiomycotina where three factors A, B and C are thought to be inherited independently *i. e.* they are unlinked. All these three factors must be heterozygous for the occurrence of nuclear migration and the formation of mature fruit bodies.

Role of matting type genes: The matting type genes of some fungi having two or multiple factor heterothallism have been isolated, cloned and their genetic sequences analyzed (Kues and Cassetton, 1992). It has been reported that 'A' factors control specific nuclear pairing, initiation of the clamp and separation of clamp by a septum. Thus, in *common 'A'* heterokaryon incompatibility irregular nuclear distribution is frequently noticed. The 'B' factors control septal breakdown and nuclear migration and as such blocking in nuclear migration is observed in *Common 'B'* heterokaryons. (In such a case if the clamp is even formed, it fails to fuse with subapical cell and thus its nucleus become trapped leaving the subapical cell uninucleate. This type of clamp is called *pseudoclamp*). They are required to complete clamp formation either alone or in conjunction with the 'A' factor. In addition to these compatibility factors, a large number of genes are also necessary for controlling some specific aspect of reproduction.

Heterokaryosis:

Heterokaryosis is usually defined as a condition in which genetically different nuclei (heterokaryons) remain associated in the same protoplasts or cell or mycelium. Heterokaryons occur from a hyphae that has only a single nuclear type (homokaryon). A homokaryon may give rise to a heterokaryon by the several ways-

i) by fusion of one hyphae (during anastomosis) with another of different genic combination followed by migration of nuclei and cytoplasm from one hyphae to another.
ii) Karyogamy of haploid nuclei to form diploid nuclei within mycelium (diploidization) followed by the reduction division and multiplication and subsequent spread.

iii) Mutation of one or more nuclei within the homokaryotic mycelium may give rise to a heterokaryotic mycelium with dissimilar nucleus.

Both mutation and diploidization occur spontaneously during sexual cycles. Those events may occur in any combinations. For examples, a heterokaryon that originated by hyphal fusion may have normal and mutated nuclei from both parents and these nuclei may exit in the haploid condition or form diploid nuclei by nuclear fusion with similar or dissimilar nuclei. These nuclei may divide mitotically for any number of nuclear generation but may finally be separated from each other during formation of uninucleate asexual spores. On germination these spores will give rise to a mycelium with a single nuclear type. However, bi- or multi-nucleate spores of many fungi give rise to heterokaryotic mycelium if the nuclei in spore are genetically different.

Heterokaryosis is a source of potential genetic variation of a given type of mycelium, since it contains cytoplasm from different sources (note cytoplasm may carry genetic determinants, for example mitochondria) as well as genetically different nuclei. Usually the phenotype of a heterokaryon is determined by the interaction of all nuclear types with the cytoplasm as well as by relative members of the various nuclei. This type of nuclear interactions sometimes become important if the nuclei are biochemically deficient and are unable to direct the synthesis of a essential compound like vitamin, amino acids, growth factors etc. Through complementation (it is the situation where two mutant alleles brought together in a cell produce a wild type phenotype) a heterokaryotic mycelium can direct the synthesis of such deficient compounds and show a wide range of physiological flexibility. According to the investigators of heterokaryosis, it enhances the chances of survival of a fungus with the help of large genetic reservoir specifically essential for adaptations and all are accomplished without delay of passing through sexual cycles.

Parasexuality:

The parasexual cycle may take place within a heterokaryotic mycelium. This cycle is usually defined as a cycle in which plasmogamy, karyogamy and haploidization take place but not at specified time or at specified points in the life cycle of an organism.

Parasexuality was first discovered in 1952 by Pontecorvo and Roper in *Aspergillus nidulens*- the imperfect stage of *Emericella nidulans*. Since then, this phenomenon has been identified in several other imperfect fungi (*A. niger, P. chrysogenum, P. expansum, P. italicum, Cephalosporium mycophyllum, Fusarium oxysporum* f. sp. *pisi, F. oxysporum* f. sp. *cubense* etc.) that possess no sexual stage as well as in some members of Basidiomycotina (*Ustilago maydis, U. violacea, U. hordei, Puccinia graminis tritici*) and in Ascomycotina (*Cochleobolus sativus*) other than the genus mentioned above. So far it has not been reported in Phycomycetous fungi. The sequence of events in a complete parasexual cycle is somewhat as follows-

i) *Formation of heterokaryotic mycelium*: There are several ways in which a heterokaryotic mycelium may be produced. The most common way perhaps is the anastomosis of somatic hyphae of different genetic constitutions. The nuclei introduced into a mycelium then multiplies and its progeny spread through the mycelium, rendering the latter heterokaryotic. Another way in which a homokaryotic mycelium may change into heterokaryotic is by mutation in one or more nuclei as has been shown to occur in some Ascomycetes. Still a third way is by the fusion of some of the nuclei and their subsequent multiplication and spread among the haploid nuclei.

ii) *Fusion between two nuclei*: Nuclear fusion may be between nuclei of like or unlike genetic constitution. The former type results in a homozygous diploid and the latter in a heterozygous diploid nucleus. Thus based on theoretical consideration, at this stage the mycelium may contain at least five types of nuclei- 2 types of haploid (A & B), 2 types of homozygous diploid (AA & BB) and 1 heterozygous diploid nucleus (AB).

iii) *Multiplication of diploid nuclei side by side with haploid nuclei*: All the above mentioned types of nuclei multiply at about the same rate. It has been found that the diploid nuclei are present in much smaller numbers than the haploid. Pontecorvo (1958) estimated a proportion of one diploid heterozygous nucleus to thousand haploid nuclei.

iv) *Occasional mitotic crossing over*: During multiplication of the diploid nuclei mitotic crossing over takes place, which results in a new combinations and new linkages of genetic material and is probably the most important phase of the parasexual cycle.

In mitotic crossing over, segments of chromosomes are exchanged for exactly corresponding segments between homologous chromosomes during their replication that is at the 4 strands stage. If dominant and recessive genes have been regrouped, subsequent haploidization may produces daughter nuclei that are genetically different from those that would be formed if this exchange had not taken place. This mitotic crossing over is a rather rare event and occur with a frequency of 10^{-2} per nuclear division in *Aspergillus nidulans* while in some fungi like *Penicillium chrysogenum* and *A. niger*, it is an frequent as during meiosis. Mitotic crossing over differs from the meiotic crossing over in a number of respects- recombination at meiosis in sexual cycle takes place during crossing over at 4 strands stage, in all chromosome pairs and random assortment of members of each chromosome pairs leads to the haploid stage while mitotic crossing over takes place as rare accidents of mitosis. Mitotic crossing over, at 4 strands stage is usually confined to one exchange in a single chromosome arm and haploidization usually occurs via aneuploidy.

v) *Sorting out of diploid nuclei*: In fungi, which produce uninucleate conidia, sorting out of the diploid nucleus occurs by their incorporation into conidia which germinate to produce diploid mycelia as in diploid strains of *Aspergillus nidulans*.

vi) *Occasional haploidization of the diploid nuclei*: Haploidization of the diploid nuclei occurs by a series of a typical and irregularly occurring successive mitotic divisions *i.e.* via aneuploidy and give rise to genetically different haploid nuclei by gradual loss of chromosomes. During anaphage, non-disjunction of the chromatids of one chromosome pair results in the formation of daughter nuclei having unequal number of chromosomes *i.e.* either (2n + 1) or (2n – 1). Both of these nuclei are aneuploids which are genetically unstable. Once the loss of chromosomes has started, the selection favours development of fully balanced haploid nuclei. As per Pontecorvo's (1958) estimation only one out of 1000 diploid nuclei in a diploid isolate of *Aspergillus niger* undergo haploidization.

vii) *Sorting out of new haploid strains*: Some diploid nuclei undergo haploidization in the mycelium and are sorted out by incorporation (of haploid nuclei) in the uninucleate conidia. Some of these haploid strains are genotypically different from their parent because of their mitotic recombinations.

After the parasexual cycle has operated or sometimes, the mycelium contain **haploid nuclei like those of both parents, haploid nuclei with various new genetic recombinations, homozygous diploid nuclei of various types and heterozygous diploid nuclei of various types.**

This parasexuality of fungi is an important tool for the fungal geneticist, because in addition to the recombination of characters, particularly in fungi which lack sexual reproduction, it provides the missing links for the imperfect fungi and could even offer a significant supplement for fungi with a perfect stage. It can also provide information on the possibilities of mitotic analysis in higher organisms in which comparable segregation process occurs. This cycle further helps in the understanding of physiological processes of perfect and imperfect fungi. It may also be useful in tissue culture, in revolutionizing genetic analysis; in progress of the planned breeding for production of improved strains as in *Penicillium chrysogenum* for production of antibiotics, *Aspergillus niger* for enzyme protease production etc. Parasexual cycle has also been successfully employed in genetic control of pathogenicity and host range in several species of *Fusarium*. Some recent studies also focused on the genetic control of aflatoxin production by *Aspergillus flavus* which lacks a sexual cycle.

MODEL QUESTION

Short answer type (5 marks each)

1. What is homothallism ? Write about primary and secondary homothallism in fungi.
2. What is heterothallism ? Discuss bipolar/ tetrapolar heterothallism with examples.
3. What is Octopolar heterothallism? Mention the role of matting type genes in heterothallism.
4. Define parasexuality. Write a brief note on parasexuality.
5. What is heterokaryosis ? How it is brought about ? Mention its importance.

Objective type (1 or 2 marks each)

1. Define homothallism.
2. Name one fungus where secondary homothallism is observed.
3. Name a fungus showing physiological heterothallism.
4. What is pseudoclamp ?
5. Mention two importance of parasexuality.

PHYSIOLOGY OF FUNGI

No fungi is able to make any increase in its dry weight in absence of organic food materials because of their achlorophyllous nature. Absence of chlorophyll make them unable to use CO_2 or to photosynthesize and thus cannot build up organic food materials. Thus fungi are chromoheterotrophic for carbon (organic) compounds which they in their natural habitats obtain by living as saprophytes or from living tissues of plants and animals as symbionts. [According to Lewis, 1974, Symbiosis is an association of usually dissimilar organisms which exhibit permanent or at least prolonged, intimate contact that is includes parasitic and mutualistic associations]. Experimental results however, indicate that some fungi are capable of synthesizing their own proteins when grown in a medium containing carbohydrates as source.

Like other organisms, the fungal growth is adversely affected if one of the essential macroelements C, H, O, N, S, P, K and Mg lack in their growing environment. Fungi also require minute amount of Fe, Zn, Cu, Mn, B, Co, Mo, etc. as trace elements or micronutrients. Calcium is not known to be needed by the fungi in general for their growth but some however, require it as a micronutrient. The organic substance usually utilized by fungi are very varied in nature for example Glucose (suitable for almost all fungi), Fructose, Xylose, Manitol, Maltose, Sucrose, Acetates etc. However, Starch, Cellulose etc. are utilized only by a fewer fungi which can synthesize the appropriate hydrolytic enzymes. Proteins, Lipids, higher alcohols are also utilized by some fungi as a sole energy source while organic acids are generally considered as poorer source of Carbon for most fungi.

Besides carbon, fungi obtain nitrogen from both organic and inorganic sources. In nature fungi decompose proteins and other materials to obtain their supply of nitrogen. During *in vitro* culture amino acids, peptides, peptones, gelatin, casein, egg albumin etc. usually serve as the organic source of nitrogen. Of the preferred inorganic nitrogen source nitrate and ammonium salts are important. A few can utilize atmospheric nitrogen directly for example *Rhodotorula, Pullularia pullans.*

Oxygen and hydrogen comes from water mainly. Water functions as a solvent in which most if not all biochemical reactions take place. It readily enters into many hydrolyting and condensing reactions also.

Most fungi utilize sulphate sulpher as source of sulpher while some require sulpher in reduced form. This element enters into the composition of enzymes, proteins, peptides and at least two vitamins. Source of phosphorus is mainly phosphate and esters, of which latter enter into a wide variety of enzymatic reactions and many coenzymes are phosphate esters. It is thought that certain phosphate esters act to transfer chemical energy to certain enzymatic reactions. Phosphorus mainly enters into proteins, mainly nucleoproteins.

Like all other organisms fungi require minute amount of specific, relatively complex organic compounds like vitamin or growth factors. Many fungi synthesize their own supply from a single nutrient solution while other (auxotrophic organisms) dependent in whole or in part on an external source because of their unability to synthesize one or more of the essential growth substances. The most important vitamins required for fungal growth are Vitamin B_1 (Thiamin), biotin, B_6 (Pyridoxin), B_2 (Riboflavin) etc. but some of them need nicotinic acid and pantothenic acid.

It has already been mentioned that fungi are chemoheterotrophic, depending on organic compounds as their principal energy and carbon sources. They must derive these compounds from either non-living organic materials or from living tissues. In the latter situation the fungi come into association with a suitable host organism and this relationship with their hosts, however, temporary, is that of a common life. The word **symbiosis,** was originally used strictly to describe this concept although it was at the same time recognized that every kind of gradation could be found in nature of fungus – host interactions. Gradually, within mycology the use of the term symbiosis has come to be erroneously restricted to associations that are characterized by **mutualism**, that is where fungus and host either wholly or partly maintain one another. However, currently there seem to be movement towards restoration of its original broad meaning. **Symbiosis**, is therefore, used here in the all-embracing sense to refer to all associations where fungi come into contact with a living host from which they obtain in a variety of ways, either major or minor metabolites or nutrients.

One fungus may be **antagonistic** towards its partner and cause it slight or severe harm either directly or indirectly. Such type of antagonistic symbionts are normally referred to as **parasite** or, if they cause damage sufficiently serve to produce easily recognizable disease symptoms in their host are termed

Pathogen. This antagonist and antagonistic are perhaps less ambiguous terms than parasite and parasitic and may eventually come to replace the latter (Cooke, 1981). The fungus may be **mutalistic** towards its partner which, as a result, benefit in some way from the association. Between these antagonism and mutualism a third possibility, that of **neutralism** may also be distinguished. A fungus may be considered to be a neutral symbiont if it is consistently found associated with a host upon which it is absolutely dependent (either long term or transient) but on which it has no obvious deletions or beneficial effect. Neutralism corresponds closely with the term **Commensalism** where the latter is used to describe a situation in which only one partner profits from an association. On the basis of the degree of fungal symbiosis, the fungus may be called obligate or facultative. The fungi that are **obligate symbionts** have no capacity for a free-living existence, other than as propagules, in absence of a suitable host. In contrast, **facultative symbionts**, while being always potential symbionts if suitable ecological condition arises, do have a well-developed free-living capability.

Fungal symbionts have certain nutritional characteristics that are determined by the source and derivation of their nutrients. Followings are the modes of possible nutrition –

i) a fungus may be **saprotrophic**, which derive organic compounds directly from the non-living components of its immediate environment.

ii) It may be **necrotrophic**, deriving its organic nutrients from the dead cells of organisms which it has itself killed. Necrotrophs are in the end behave in a manner similar to saprotrophs but are continually create their own dead organic substrates throughout the duration of the symbiosis.

iii) A fungal symbiont may be **biotrophic** and be capable of deriving its organic nutrients only from the living cells of its host.

iv) The term **hemibiotrophic** has been applied to a pathogen that initially invades a plant in a biotrophic mode but later switched to a necrotrophic mode.

Biotrophs:

This type of fungi entirely lack or have only a poorly developed capacity for a free-living existence in nature and require a symbiotic association for normal development. They are also called as **antagonistic obligate symbionts**.

This group of fungi are physiologically extremely specialized. Interactions with their host (particularly higher plants) are distinguished by a number of characteristic features –

i) intracellular penetration of the host by the fungus;

ii) nuclear disturbances within both the penetrated and adjacent unpenetrated cells. There is normally minimal tissue damage, although major morphological changes is the host plant may occur;

iii) Induction of translocation of host metabolites into the infected regions.

Major informations on the host-biotrophs come from the studies on infections by members of the Uredinales mainly *Puccinia* sp., Erysiphales like *Erysiphe* sp. on graminaceous plants as well as from 'lower' biotrophic fungi – Plasmodiophoromycetes, Chytridiomycetes and Oomycetes. At an early stage of successful establishment of a biotroph, a sequence of events occur during which normal metabolism of host cells alter as they respond to demands made on them by the fungus. For example local hydrolysis of starch etc. *Uromyces phaseoli* contain 2-amylase activator which diffuses into the mesophyll and activate host's hydrolytic enzymes, and soluble products thus obtained are utilized subsequently by the biotroph after successful penetration. Antagonistic biotrophs usually produce some lateral hyphal branches of determinate growth which intrude through the host cell wall into the cytoplasm and act as organs for the absorption of major nutrients from the host cell or sometimes involved in more subtle biochemical exchanges which lead to specific modifications in host metabolism that are favourable to the biotrophs. It has been reported that during early stage of infection of wheat leaves, *Erysiphe graminis* never produce extracellular mycelium until and unless the primary haustorium reach a particular stage of development. Indirect evidences indicate that some haustorial biotrophs may have a limited ability to produce their own

proteins and that in these cases during establishment, early haustorium formation may be essential to alleviate this deficiencies.

Unlike necrotrophs, the biotrophs produce different CWDE (Cell Wall Degrading Enzymes) like cutinases, cellulases (as in *Erysiphe graminis*), hemicellulases and pectinolytic enzymes (*Puccinia graminis*) but their action is extremely confined so that there is only localized degradation instead indiscriminate cell wall degradation and cell collapse.

Studies on changes in nucleic acid content indicate that in young rust infections both haustorial mother cells and the haustorial bodies are rich in RNA. At the same time the enlargement of host nuclei and nucleoli along with enhancement of uptake of labeled precursors into RNA in host tissue takes place. These evidences indicate a host mediated induction of RNA synthesis in the biotrophs and also indicate induced host protein synthesis, the products being then utilized by the biotroph. Similarly, studies on non-rust fungi, principally *Plasmodiophora brassicae* indicates that plasmodial development within a root hair is paralled by a two-fold increase in the size of the root hair cell nucleolus of cabbage – the host, which is accompanied by an increase in nuclear RNA and protein but there is no increase in nuclear DNA, decrease in nuclear histone and an increase in non-histone protein. This increases in nucleolar size, RNA, total protein suggest the host ribosome synthesis stimulated by biotroph while the increase in non-histone protein may indicate that the normal transcriptional processes of the host nucleus are being altered by the presence of the fungus.

During the development of biotrophs on the photosynthetic parts of plants, a decrease in photosynthetic activity due to loss of chlorophyll as well as loss in available photosynthetic areas takes place. However, an enhanced fixation of CO_2 in dark is noticed within green islands induced by rusts, powdery mildew and some of the Peronosporales but this enhancement seems to be due to the ability of the biotroph itself to dark fix, particularly during sporulation. The malic enzymes involves in dark fixation has also been found in rust Urediospores. It should be noted that both saprotrophic and necrotrophic fungi are capable of this process and it is not unique to biotrophs. However, its significance in biotrophic symbiosis is not clear.

An almost constant feature of infections by obligate biotrophs is an increase in the respiration rate of host tissue. Available evidence suggests that, in rust and powdery mildew infections, the respiration pattern of the host tissue is shifted from a system that is predominantly channeled through glycolysis and the Krebs cycle to one which is dependent on the pentose phosphate pathway. This increase in the activity of pentose phosphate pathway results in increased amount of $NADPH_2$. This could participate in the enhancement of synthetic processes particularly lipid and sugar alcohol synthesis, which are important to the metabolism of rusts and powdery mildews. Furthermore, this pathway provides an increased supply of pentoses for the synthesis of nucleic acids and ultimately for protein synthesis.

Another interesting point to be noted that the most, if not all, biotrophs have a capacity to influence the distribution of carbohydrates within host tissues so that a typical feature of infections involving rust fungi, powdery mildews, *Albugo* sp., *Plasmodiophora brassicae*, is the accumulation of one or most host carbohydrates (glucose, fructose, sucrose, polysaccharides mainly) at infection sites prior to sporulation of the fungus. These are then disappear as spore production progresses. After hydrolysis of polysaccharides, the product sugars are then taken up and metabolized by the biotroph but in addition, where there is a surplus of soluble carbohydrate, particularly sucrose, hydrolysis provides hexoses for starch, synthesis within the chloroplast. When a host normally stores insoluble fructans rather than insoluble glucans the activity of invertase similarly leads to fructan synthesis and deposition of these compounds within the vacuoles of host cells. Thus, invertase mediates a system by which the excess soluble carbohydrate occurring at infection sites is converted to polysaccharides that are less osmotically active. Potential osmotic interference with the physiology of the biotroph is in this way prevented.

It is now well established that carbon compounds moving from host to biotroph accumulate within the hyphae in the form of specific fungal products. In infections by species of Basidiomycotina and Ascomycotina these are usually acyclic polyols, principally arabitol and mannitol together with trehalose and glycogen. Since these fungal products cannot be metabolized by host cells, their re-utilization by the host is prevented. Species of Plasmodiophoromycetes and Oomycetes do not appear to accumulate polyols, although some are capable of synthesizing trehalose, in others it is entirely lacking. In those organisms source-sink movement of host carbohydrates may be maintained by means of the conversion of soluble sugars to glycogen or to lipid within the cytoplasm of the fungus.

Now-a-days, rust fungi can be grown axenically with relative ease, in at least some cases. The entity which grows and develops saprophytically on nutrient agar often differs in some important respects from the biotrophic material from which it originated. When growing axenically rust fungi can utilize a wide range of soluble carbohydrates as sole sources of carbon. They are self-sufficient for vitamins, but lack the ability to utilize inorganic sources of nitrogen and may require growth factors present in the yeast extracts or peptone (Jones, 1973). The major feature of their nutrition in axenic culture is an inability to incorporate inorganic forms of sulphur into amino acids, so that they have a requirement for an exogenous supply of sulphur containing amino acids. Cysteine, homocysteine or methionine all support growth in *P. graminis tritici* but in *Melampsora lini* methionine can not be substitute for cysteine. Mycelium of *P. graminis tritici* takes up ^{35}S-Sulphite but is unable to reduce it. This species however, incorporate ^{35}S-Sulphite but still show a requirement for a sulphur containing amino acid. Incorporated labeled sulphide can be detected in culture filtrates in Cysteine, S-methylcysteine, glutathione and cystein glycins. Appreciable amounts of methionine are found in protein within the mycelium yet little methionine appears in culture filtrates. This implies that the requirement of this species for exogenous sulphur compounds may be caused by continual and excessive loss of cysteine and some of its derivatives from the hyphae. This explain why in some strain of *P. graminis tritici* increased Urediospore density increases the chances of successful establishment of axenic colonies, since in this situation leakage would be at least partially alleviated by reabsorption. Where Urediospores are relatively widely separated. Sulphur amino acids would rapidly diffuse away with respect to this possibility it has been observed that sometimes application of Cysteine aids axenic growth in areas of low spore density.

Necrotrophs:

It has already been mentioned that the necrotrophs destroy their hosts relatively rapidly and are able to exist, normally very successfully, as free-living saprotrophs. Some obligate necrotrophs, like facultative necrotrophs rapidly colonize their hosts and death of the latter is accompanied by massive tissue destruction while other cause only localized damage. In either case death of the host/part terminates necrotrophic activities and a purely saprotrophic phase then ensures. However, in contrast to facultative necrotrophs, obligate necrotrophs have a poorly developed capacity for a free-living saprophytic existence under natural conditions. They can be grown on relatively simple media rather than being determined by

specialized nutrient requirements. Within the dead organic substrates obligate necrotrophs are able to survive until new hosts become available for infection. Since, their saprotrophic phase are so severely limited and the provision of substrates within which they can survive depends on a preceding necrotrophic phase, they are thus may be termed obligately symbiotic requiring association with a host for their normal growth. Many of these necrotrophs like uniquitous *Rhizoctonia solani*, vascular wilt fungi, *Pythium* sp. can spread rapidly through the soil from host to host when these are in close proximity to one another, use dead host remains as food bases for further infections and can survive for considerable periods in dead organic substrates i.e. in relatively simple nutrient media.

Hemibiotrophs:

During symbiotic mode of nutrition hemibiotrophic fungi passes through two distinct phases, being at first biotrophic then necrotrophic. In biotrophic phase little or no host cell wall degradation takes place and thus, host protoplasts remain alive. In addition, if an infected leaf dries and then falls from the host then the necrotrophic phase is followed by one of saprotrophy, which usually remain restricted to tissues that were previously occupied by the fungus when it was necrotrophic. So the hemibiotrophs have only a restricted or transient capacity for a free-living existence. This type of behaviour is exemplified by many leaf-spotting fungi of which the Pyrenomycete *Guignardia bidwellii* on *Vitis rotundifolia* is a typical representative (Luttrell, 1974).

Hemibiotrophy is widespread amongst Ascomycotina, Hyphomycetes, some members of Peronosporales. *Phytophthora infestans* is usually treated in discussions on physiology of biotrophy, as a typical biotroph yet it has a distinct and important necrotrophic phase. Hemibiotrophs differ from biotrophs in one major respect that hemibiotrophs are normally easily grown *in vitro* on chemically defined media upon which their growth remain more or less constant along with their infectivity (except *P. infestans*).

Although it is probable that many hundreds of species are hemibiotrophs, detailed investigations on their physiology particularly during the biotrophic and necrotrophic phases have been infrequently made and these have been mainly confined to three fungi – *Venturia inaequalis* on apple, *Rhynchosporium secalis* on barley and *Phytophthora infestans* on potato.

In *R. secalis* time interval between initial infection and appearance of symptoms is short that is 3-7 days. During this period the nutrition of the fungus is strictly biotrophic and growth is mainly subcuticular and utilize the soluble nutrients present in the free space of the leaf but an increase in permeability of the underlying host cells increase the concentration of nutrients in the free space. In course of time the fungus becomes necrotrophic and produce pronounced symptoms subsequently, particularly when the mesophyll cells become affected. There is no direct lateral or longitudinal spread of the fungus within deeper leaf tissue, lesions increasing in diameter as a result of marginal growth of the overlying subcuticular mycelium.

The interval between infection and the appearance of visible symptoms in infected apple plants with *Venturia inaequalis* is extensive during which the growth of mycelium is subcuticular. As infection progresses, the death of hosts epidermal cells take place and the fungus switches over to necrotrophic mode from the biotrophic one. In the late growing season the fungus moves into deeper leaf tissues particularly before leaf fall. Nutrition of the fungus in the mesophyll is thus finally saprotrophic. It has been observed that the fungus can confine host translocates to the vascular network (i.e. not interveined tissue) before the lesion becomes visible, with the help of melanoproteins (MW – 10,000-70,000).

The nutrition of *Phytophthora infestans* just after infection is biotrophic but hyphae quickly ramify through all the leaf tissues and within a short time (2-3 days) the lesions appear which become necrotic at their centers and rapidly increase in size. Within the necrotic host tissues the fungus is necrotrophic, but at the margins of the developing lesions the fungus is growing biotrophically 3-6 mm in advance of the necrotic regions.

Mycoparasites may be necrotrophic and biotrophic:

Mycoparasites (fungi parasitic on other fungi) are separated into two major groups based on the mode of parasitism i.e. the necrotrophic and biotrophic.

Necrotrophic mycoparasites are capable of indefinite saprophytic existence and are characterized by relatively rapid growth on a variety of fungi. The most destructive necrotrophic mycoparasite is *Gliocladium roseum*, *Trichoderma* sp., *Rhizoctonia solani*, *Polyporus adustus*, *Ampelomyces quisqualis*,

Sepidonium sp. etc. These fungi make contact by means of short branches which touch or curl around the host fungal hyphae or spores, the contents of which soon begin to disintegrate and the parasite then draw its nutrient from that.

Biotrophic mycoparasites differ basically from the necrotrophic mycoparasites in their ability to obtain nutrients from the living hyphae. Usually chitrids grow internally within cells of other fungi and draw nutrients, while the contact parasites like *Gonatobotrys simplex*, *Stephanoma phaeospora* parasitic on Asco – and Deuteromycetous fungi, do not produce haustoria or other internal hyphae for their (temporary possession) sequestration of nutrients. The haustorial parasites which produce distinct haustoria within the host hyphae, belong to the merosporangiferous Mucorales (produce rod like spores within sporangia) are *Syncephalis*, *Piptocephalis*, *Dispira* etc.

Symbionts:

One partner of a symbiotic association is called the symbiont. Fungal symbionts of antagonistic symbioses and their modes of nutrition have already been discussed earlier. In this brief resume another group of fungi called *mutualistic* symbionts will be discussed. Fungi of this group have either no capacity or a poor capacity for free living saprotrophy in environment. Those associated with plants normally exist as biotrophs either in a lichenized form or mycorrhizal association.

Lichen is a stable symbiotic association between a fungal partner and a simple photosynthetic autotrophy (Phycobionts) that results in the development of a morphologically distinct thallus. The two symbionts interact in such a way that the biology of the lichen is distinctive and not comparable to that of either partner, when isolated. The largest number of lichenized fungi occur in the Ascomycotina (Loculoascomycetes and Discomycetes). Overall, the lichens having an ascomycete represent about 98% of all lichens. Next to the Ascomycotina, the largest number of lichenized fungi occur in the Deuteromycotina while comparatively few basidiomycotina (Hymenomycetes) are lichenized. There are no lichens known belonging to the lower fungi, Hemiascomycetes, Plectomycetes and Laboulbeniomycetes.

Living as a symbiont in a lichen appears to be a very successful way for a fungus to drive essential nutrients. The autotrophic symbionts occurring in lichens are simple photosynthetic algae. The algal symbionts includes the members of Cyanophyta (example – *Nostoc, Calothrix, Gleocapsa, Scytonema* etc.), chlorophyta (example – *Trebouxia, Pseudotrebouxia, Trentepohlia, Coccomyxa* etc.) and Xanthophyta. The most commonly occurring genus is *Nostoc, Trebouxia* and *Trentepohlia*. Within the lichen, the algal cells represent only 3-10% of the total mass of the lichen by weight while the fungus make up the remainder of the mass. The algae are important in nutrition of the lichen because they are responsible for photosynthesis. Typical organization of lichen thallus permits CO_2 to readily reach the algal cells and helps to carry out photosynthesis. The fate of the fixed carbon can be traced by using $^{14}CO_2$. About 90% of the carbondioxide in photosynthesis is not retained by the algae, but instead massive amounts of carbohydrates are secreted into extracellular matrix. Usually cyanophytes excrete glucose, while green algae excreted a polyol (ribitol, erythritol or sorbitol). These carbohydrates are taken up by the fungus, which subsequently converts either glucose or polyol into mannitol- a storage sugar alcohol. Once formed, the mannitol is stored by he fungus and used later as an energy source. It is usually thought that the fungus in a lichen thallus act as a sink and by rapidly converting glucose to mannitol induce the alga to continue leakage across this sharp gradient.

The mechanism of carbohydrate release are not yet fully understood but is thought that fungus induce leakage by some unexplained biotrophic effect related to haustorial or appressorial contact. In some primitive lichens, haustoria penetrate the cell wall of the algal cell from which nutrients are derived; while in more advanced lichens they are associated with degenerating and decaying cells. In some other forms cell walls of the fungus and alga adhere tightly together for transport. However, recently it has been proposed that this movement is accomplished by facilitated diffusion (Galun, 1998).

Nitrogen is an essential metabolite for synthesizing proteins in both the alga and the fungus. In general, lichens can utilize exogenous urea, ammonia, nitrates and amino acids as nitrogen source. Many lichens are able to hydrolyze urea to ammonia and CO_2. The ammonia then converted into amino acids and proteins while the CO_2 can also be used in the synthesis of organic compounds. The lichens having green algae as symbionts depend entirely upon exogenous sources of nitrogen. On the other hand, Symbiont Cyanobacteria can fix atmospheric nitrogen with help of heterocysts and redistribute the fixed

nitrogen throughout the thallus so that both the fungus and the green algal symbiont (if present as third component) can utilize the ammonia to produce amino acids and proteins (Rai, 1988).

Minerals need of lichens are apparently satisfied by air borne input to the thallus and to a lesser but as yet undetermined extent from the substratum. Both algae and the fungus are involved to some extent in the uptake of the minerals.

Vitamin deficiencies are probably widespread in lichenized fungi. On the basis of more recent studies, requirements for both thiamine and biotin have been positively identified. It is known that the algae synthesize thiamine, biotin and many other vitamins, sometimes in large amounts and presume that they are made available to the fungus in the symbiont thallus (Hale, 1983).

Thus, from the above it is clear that the algal symbionts supply organic metabolites synthesized so far including vitamins. On the contrary the fungal symbionts provides protection to injury i.e. mechanical as well as from high light intensities, helps in absorption and retention of water. This is why these lichens are often able to live under extreme environment where free living algae could not thrive.

Mycorrhizae:

Fungi can be found in association with roots of almost all kinds of plants. This fungus – root combination is simply termed as mycorrhizae. The fungus and plant live together in a mutually beneficial symbiotic relationship under most conditions. Different types of mycorrhizae that occur are distinguished by their morphology and to certain extent physiology.

Ectomycorrhizae consists of a dense sheath of fungal cells surrounding the root. The fungus often penetrates between the cells of the epidermis and the first few cells of the cortical region forming an intercellular hyphal network (Hartig-net) but does not penetrate the cells themselves. Fungi that form ectomycorrhizae are usually members of Basidiomycotina especially members of the Agaricales and Hymenogastrales. Some members of the Ascomycotina (Orders – Eurotiales and Pezizales) also form ectomycorrhizae. Species of *Boletus, Russula, Tricholoma, Entoloma, Amanita, Lactarius, Rhizopogan* are the common fungal partner in this type of symbiotic relationship. Families of flowering plants which

belongs to Pinaceae, Salicaceae, Betulaceae, Caesalpinioidae, Tiliaceae harbour this symbionts. *Pinus, Picea, Pseudotsuga, Quercus, Fagus, Betula, Alnus* have ectomycorrhizal infections.

Futile attempts to culture the fungal partner of ectomycorrhizae indicate the existence of intricate nutritional relationships with the host. In culture, these fungi are likely to demonstrate a requirement for relatively high sugar concentrations. Germination of fungal spores and growth of mycelium in the vicinity of root are stimulated by exudates from the root which contain a variety of amino acids and vitamins. The fungus responds chemotactically to exudates, grows towards the roots, contact it and adheres to it. There is a specific recognition event, possibly involving binding of fungal lactins with polysaccharides in the root cell walls. The fungus forms a weft of hyphae over the surface while some hyphae invade the root mechanically penetrating between the cells of the cortex. Further, invasion is inhibited by endogenous volatile compound. Hormones produced by the fungus probably influence the development of the root, in part causing the distinctive morphological changes. In culture they secrete auxins, ethylene, Gibberellic acids while a few can produce cytokinins. These hormones are homologous to those formed normally by the plant and that regulate cell division, growth and other physiological processes, such as the mobilization and control of nutrient translocation.

Use of radioisotope (^{14}C) indicate that after the fungus become established within the root the soluble carbohydrates fixed in photosynthesis pass from the root into the fungal sheath are converted to insoluble storage polysaccharides (mannitol, trehalose, glycogen) which are used by the fungus later but not readily absorbed or used by the plant. Thus, the fungal sheath acts as a storage site for carbohydrates. Although these are generally unavailable to the root, there is some leakage so that some carbohydrates are returned to the root. Consequently the enhanced uptake of phosphates, ammonium and potassium by ectomycorrhizal fungi is significant. It has been observed that 80-90% of the phosphate absorbed may remain in the fungus sheath while 10-20% passes into the root. Stored phosphates can be translocated from the sheath to the host if the phosphate supply in soil becomes deficient. Similarly, these mycorrhizal fungi can use the ammonium ion and simple amino acids as nitrogen sources but also hydrolyze nitrogenous compound in humus and use the products of its hydrolysis. After its absorption by the hyphae, nitrogen ion or compound is converted to glutamine and is translocated to the host in this form.

Furthermore, Ectomycorrhiza can also increase water absorption and thus, particularly valuable as symbionts in dry habitats.

***Vesicular-Arbuscular Mycorrhizae*:** This type of fungi are endomycorrhizal one which invade deeply into the root. It has been observed that Vesicular-Arbuscular (VA) mycorrhizae are abundantly present in herbaceous plants growing in cultivated soils and grass lands. They are more common than the other mycorrhizal types and occurs almost universally in plants (Legumes, grasses, tomatoes, apples, strawberries, coffee etc.) lacking other types including some forest trees. The VA association is obligate for some plants that die if deprived of their mycorrhizal fungi while, in contrast some other plants can live either with or without these fungi. The VA fungi are usually the members of Zygomycotina belonging to the Order Glomales (*Glomus* sp.).

The VA fungi grow as individual strands or loose wefts over the roots and extend into the soil and ultimately penetrate host cells by forming cellulosic enzymes that digest small portions of the cell walls. The fungus maybe limited to the root hairs or other epidermal cells but also may penetrate cells of the cortex. Within the cells, the fungus produces enlarged or globular vesicles and clusters of dichotomously branched hyphal endings (called arbuscules). The vesicles develop as swellings along the hyphae or at the tips. Their walls thicken and lipids are deposited in their cytoplasm as they mature and thus probably function as storage organs. The arbuscules store large amount of phosphate (as storage polyphosphate granules) and thus, indicate their role in transportation of polyphosphate. Transfer of minerals from the fungus to the host and of carbohydrates from the host to the fungus probably occurs largely across the plasmamembrane of the arbuscules. The arbuscules lyse when they become senescent, also release nutrients into the plant cells.

The VA relationship can increase the growth of the plant by enhancing phosphate uptake and perhaps that of other minerals, such as potassium, iron, copper, calcium and zinc. The increased uptake of phosphate is especially important. This ability to increase minerals uptake favour plant growth in poor soils.

Furthermore, VA roots have a greater capacity to uptake water and may withstand the stress of low levels of available moisture better than do non-mycorrhizal roots. For examples, they recover from

wilting better than non-mycorrhizal plants do and are better able to withstand the shock of being transplanted.

Orchid Mycorrhizae: Almost all members of Orchidaceae, the orchid family, enter into a symbiotic relationship with fungi that produce clamps and are therefore basidiomycetes, are members of genus *Rhizoctonia*, non-sporulating Deuteromycetes with affinities to the Basidiomycetes.

Endomycorrhizal formation is obligate for the orchids under naturally occurring conditions. The length of symbiotic period varies from a few months or years to their entire lifetime in the case of some non-chlorophyllous orchids. Prior to symbiotic period, orchid seeds are invaded by hyphae or basidiomycetes and *Rhizoctonia* sp. that forms the endomycorrhizae with the developing roots or other absorbing organs. In the absence of fungal invasions, orchid fails to grow unless they are supplied with an external source of organic carbon compounds and sometimes vitamins. Fungi provide carbohydrates and possibly other accessory metabolites such as vitamins to the orchids, so in reality the orchids are parasitic on the fungi. Unlike those fungi that form ectomycorrhizae, the orchid fungi are able to utilize lignin and complex carbohydrates often including pectins and cellulose. The fungus hyphae digest organic materials in the surrounding environment (either the soil or supporting tree, in the case of epiphytes). The fungi assimilate these nutrients to support their own growth and to produce glucose, ribose, and other simple carbohydrates. The carbohydrates and other nutrients are translocated within the hyphae and are finally released into the host. It is to be noted here that the fungus also derive nutrients from the orchid. The fungi sometimes depends upon the orchid as a source of amino acids or exogenous vitamins such as thiamine or only one of the moieties or thiamine. However, experiments with radioactive carbon have indicated that carbon is not transferred from the orchids to the fungus (Arditti, 1979).

An additional resistance mechanism is the formation of at least three anti-fungal substances that are produced by some orchids in response to fungal invasion. These antifungal substances include *Orchinol*, a high molecular weight phenolic compound that inhibits fungal growth and thus prevent the fungus from completely parasitizing seedling and killing of host cells.

Endophytes: These are fungi that live through their life cycle within stems or leaves of their host plant. They establish a systemic infection and usually intercellular. Unlike the parasites, they cause little or no apparent harm to the host and can be considered to be either mutualistic or neutral symbionts.

Fungi found to be living as endophytes include numerous species of Ascomycetes and Deuteromycetes. Some Basidiomycetes and a few members of the Oomycetes. Plants capable of hosting endophytes can be found in mosses, liverworts, ferns, gymnosperms and flowering plants. It is usually believe that the biological fitness of fungi is enhanced because they are provided with nutrients, protection and a means of dissemination (through seeds) by the plant hosts. As noted above, this relationship is obligate for the fungi.

There are both specific disadvantages and advantages derived from this relationship. On one hand the endophyte can harm their plant hosts by interfering with their sexual reproduction and sometimes yellowing and necrosis (in *Balansia* infected grasses) of leaves, while potential advantages of the plants are – (i) increased growth of infected grasses, (ii) plant/grasses may be tolerant to drought, (iii) toxins produced by the endophytes make the plant unattractive to the herbivores. These toxins result in decrease in reproductive capability, poor larval growth and development and perhaps death. This endophyte – grass mutualism maybe exploited for agriculture purpose and for commercial purpose.

Fungus as insect symbionts: A number of fungi have mutualistic symbiotic relationships with insects. Some of the fungi occur within the insects (endosymbionts -for example, yeast or yeast like *Candida, Torulopsis* etc.) or occur externally to the insects (exosymbionts – for example, *Septobasidium*). Normally the insects depend upon higher plant materials ultimately for their nutrition but lacking the ability to break these down and digest themselves, allow fungi to perform this function and then feed on the fungal mycelium. Some insect species are always found in association with certain fungus and some fungi only with certain insect when move from one place to another, fungi are transported with them. Efficient adaptations have evolved in both the partners in the symbiosis to ensure successful transmission of the fungus.

MODEL QUESTION

Short answer type (5 marks)

1. Write a brief note on type of fungi based on nutritional chracters.
2. Give an idea about the hemibiotrophy with examples.
3. What is mycorrhizae ? With suitable examples write a note on ectomycorrhizae.
4. What is VAM fungi ? Write a concise account of VAM with reference to its importance.
5. What do you mean by endophytic fungus ? Mention advantages and disadvantages of endophytic relationship.
6. Give a brief account of different mycorrhizae with examples.

Objective type (1 or 2 marks)

1. Name one fungus which can utilize atmospheric nitrogen directly.
2. What is mutalism ?
3. What do you mean by necrotrophism ?
4. Give one example of hemibiotrophic fungus.
5. Write down the basic differences between biotrophic and necrotrophic mycoparasite.
6. Name one VAM fungi.

ROLE OF FUNGI IN INDUSTRIES

Fungi were exploited for useful purposes long before anything was known about their existence or their characteristics. As early as 6000 B.C. the Babylonians and Sumerians used yeast to make alcohol. Now-a-days many other fungal metabolites form the useful products and are separated from the substratum for further use in the pure form. Fungal products used commercially include organic acids, alcohol, antibiotics, vitamins, enzymes, pigments etc. For industrial purposes specific fungal strains must be developed to yield high yielding desirable strains and then meticulous attention must be given to ensure that favourable cultural conditions are maintained that will provide the maximum yield of the desired end product.

Some of the prerequisites to an economically practicable industrial microbial process are the following in terms of organisms, medium and the products. Fungal strains to be used must be able to produce appreciable amounts of the product. It should have relatively stable characteristics and the ability to grow rapidly and vigorously. Modern industrial fermentation techniques make it possible to produce millions of tons of useful compounds annually, through introduction of new fungal strains with favourable characteristics constructed through genetic manipulations, for example, mutation, genetic recombinations, genetic engineering (recombinant DNA-techniques) etc. Since the metabolites are formed along diverse pathways and as we might expect, there is no single set of environmental or nutritional conditions common to all commercial syntheses. In addition, a single fungus is usually capable of producing several metabolites. Attaining a metabolite of choice is accomplished by forcing the fungus to favour certain biosynthetic pathways over the others. This is often achieved by, for example, pH adjustment so that some enzymes act normally while others are inhibited, by adding antagonists that affect certain enzymes only, by adding nutrients or maintaining conditions that do not favour growth and thereby divert assimilated nutrients into metabolic waste products rather than into protoplasm, or by adding precursors that favours certain pathways.

In this part only an account of the products, their sources and uses will be discussed and as such for detail account of the concerned industrial processes consult other books.

A. Organic acid production:

Citric acid: It is one of the most important organic acids produced by fungi. Of the total production (about 350,000 tons) about 70% used in food industry (as food additives) while 10% is used to produce cosmetics and pharmaceutical products and 20% in other processes like manufacturing of ink, dying, engraving, electroplating, leather tanning, reactivating old oil wells where the pores of the sand become clogged with iron.

Citric acid can be produced commercially from selected strains of *Aspergillus niger*, which actively produce citric acid under certain conditions. The most important of these condtions is utilization of a medium deficient in some essential metallic nutrients (like Mn, Zn, Fe) and generally conditions which limits growth.

The probable origin of citric acid is directly from TCA cycle where the cycle is inhibited by the enzymic inhibition of low pH or by specific inhibitor like copper. Biosynthesis of citric acid is not inhibited but those involving its conversion are inhibited leading to the accumulation of citric acid as an "overflow" product.

Gluconic acid: It is marketed usually as the calcium or sodium gluconate salt. The former one is employed as the pharmaceutical to supply calcium while calcium and sodium gluconates in alkaline solution are good metal sequestering agents particularly for iron, aluminium and copper. In addition, ferrous gluconate supplies iron for treatment of anaemia.

Several fungi including *Penicillium* and *Aspergillus niger* are employed in commercial production. The fermentative production of gluconic acid by *A. niger* is a one step fermentation of glucose to gluconic acid, where the aldehyde of glucose is oxidized to the carboxyl of gluconic acid by the enzyme glucose-oxidase.

Itaconic acid: This organic acid is frequently used for manufacture of plastics and synthetic detergents. Itaconic acid is produced commercially by *Aspergillus terreus* in submerged fermentation process at 30-35^{0}C.

Fumaric acid: Demand for fumaric acid in the manufacture of resins and for some wetting agents. This acid can be converted to maleic acid by heating in acid solutions and the demand for maleic acid is increasing for the manufacture of alkyd resins, unsaturated polyester coating compounds, resin adducts and plasticizers.

Most microorganisms produce small amounts of fumaric acid as an intermediate of the TCA cycle but a few microorganisms (like various members of Mucorales, particularly *Rhizopus nigricans*) accumulate this acid as an end product of metabolism.

Kojic acid: It is produced by fungi of the *Aspergillus flavus – oryzae* and *Aspergillus tamarii* groups in surface culture. High yield of Kojic acid occur during growth on a medium containing ammonium nitrate, glucose or sucrose or xylose.

Glutamic acid: Salt of monosodium glutamate is used as a flavour enhancing agent. It is formed from K-ketoglutaric acid by transamination or reducing amination by glutamic acid dehydrogenase. Usually *Saccharomyces, Aspergillus, Penicillium* and *Rhizopus* produce glutamic acid by reductive amination of K-ketoglutarate.

Similarly, Gallic acid, Ustilagic acid, Lactic acid etc. are also produced commercially by exploitation of fungi.

B. Food processing:

Use of fungi in the processing of food has reached its greatest diversity in Asia, especially in Japan, where different types of foods are produced.Some of the food production techniques have been practiced for centuries on a small scale in the home and continue to be utilized in the traditional fashion. Other foods are now produced on a large scale using modern techniques such as those previously described.

During food processing an edible food is deliberately inoculated with one or more microbes which then grow on food and favourably alter the flavour, texture and nutritional value of the original food and also enhance the storage capabilities. Food prepared worldwide with the direct involvement of yeasts or filamentous fungi include bread, cheese, etc.

Baker's yeast and Bread production: Breads, buns, pizza dough, rolls etc. are most widely consumed food processed with the aid of fungi that is strains of *Saccharomyces cerevisiae*, which ferment glucose to yield ethyl alcohol and carbondioxide. The baker takes advantage of that carbondioxide gas production to make his bread porous and of a light texture.

$$C_6H_{12}O_6 \xrightarrow{\text{Yeast enzyme}} 2\,C_2H_5OH + 2\,CO_2$$

Now-a-days, large scale production of baker's yeast begins with the selection of a suitable strain (which is able to produce abundant CO_2 quickly, viable during storage, performances related to flavour etc.). The selected strain of yeast then grown in the laboratory to make large volume of inoculum. The already built inoculum then added to a medium containing molasses and corn-steep liquor containing minerals like Mg, Zn, vitamins (such as Thiamine, pantothenic acid, biotin etc.), ammonia, phosphoric acid etc. pH of the medium during this is maintained at 4.4 to 4.6, incubated at 30^0C and aerated vigorously. At the end of incubation (12-20h) the yeast cells are removed from the fermented medium by centrifugation, washed and refrigerated. Further processing involves removal of excess water and the pressed yeast is then used to make specific yeast products for bakers, that is *compressed yeast cakes* (with 70% moisture and an emulsifier added to it), *crumbled yeast* (it lacks an emulsion), *active dry yeast* (obtained by drying compressed yeast and is stable in room temperature for long period), *instant dry yeast* (packed in an inert gas or vacuum). Of the four types of commercial yeasts instant dry yeast is advantageous because it can be added directly to the dry ingredients without being previously rehydrated while active dry yeast must be rehydrated before being added to the dry ingredients.

During preparation of dough in bakery a combination of yeast, water, flour, salt and possibly other ingredients (of trade secrete) is made. The mixture is then kneaded and allowed for fermentation. After fermentation CO_2 bubbles are trapped in the gluten (flour protein) network and produce large number of pores that make the bread rise. Immediately before baking the alcohol content of the bread is as high as 0.5% about $1/6^{th}$ of the ale. This alcohol is driven off during baking and give bread a pleasant aroma.

Cheese: Cheese is a food product made from milk which contains butter fat, lactose and proteins – albumin, casein and the water whey. Caesin will coagulate to form a curd when it is acted upon by an acid or by the enzymes pepsin and rennin. Fungi produce extracellular enzyme rennin which has become

increasingly important in cheese manufacturing. Fungus which is commercially important as a source of rennin is a plant pathogen *Cryphonectria parasitica*. The coagulated curd is the newly formed cheese that separate from a watery fluid, the whey. The cheese may be eaten in fresh form or may be further processed with some bacteria or fungi. Fungi are used to process cheese of 2 principal types -

(1) Camembert type and

(2) Roquefort type

During preparation of *Camembert cheese* the curds are placed in hoops and allowed to drain overnight and then turned over to drain evenly. After 2-3 days, each cheese is rolled in salt and sprinkled with spores of *Penicillium camemberti*, a white mold. The layer of salt extracts the whey from the curd and forms a shiny cover of brine, the surface of cheese becomes hardened and forms a rind. In this stage the cheese consists of a sour curd, surrounded by a salty rind, both of these inhibit growth of bacteria and the fungus *Geotrichum candidum*. The spores of *P. camemberti* germinate and form mycelium which penetrates the rind and finally produce spores on its surface. The proteolytic enzymes secreted by *P. camemberti* then make curd soft, buttery and develops a mild flavour. Acidity of the curd ultimately disappears and allows the establishment of *G. candidum* and bacteria again during later stages of ripening and accounts for its ammoniacal flavour.

During preparation of *Roquefort cheese*, milk is curdled first with rennin and curd is broken into small masses, drained to make it dry and heaped loosely. It is then inoculated with spores of *Penicillium roqueforti* (grown on bread). During its growth in the cheese the fungus secretes protein and fat – digesting enzymes which gives pungent flavour resulting from hydrolysis of fats and the liberation of high molecular weight fatty acids (Capric, Caproic, Caprylic) and accompanying methyl ketones and alcohols.

Tempe: It is a meat substitute made by Indonesian because it provides about 35% protein. Tempe is prepared from soybean with the help of *Rhizopus oligosporus*. The inoculated soybeans form a mass like a firm cake which is typically cut into thin strips, sun dried and then fried in deep fat. During fermentation crude protein (which is difficult to digest) are degraded and increase in quantity of readily available soluble nitrogen compounds and amino acids. There is also an increase in vitamins like

riboflavin, Vit. B_{12}, pyridoxin, niacin etc. Antioxidants are also produced, stabilizing the oils and improve nutritive value.

Shoyu: Shoyu or soya sauces are made from a mixture of whole soybeans or defatted soybean grit, wheat kernels, wheat flour or wheat bran in various ratio with the help of *Aspergillus* sp., (specially *A. oryzae & less commonly A. soyae*) which is responsible for production of proteases essential for digestion of soybean and wheat proteins. During incubation (10 weeks to 1 year) different microbes develop in succession but maturation begins with lactic acid bacteria *Pediococcus halophylus* (carry out lactic acid fermentation) and later yeast like *Saccharomyces rouxii* or species of *Torulopsis* (both of which carry out alcoholic fermentation).

Tofu and Sofu: Tofu (Japanese) and Sofu (Chinese) are cheese-like product produced from fermented soybeans. For this purpose *Mucor* sp. are used. Usually a paste of soybean is curdled by adding Ca or Mg salts. Pressed, curd blocks are incubated at 14^0C for one month.

C. Nutritional supplements:

Various fermentations products find commercial application for their use in animal and human nutrition. Several vitamins, enzymes, proteins, amino acids etc. are produced during their growth and metabolism of which some find their way into the commercial field.

Vitamin: Riboflavin (Vit.B_2) is essential for growth and reproduction of human as well as animals and thus used as a feed additive for nutrition. Vit. B_2 is produced commercially by direct fermentation utilizing members of Ascomycotina *Eremothecium ashbyii* and *Ashbya gossypii*. Except these *Candida guilliermondia* and *C. flareri* also produce sufficient amount of riboflavin but are not employed commercially.

Vitamin B produced by yeast also used as nutritional supplement.

Provitamin A that is 2–carotene occurs naturally as a component of various agricultural products and particularly as a components of green plants. It is also produced commercially by strains of

Phycomyces blackesleeanus, Choanephora cucurbitarum and *Blackeslea trispora* of which former one is widely used.

Enzymes: Molds and some other fungi synthesize and excrete large quantities of enzymes in their growth medium which is then concentrated and purified and sold in market. A list of industrially produced enzymes from fungal sources are given below-

Enzymes	Source(s)	Applications
Amylase	*Aspergillus oryzae, A. niger, Mucor* sp., *Rhizopus* sp.	Hydrolysis of starch, desizing textiles, syrup, alcohol, fermentation industry. Acid resistant amylase from *A. niger* is used as digestive acid.
Invertase	*Saccharomyces cerevisiae, Aspergillus* sp.	To increase sweetness in confections, yields soft center in chocolate-covered candies, to prevent crystallization of sugar.
Amyloglucosidase	*Rhizopus niveus, A. niger*	Used in glucose production.
Pectinase	*Coniothyrium diplodiella, A. oryzae, A. niger, A. flavus, Sclerotina libertina, Botrytis cinerea*	Increase yield and for clarifying fruit juice, removal of pectin, coffe concentration to decrease wine turbidity.
Proteases	*A. oryzae, A. niger, Mucor pusillus*	Flovouring of sake, haze removal in sake, feed and digestive aid, meat tenderizer, remove bitter flavour, replace rennin in cheese, reduces elasticity of glutin protein in bread production
Taka diastase	*A. oryzae*	Digestive aid, supplement to bread and syrup.
Glucose oxidase	*A. niger, P. chrysogenum*	For removal of oxygen or glucose from various food, dried egg manufacture, for glucose determination.
Laccase	*Coriolus versicolor*	Drying of lacquer
Lactase	*A. niger, A. oryzae*	Hydrolysis of lactose in milk to be used for lactose-intolerant individuals.
Rennet	*Mucor* sp., *Cryphonectria parasitica*	Cheese manufacturing
Lipase	*Rhizopus* sp.	Digestive use, flavouring of milk product.
Cellulose	*Trichoderma koningi*	Digestive aid.

Protein: Mass cultivation of yeasts offers a possible source of food supplements or substitute for human and animal consumption. Yeast cultivated for food purpose is known as *food yeast* while yeast produced chiefly to feed animals is called *fodder yeast*. These masses are used as sources of protein. Yeast can be cultivated on industrial wastes or by products to yield a large cell crop rich in protein. Sometimes name SCP (Single Cell Protein) is given to such a microbial products because of their richness in proteins. It has been estimated that 100 lbs. of yeast will produce 250 tons protein in 24 hrs. and as such massive production of cells may provide the way of bridging the "**protein-gap**" in our protein-hungry world.

Amino acid: Now-a-days there has been a rapid development in production of particular amino acids by fermentation. Microorganisms mainly can synthesize amino acids from inorganic nitrogen compounds. Fungi like *Saccharomyces, Aspergillus, Penicillium* and *Rhizopus* produce glutamic acid by reductive amination of K-ketoglutarate. Many commercial processes have now been developed for the production of lysine, threonine, methionine, tryptophan, alanine etc.

D. Alcohol and alcoholic beverages:

Ethanol is a good solvent used in several industrial processes and also as a fuel for automobiles. This alcohol can be derived from petroleum by chemical means or as an end product of the alcoholic fermentation carried out by yeasts. It can be produced by fermentation of any carbohydrate containing a fermentable sugar or a polysaccharide that can be hydrolysed to a fermentable sugar.

$$C_6H_{12}O_6 \xrightarrow[\text{Enzyme}]{\text{Yeast}} 2\,C_2H_5OH + 2\,CO_2$$

For fermentation purpose selected strain of *Saccharomyces cerevisiae* having high tolerance for alcohol are commonly employed.

Production of alcoholic beverages follow the same principle applied for the production of industrial alcohol. In beverage production refinements are introduced with respect to flavour, aroma and

sanitation that are not necessary in the making of industrial alcohol. However, the type of beverage produced is determined by the nature of the plant material involved for fermentation.

Beer: It is prepared from fermented grains mainly barley. The chief raw material is malt which is germinated barley that has been dried and ground. It contains starch, protein and high concentration of amylases and proteinases. Amylase derived from *Aspergillus oryzae* convert starch into fermentable sugars (maltose) and make the medium nutrient rich. Then the selected strain of *S. cerevisiae* and or *S. carlsbergensm* is added to such nutrient medium for vigorous fermentation. In some country *S. diastaticus* is also used which utilize starch to produce light beer but this yeast produces an unpalatable medicinal flavour

Whiskey: Whiskeys are made from fermented grains like corn, wheat, barley malt or rye malt etc. which are mixed in varying proportions according to the type of whiskey being produced and fermented by yeasts. In this case also amylase derived from *A. oryzae* is added to the medium to hydrolyze starch and then selected strain of yeast is added for fermentation.

Wine: Wine production begins with the pressing of the fruit and juice extraction. Pectic enzymes derived from *A. niger* are added routinely to degrade the pectin of middle lamella which help to increase yield of fruit juice and also help in increasing clarity of the finished wine. Fermentation is usually initiated by species with a low tolerance for alcohol like *Kloeckera apiculata, Hanseniaspora guillermondi, Torulopsis* sp., *Candida* sp., These yeasts are eventually replaced by more alcohol tolerant *Saccharomyces* yeasts. Commercial producers of wine however, utilize cultures of selected yeast strains of *S. cerevisiae* as well as *S. bayanus.*

E. Antibiotics and other drug production:

Production of antibiotics is one of the largest and most important among different microbiological industries. Antibiotics derived from fungi that have sufficient toxicity towards specific microbes to be clinically effective are the Penicillins, Cephalosporins, Griseofulvin and Fusidic acid etc. Fungi produce these compounds as *secondary metabolites.*

Antibiotic	Source	Antimicrobial activity
Penicillin	*Penicillium notatum, P. chrysogenum*	It is a oral parenteral antibiotics used against gram positive bacteria. Synthetic penicillins derived from it are however, also active against gram positive bacteria.
Cephalosporins	*Cephalosporium acremonium*	It is a oral and parenteral antibiotic used against both gram positive and gram negative bacteria.
Griseofulvin	*Penicillium griseofulvum, P. nigricans, P. urticae*	Oral and topical antibiotic used against fungal infections mainly against dermatophyte infections.
Fumagillin	*Aspergillus fumigatus*	It is a polyene antibiotic used to control amoebae and to some extent bacteriophage.
Fusidic acid	*Fusidium coccineum*	Active against gram positive bacteria.
Other Drugs		
Ergot alkaloids (ergometrin, ergotamine, ergotaminine etc.)	*Claviceps paspali, C. purpurea*	These alkaloids stimulate smooth muscles and selectively block sympathetic nervous system. Used in stimulating uterus to contract to initiate child birth, in treatment of certain peripheral circulating disorder and also in treatment of migraine headache.
L-Ephedrine	Yeast	Drug for treatment of asthma. Ingredient for nasal drops and inhalants.
Antihypercholesterolemic agents	*Cephalosporium caerulens, P. citrinum*	Used to lower cholesterol level.

F. Bioconversion of steroids:

Steroids are important complex organic compounds that have a common 4-membered ring structure.

The most striking examples of the use of microorganisms to carry out chemical transformation is that of the steroid transformations. Chemotherapeutically important steroids are now a days prepared commercially by multistep chemical and microbiological transformations of naturally available steroids. A list of steroid transformation are given below-

Fungi	Reaction	Substrate	Product
Rhizopus nigricans, R. stolonifer, R. arrhizus, Aspergillus sp.	11 K-hydroxylation	Progesterone	11 K-OH-Progesterone
Curvularia lunata, Cunninghamella blackesleeana	11 2-hydroxylation	Cortexolone	Hydrocortisone
Aspergillus sp., *Penicillium* sp.	Side chain degradation of ring D	Progesterone	4-Androsterone-3-17-dione
Aspergillus sp., *Penicillium* sp., *Gliocladium* sp.	Side chain degradation of ring D	Progesterone	Testolalactone
Fusarium solani	Nuclear double bond in ring A and side chain	Progesterone	1,4-Androstadiene 3-17-dione
Saccharomyces sp.	172 –hydroxylation	Androstenedione	Testosterone
Ophiobolus herpotrichus		Progesterone	Cortexolone

G. Plant Growth Factor:

The Gibberellins (GAs) are plant hormones that promote growth of plant by both cell enlargement and cell division. This growth promoter is produced by only one fungus *Gibberella fujikuroi*, a pathogenic fungus for rice seedlings and is the conidial or imperfect stage of *Fusarium moniliforme*. Gibberellin production is usually conducted in aerated submerged fermentation culture at 25^0C for a period of 2-3 days. A simple glucose-salt medium of acidic pH is used for maximum GA production.

H. Cultivation of Mushrooms:

A wide variety of mushrooms are cultivated commercially in Asia as well as United States and other Western countries. In all more than two dozens of mushrooms have been cultivated for edible purposes in different countries. The world production of all types of fleshy edible fungi is around 2 million tons.

Mushrooms provides higher protein than many vegetables and fruits and accounts for about 3-4% fresh weight basis. They are of intermediate type between that of vegetables and meat because it has lower quantities of some amino acids (like methionine and cysteine) than meat but higher concentrations of lysine and tryptophan than most plant proteins. Since they provide low carbohydrates, are used for attractive low-carbohydrate diet for diabetic patients. They are also excellent source of some vitamins A, D, E, K specially riboflavin and nicotinic acid, essential fatty acids- linoleic acid and minerals like phosphorus and iron.

In Asian countries jelly fungi for example- *Auricularia polytricha, Tremella fusiformis*, Shiitake mushroom *Lentinus edodes* are commonly cultivated. Besides these, *Agaricus bitorquis* (*edulis*), *Pholiota nemeko, Tricholoma matsutake, Stropharia rugosoannulata, Coprinus fimetarius, Agrocybe aegerita, Tuber melanospermum* are also cultivated in different countries.

In India paddy straw or Chinese mushroom *Volvariella volvacea, V. diplasia, V. esculenta*; Oyster or tropical mushroom like *Pleurotus sajor-caju, P. sapidus, P. ostreatus* etc.; button mushroom like *A. bisporus*; milky mushroom *Calocybe indica* are cultivated widely. Another possibly less well known mushroom, the morel mushroom-*Morchella hortensis* (a number of Ascomycotina) are also cultivated commercially.

MODEL QUESTIONS

Short answer type (5 marks)

1. Write short note on industrial use of fungi in organic acid production.
2. Write notes on importance of fungi in production of alcohol and alcoholic beverages.
3. Give an outline idea of useful activities of fungi.
4. Discuss the role of fungi as food and food producers.
5. Write an essay on the role of fungi in food processing.
6. Write about role of fungi in conversion of steroids.

Objective type (1 or 2 marks)

1. Name the fungus used for commercial production of citric acid.
2. Name the organisms used for production of cheese.
3. Which fungus is used for preparation of Tempe ?
4. Name the organism used for production of amylase.
5. Name two edible fungi cultivated in India.
6. What do you mean by SCP ?
7. From which fungus gibberellin is isolated ?

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BOTANY AND FORESTRY
Module No. -10
Part - I, Paper - I (2nd Half)
Bryophyta

CONTENTS

LESSON – 1

1.1 INTRODUCTION

The group Bryophyta stands between Thallophyta and Pteridophyta in Plant Kingdom. As the name indicates (Bryos = both, phyton = plant) members of this particular plant group are amphibious in nature because they essentially require water for successful completion of their life cycle inspite of their useful terrestrial habitat on swampy soils or rock surface. Of course, a few members are absolutely aquatic viz., *Riella* sp., *Riccia fluitans, Ricciocarus natans* and a few others prefer to grow in bog viz., *Sphagnum* sp., *Leucobryum glaucum, Drepanocladus fluitans, Calypogeria fissa* etc. Most bryophytes are autotrophic but a few are more or less saprophytic and grow upon organic matter especially rotten wood viz., *Buxbaumia aphylla, Cryptothallus mirabilis* etc. Morphologically the vegetative body is a small thalloid or leafy structure which rarely exceeds 5 inches. The thallose members grow prostrate on the ground and get attached to the substratum by delicate rhizoids. In higher bryophytes (mosses) the plant body is erect consisting of stem-like (cauloid) and leaf-like (phylloid) structures. It is attached to the substratum by branched multicellular rhizoids. The bryophytes are distinctly advanced over algae specifically because of (i) multicellular sex organs with an outer layer of sterile cells; (ii) permanent retention of the zygote within archegonium (the female sex organ), (iii) retention of embryonic sporophyte within the archegonium and (iv) parasitism of the sporophyte upon the gametophyte for at least early stages in development. And the major reasons for considering the bryophytes at a lower evolutionary status than pteridophytes are (i) absence of true root, stem and leaf in vegetative body, (ii) absence of xylem and phloem in conducting system, (iii) dependence of sporophyte on gametophyte and (iv) domination of hoploid gametophytic generation over sporophytic one in life cycle.

A distinct and sharply defined heteromorphism is observed in the life cycle of bryophytes, and the haploid and diploid phases follow one another in regular succession. The haploid gametophytic phase is the conspicuous one and perform the major functions in its cycle viz., photosynthesis, production of sex organs and some other propagating units for vegetative reproduction, absorption of water and minerals etc. All members exhibit oogamous mode of sexual reproduction. The male sex organ antheridium usually consists of a single layer of protective sterile jacket cells investing a mass of antherozoid mother cells, each of which gives rise to a single ciliated motile antherozoid. The female sex organ archegonium is a multicellular, more or less flask-shaped organ, the swollen basal portion of which is called venter and the slender elongated upper portion is called neck. Within neck, the axial row consists of a variable number of neck canal cells, and within venter a single ventral canal cell and a basal larger cell, the egg or oosphere are

present. The archegonium provides nourishment and protection to the egg and, after fertilization, to the developing embryo. Fertilization is effected in presence of external water. Immediately after fertilization the fertilized egg undergo quick successive division of produce a multicellular embryo which is retained within archegonium. It obtains its nourishment directly from the gametophyte to which it is organically attached.

The embryo, thus formed, subsequently develops into a mature sporophyte (the sporogonium) which still depends on the gametophyte for its subsistence, The sporophyte exhibit considerable differentiation in most of the members. Usually it consists of a bulbous foot, a projecting seta and a globose capsule. The foot absorbs nutrition; the seta proliferates quickly and conducts food to the capsule and also causes dispersal of spores; the capsule produces spores, alongwith some sterile cells, which upon germination gives rise to succeeding gametophytic generation.

LESSON – 2

2.1 Origin of bryophytes

The question of theorigin of bryophytes is a debatable one. Fossil records, unveiled so far, afford very little help as to the origin of bryophytes because of the fact that most of the fossil members are similar to the living forms and also because of limited fossil data. A comparative study of the Plant Kingdom reveals that the bryophytes are more complex algae but they are less developed than the pteridophytes. So, if it is taken for granted that the more complex forms developed from simpler ones, it may be assumed that the bryophytes originated out of algae and the pteridophytes developed out of bryophytes. But interestingly fossil pteridophytes were found to be existed during the Cambian period while there was no evidence of the presence of any bryophyte before the Devonian period. Thus, all the views about the origin of bryophytes must be based on comparative morphology and ontogeny of living forms and analogies with the living plants of other groups. There are two diametrically opposite schools of thought regarding the origin of bryophytes. The supporters of one school hold that the bryophytes have descended from pteridophytes (pteridophytean origin). The ardent supports of this view are Scott, Lang, Kidston, Haskall, Kashyap and others. The adherents of the second school believe in the aquatic ancestry of the bryophytes from algal stock (algal origin). Bowere, Cavers, Smith, Campbell, Fritsch and others are the eminent supporters of this school.

Pteridophytean origin of bryophytes: There are some evidences in the origin of bryophytes by degeneration of pteridophytes. According to Kidston and Lang (1971, 1921) the simple pteridophytes (Psilophytales) in which the sporophyte was a rootless, leafless, dichotomously branched shoot with terminal sporangia could

be compared with the bryophytes especially with Anthocerotales. The Psilophytales differ from the bryophytes in the physiological independence of the sporophyte and in presence of vascular tissues. There is a similarity between the sporangia of some genera of Phylophytales (Horneophyton, Sporogonites) with the capsule of the sporophytes of *Anthoceros, Sphagnum* and *Andreaea.*

Andrews (1963) observed that *Sporogonites exuberans* lack vascular supply and have a flat thallus bearing slender, upright axis showing a close resemblance with *Anthoceros sporophyte.*

Scott (1911) came to the conclusion of pteridophytean origin of bryophytes on the basis of similarity of stomata in the land plants, in the sporophyte of Anthoceros and on the neck of the apophyses of mosses. Kashyap (1919) opined that the liverworts originated from pteridophytes by reduction. He came to this conclusion on the basis of similarities of certain thalloid forms of *Anthoceros erectus* with the proghalli of *Lycopodium cernuum.* According to him the first step of simplification was a change from erect radial symmetry to a prostrate and dorsiventral habit. Such a stage is clearly found in the prothallus of Equisetum debile. He further reported that the liverworts have more close resemblance to *Equisetum* than any other groups of pteridophytes.

Haskell (1949) advanced the origin of bryophytes from the alga through the Psilophytales by progressive simplification. He stated, "The origin of the Bryophyta from an algal ancestral is open to question and they may represent a group originating from phylophytalean ancestry, following reduction due to their habitat." Christensen (1954) believes that bryophytes descended from pteridophytes and put forward three alternative sources : (i) from pteridophytes with leaves on the stem of the gametophyte as well as that of sporophyte, (ii) from leafless pteridophytes like members of Rhyniaceae, and (iii) from different types of pteridophytes, some leaf-bearing and others not, in a polyphyletic manner.

Proskauer (1960, 1961) suggested the origin of *Anthoceros* like bryophytes out of *Homeophyton* pointing out to the presence of nonlignified spiral thickenings in the cells of the outer layers of Dendroceros columella which is similar to the tapetum of Horneophyton.

Algal origin of bryophytes : It is the predominant view of the bryologists that the bryophytes arose out of algae. According to Lignier (1903), the hypothetical algal ancestry which gave to the group Bryophyta in one hand and Pteridophyta on the other is "Prohepatics" or Protobryophyta (Cavers and Smith). Bower (1908) assumed that the Archegoniatae originated from the aquatic ancestors inhabiting shallow fresh water or the higher levels between marine tedi-marks. In such forms, sexual reproduction was effected through the medium of external water but certain forms established themselves on land where excess water was only occasional occurrence. In such forms sexual reproduction would only be effected at times of rain. Thus, production of a new structure, the sporophyte, was initiated from zygote for safer propagation on land.

photosynthetic pigments in the assimilatory tissue. such as a metabolic product and cell-wall frequently consisting of cellulose. Thus. it seems probable that the nearest algal relative of bryophytes were the chlorophyceal. Although it is generally agreed that the bryophytes originated from algae, the manner in which and from which they have arisen is still a matter of speculation. Campbell (1940) stated that there is no form known at present where the female sex organ of an algae can certainly be compared with the archegonium of bryophytes, although the oogonia of *Chara* do it to some extent.

Strasburger (1943) remarked that the morphological comparison points to a connection between bryophytes and brown algae. The multicellular gametangia of brown algae may be regarded as homologous structure leading to the antheridia and archegonia of Archegoniate including bryophytes.

Fritsch (1916, 1945) opined that Chaetophorales contain all the requisites for the development of more complex types, and *parenchymatous* Phaeophyceae, in particular, can show what could be attained starting from such beginning. According to him the evolution of the archegoniate plants from algae may have followed the following course:

1. The development of heterotrichous habit which is well-established in Chaetophorales.
2. Development of paranchymatous structure in the initial up right filaments as in Fucales and Laminariales.
3. Establishment of apical growing point in the erect branches as in Trentepohlia and in some brown algae.
4. Establishment of dichotomous branching habit.
5. Disappearance of prostrate portion.
6. Differentiation of vascular system in aerial part as indicated in the trumpet hyphae of *Chord,* sieve tube-like elements in *Macrocystis* and in Scalariform thickenings of medullary cells of *Sargassum*.
7. Development of cuticularized surface layer.

According to Fritsch (1945) Bryopsida appears to be the only taxon of Archegoniatae, in which both the parts of heterotrichous algal ancestors have persisted in the gametophytic phase, the filamentous protonema being equivalent to the prostrate region and the leafy shoot equivalent to the erect portion. Allsopp and Mitra (1958) observed that the fully developed protonema of a number of Bryales possess marked hetrotrichous habit. Thus, the similarity with cfhaetophoraceae is considered to be even greater than Fritsch supposed.

Conclusion : From the above discussion and the views put forward by various workers in this field

a conclusion can be drawn that the actual mystery of bryophytean origin is still to be unveiled. Probably, a solution to this vexing question may be obtained only after further discovery of allaied fossil forms which could point out krnship with some living bryophytes and thus could establish a connecting link sufficient enough to trace the origin. However, it is not wise to be biased on a particular school of though at least at the present state of our knowledge and the data on this field available till date.

2.2 Primitive and advanced features of bryophytes:

The bryophytes occupy a position in between Thallophyta and Pteridophyta. It is accepted that the members are at an evolutionary higher level than algae and other thallophytean taxa and lower that of Pteridophyta. Thus, it is likely that the members will possess some characters which are of primitive nature, and alongwith these they also show some advanced characters which are encounted in pteridophytes. Of course, they have a number of features specific to its own group. A few ditinctly primitive and advanced features of bryophytes are enumerated below:

Primitive features:

1. Presence of filamentous plant body as found in moss protonema.
2. Undifferentiated thallus-like gametophyte as found in *Sphaerocarpus*.
3. Predominating phase in life cycle is gametophyte.
4. Retention of swimming habit of sperms indicating their algal ancestry.
5. Presence of very simple absorption system i.e. rhizoids and scales.
6. Very little tissue differentiation in sporophytes as found in *Riccia* where spore case or capsule represents the while sporophyte without any dehiscence mechanism.
7. Fertilization is effected in presence of water.

Advanced features:

1. Presence of leafy plant body with distinct morphogenesis showing stem-like, leaf-like and root-like structures as found in mosses.
2. Presence of stomata on sporophytes of *Anthoceros* and mosses.
3. Semiindependence of sporophytes upon gametophyte as noted in Anthoceros.
4. Appearance of root-like projections from the foot of the sporophytes of some members of *Anthoceros*.
5. Indeterminate growth of the sporophyte of *Anthoceros* by virtue of the action of meristematic zone in between foot and capsule.

6. Presence of hydrome and leptome cells as found in higher mosses (eg. *Polytrichum*) which are forerunners of xylem and phloem of higher plants.
7. Presence of leaf-trace in higher mosses.
8. Definite dehisence mechanism of true mosses by virtue of having peristome teeth.

2.3 Classification of Bryophyta

The group Bryophyta comprises of more than 24,000 species and 960 genera. Schimper (1879) gave Bryophyta the rank of a division while its name was first proposed by Braun in 1864.

Since then attempts have been made by many botanists to classify bryophytes in a convenient manner keeping in mind the resemblance of characters and possible evolutionary sequence of the taxa. Eichler in 1883 recognized two groups, Hepaticae and Musci in the division Bryophyta. In classical taxonomy, Hepaticae and Musci are considered as two classes under Bryophyta. Engler (1892) subdivided each of the two classes in the following three orders :

Bryophyta

Class 1. Hepaticae (Liverworts)	Class 2. Musci (Mosses)
Order 1. Marchantiales	Order 1. Sphagnales
Order 2. Jungermanniales	Order 2. Andreaeales
Order 3. Anthocerotales	Order 3. Bryales.

However, the anomalous position of Anthocerotales as an order of the class Hepaticae was pointed out by many Bryologists. Hence, subsequencely Howe (1899), Campbell (1918, 1940), Smith (1955), Takhtajan (1953) and others divided the Bryophyta into 3 classes:

Hepaticae, Anthocerotae and Musci Rothmailer (1951) suggested the following class taxa for the old ones : Hepaticopsida for Hepaticeae, Anthoceropsida for Anthocerotae and Bryopsida for Musci using the suffix-opsida. The new names have been recognised by the International code of Botanical Nomenclature. Proskauer (1957) suggested the name Anthocerotopsida for Anthoceropsida. Modern bryologists thus classify bryophytes as such :

Division : Bryophyta

Class I Hepaticopsida (=Hepaticae)

Class II Anthocerotopsida (=Anthocerotae)

Class III Bryopsida (=Musci)

The current trend of classification of the classes into orders and families is outlined here mentioned a representative member of each family/order.

Hepaticopsida

Order (5)

1. Sphaerocarpales
Families (2)
(i) Sphaerocarpaceae
(*Sphaerocarpus*)
(ii) Riellaceae
(*Riella*)

2. Marchantiales
Families (5)
(i) Ricciaceae
(*Riccia*)
(ii) Corsiniaceae
(*Corsinia*)
(iii) Targioniaceae
(*Targionia*)
(iv) Monocleaceae
(*Monoclea*)
(v) Marchantiaceae
(*Marchantia*)

3. Jungermanniales
Suborder I
Metzgerineae
(=Jungermanniales Anacrogynae)
Families (3)
(i) Aneuraceae
(*Aneura*)
(ii) Blyttiaceae
(*Blyttia*)
(iii) Codoniaceae
(*Blasia*)
Suborder – II
Jungermannineae
(=Jungermanniales Acrogyneae)
Families (8)
(i) Lophoziaceae
(*Lophozia*)
(ii) Cephaloziaceae
(*Cephalozia*)
(iii) Ptilidiaceae
(*Ptilidium*)
(iv) Scapaniaceae
(*Scapania*)
(v) Radulacea
(*Radula*)
(vi) Pleuroziaceae
(*Pleurozia*)
(vii) Porellaceae
(*Porella*)
(viii) Lejeuneaceae
(*Lejeunea*)

4. Calobryales
Family (1)
(i) Calobryaceae
(*Calobryum*)
(*Takakia*)

5. Takakiales
Family (1)
(i) Takakiaceae

Order 1. Anthocerotales

Families (2)

(i) Anthocerotaceae

(*Anthoceros*)

(ii) Notothylaceae

(*Notothylas*)

Bryopsida

Subclasses (3)

I) Sphagnidae
Order (I)
Sphagnales
Family (I)
Sphagnaceae
(*Sphagnum*)

II) Andreacidae
Order (I)
Andreaeales
Family (I)
Andreaeaceae
(*Andreaea*)

III) Bryidae
Section I
Nematodonteae
Orders (4)
(i) Tetraphidales
(*Tetraphis*)
(ii) Buxbaumiales
(*Buxbaumia*)
(iii) Polytrichales
(*Polythrichum*)
(iv) Dawsoniales
(*Dawsonia*)
Section II
Arthrodonteae
Orders (12)
(i) Archidiales
(*Archidium*)
(ii) Dicranales
(*Dicranum*)
(iii) Fissidentales
(*Fissidens*)
(iv) Syrrhopodontales
(*Syrrhopodon*)

(v) Pottiales
(*Pottia*)
(vi) Grimmiales
(*Grimmia*)
(vii) Encalyptales
(*Encalypta*)
(viii) Funariales
(*Funaria*)
(ix) Eubryales
(*Bryum*)
(x) Isobriyales
(*Erpodium*)
(xi) Hookeriales
(*Hookeria*)
(xii) Hypnobryales
(*Hypnum*)

LESSON – 3

3.1 General Characters of Calobryales:

The order includes two genera *Haplomitrium* and *Calobryum* and 13 species, the former genus is anacrogynous and the latter one is acrogynous in nature. The order is characterized as such ;

1. Cametophytic plant body is erect and leafy, leaves being arranged in three vertical rows and all are more or less alike.
2. Creeping rhizomatous portion devoid of leaves and rhizoids.
3. Anatomy of the stem shows thicker corticla cells and thinner central cells resembling some mosses.
4. Antheridia are borne in clusters at the apex of the stem but archegonium is borne singly. Members are hetrothallic.
5. Sporophyte is represented by a very short a cuminate foot, a long slender sota and an elongated capsule whose jacket layer is only one-cell in thickness except at the apex.
6. Capsule contains very elongated double spiral elaters and dehisces by a single longitudinal cleft.
7. The dominant chromosome numbers are 8 and 9 found in cells of gametophytes.

3.2 General Characters of Sphaerocarpales :

The order Sphaerocarpales is represented by two families - (i) Sphaerocarpaceae which includes two genera Sphaerocarpus and Geothallus, and (ii) Riellanceae with the only genus Riella. Sphaerocarpus includes 7 species (e.g.S. Caliciformis, S. Cristatus, S. donnelii) while Geothallus is monotypic. But the submerged aquatic genus Riella in constituted of 17 species (e.g.R. affinis, R. americanum, R. vishwannathai). The general characters fo this order are enumerated below.

1. Gametophyte body simply thallose, dorsiventral(Sphaerocarpus) or erect with thickened stemlike lateral axis bearing plate-like thin spirally twisted wings (Riella).
2. Scales are usually absent and rhizoids are of smooth-walled type.
3. Thallus without any tissue differentiation.
4. Presence of a globose or flask-shaped involucre around each of the sex organs.
5. Archegonial neck composed of 6 vertical rows of cells.
6. Sporophyte usually shows an insignificant foot, a slender seta and a very simple one- cell layered jacketed capsule.
7. The chromosome numbers of gametophytic cells are 8.

3.3 General characters of sphagnales :

The order includes the sole family sphegmaceae and the sole genus *Sphagnum* which includes 207 species of which 22 species are known from India. They grow in extensive masses on boggy or peatly soils and also as submerged aquaties in peatly pools. This order is characterized by the following distinctive features:

1. A simple, flat, plate-like thallose protonema is fixed to the substratum by multicellular rhizoids; the septa of the rhizoids are oblique.
2. The protonema produce a single gametophore, which is the adult or mature plant.
3. Leaves of gametophore devoid of midrib and usually composed of two markedly different types of cells - The narrow, living greend assimilatory cells and the large, colourless, dead capillary cells.
4. The antheridia and archegonia are always borne on special separate antheridial and archegonial branches.
5. The archegonia are always terminal in position (acrogynous) and occur in clusters.
6. The mature sporophyte is differentiated into an enlarged foot, a rudimentary constriction-like seta and a large globose capsule.

7. The capsule is not invested by calyptra.

8. A distinct stalked leafless structure, the pseudopodium, elevates the mature sporogonium at its apex.

9. Unlike other mosses, the sporogenours tissue of a sporophyte is formed from the amphethecium of capsule.

10. A broad/central columella is developed from the entire endothecium and it occupies the major part of the cavity of the capsule.

11. The hemispherial columella is arched by a semilunar spore sac which is capped annulus and operculum.

12. The capsule lacks peristome teeth and opens by the oporculum.

13. The chromosome number of Sphagnales is n = 19 + m bivalewts (or the polyloid n=38 + m bivalewts (m=diminutie chromosomes).

3.4 General Characters of Bryales:

This group includes aproximately 14,000 species grouped in about 675 genera and the members are commonly called true mosses. They are the most advanced broyophytes and are clearly distinguished from other Broyopsida in their usually filamentous protonema, absence of speudopodium and usual good development of seta. This gruoup is characterisex by the following features :

1. Plant body with prominent stem-like, leaf-like structure young leave at the apex of the stem are arranged in a definite manner.

2. The leaves or the gametophore usually have a distinet midrib,more than one cell in thickness.

3. Early growth of the embryo by means of a two-sided apical cell situated both at the anterior and posterior ends.

the columella.

5. The columella usuallypenetrates the cylindrical spore-bearing layer, and continues up to the apex of the capsule.
6. The spore sac is usually separated from the wall of the capsule by an intercellular space traversed by trabeculae.
7. The sporogonium is never elevated a: maturity on a pseudopodium but by a seta.
8. Presence of pecistome teeth around the mouth of the spore cavity and these structures ensure spore dispersal by instalments.
9. Opening of the capsule is effected by an operculum.
10. Calyptra is well developed.
11. Protonema, with a few exceptions (thallose in Tetraphidales and *Diphyscium*), is filamentous.
12. Haploid number of chromosomes vary from 5 to 66. Lowest number is reported from *Physcomitrium* (n = 3).

LESSON – 4

Experimental studies on bryophytes:

4.1 Spore germination

Spores of bryophytes are haploid propagating units which are produced within capsules of mature sporophytes by meiotic division of spore mother cells. Upon liberation from the capsules they fall on a substratum and start germinating in favourable environmental conditions viz., sufficient moisture, optimum temperature, optimum light intensity, minerals etc. Most of the spores are reported to remain viable upto certain period depending upon the genetic make-up of spore types and thereafter became nonviable, if germination is not stimulated within their life-span.

Whether protein synthesis is an essential prerequisite for the onset of spore germination is not still established. Application of protein synthesis inhibitor; cycloheximide strongly retards spore germination and prevents normal growth of germ tubes. If dormant spores are treated with an enzyme solution of β-glucuronidase or arylsulfatase and then planted on a medium containing cycloheximide they germinate earlier than the non-enzyme treated dormant spores (Kurz, 1976).

Factors affecting spore germination : The factors can be divided into two groups - extrinsic factors and intrinsic factors. The former ones include temperature, moisture, light, minerals etc and the letter ones include growth promoters, inhibitors, enzymes and above all genetic factor. The factors are discussed below:

(i) **Light** : It has been well-established that bryophytic spores generally do not germinate in dark and they are photosensitive in nature. Heald (1898) reported that *Marchantia* spore failed to germinate in dark and normal sporelings and thalli developed only at moderately high light intensity. For *Plagiochasma* and *Targionia* light of 1300 lux is optimum and at this level 100% germination occurred. In *Riccia crystallina* germ rhizoids develop in 2000 to 2500 lux, whereas in lower (50 to 500 lux) or higher (4000 lux) light levels no germ rhizoids are produced. In optimum light the percentage of spore germination is fairly high in many species.

Photoperiod is also known to affect spore germination in Hpaticopsida. In *Targionia* a light duration of more than 2 hours is essential for appreciable spore germination and with increase in light period percentage germination is substantially increased (Kaul, 1974).

Alongwith intensity and duration of light, quality of light was also reported to be significant factor for spore germination in some species. In general, moss spores grown in yellow light produce longer and thinner protonema than those grown in white light. Red light promotes spore germination in Plagiochesma intermediaum (Ahmad et al, 1977). Phytochrome also seems to be involved since spore germination of *Funaria hygrometrica* is enhanced by red light and its effect is reversed by far-red light (Bauer & Mohr).

(ii) **Temperature** : In *Cryptothallus mirabilis* spore germination is retarded at higher temperature (Benson-Evans, 1960). On the other hand, for 100% spore germination of *Sphaerocarpus donnellii* requires a long pre-treatment with relatively high temperature of 25°C (Stelner, 1964). However *Ricci crystallina* requires a fairly low temperature (8 – 15°C) for germination. Cold treatment shortens the dormancy period and also increases the percentage germination.

(iii) **Sugars** : Addition of certain organic substances, like sugar (glucose, fructose, maltose or sucrose) to distilled water or to the inorganic media stimulates spore germination in dark in some systems (Hoffman, 1964). In light of 100 and 30 lux, disaceharides such as sucrose, lactose and maltose and aldohexoses such as glucose, galactose and mannore are effective. Sucrose and maltose are more effective in *Funaria* as compared to glucose and fructose. Sucrose can in part replace the effect of light on spore germination.

(iv) **Minerals** : Very little is known about mineral requirement during spore germination (Inoul, 1960), Rashid (1970) observed that on N_2 deficient medium spores form globular cell masses. In *Asterella angusta, Targionia hypophylla* and *Plagiochasma intermedium*, ½ Knop's and ½ Hoagland's solutions prove better than their full strength (Kaul, 1974). Higher concentrations of $KHNO_3$ inobit spore germination in *Asterella*. In *Targionia* and *Plagiochasma* germination percentage is quite high in nutrient medium comprising KH_2PO_4, $CaSO_4$, $MgSO_4$ and KNO_3 (Kaul, 1974). In Marchantia

polymorpha Fe and $Ca(NO_3)_2$ induce germination but optional germination and growth have been observed with $Ca(NO_3)_2$, KNO_3 and $MgSO_4$ (Gemmrich, 1976).

(v) **Moisture** : The role of moisture or spore germination needs no introduction as without sufficient moisture the germ tubes never emerge. However, certain spore types can remain in dorrant state in absence of adequate moisture and when moisture is supplemented onset of germination commences till a gametophyte is formed. Hence mass scale germination of spores occur during monsoon months.

Intrinsic factors

(i) **Growth promoters** : IAA stimulates germination of mature and immature spores in some bryophytes (Kofler, 1959). (CA_3 promotes spore germination in some moss species like *Dicranum scoparium, D. undulatum*. NAA inhibits spore germination in *Marchantia polymorpha* (Rousseau, 1952). Gunther (1960) observed that IAA strongly increases the site of cellulose fibrils which affect the internal polarity in germinating spores. In *Cryptothalles mirabilis* application of IAA and 2, 4-D at 10 ppm results in enhanced spore germination with a slight increase in cell size in the sporelings. Light-induced spore germination is further stimulated in GA_3, IAA and kineten in *Entodon myurus*. IAA is found to be most effective followed by GA_3 and Kinetin (Sood, 1972).

(ii) **Growth inhibitors** : Inhibitors like abseissic acid retard spore germination in many species. Phendie inhibiters at supraoptimal concentrations temporarily suspend germination. The weedicide 2, 4-D at a low concentration stimulate germination but at high concentration beyond 500 ppm strongly retard germination.

(iii) **pH :** Inow (1960) studied the effect of different pH values (5.0, 5.6, 6.2, 6.8, 7.4, 8.0 and 8.8) on the spore germination of 5 liverworts. Spores of *Marchantia polymerpha* show 100% germination in pH ranging from 5.0 to 7.4, whereas in *Reboulia hemispherica* such a response is observed upto pH 6.8. At pH 8.0 spores do not germinate at all (Kaul, 1974). Thus, in general, pH beyond 7 is not favourable for spore germination in the investigated bryophytes.

(iv) **Gene :** Of all the factors, gene factor plays the prime role. In fact, specific genes trigger the onset of some enzyme action for effecting germination. Suppression of such genetic action by any means leads to non germination of spores in spite of all conditions favourable for germination.

Mode of spore germination in some bryophytean taxa :

In Sphaerocarpales a germ tube emerges either from the distal surface as in *Sphaerocarpus* and *Riella* or from the proximal surface as in *Geothalus*. The germ tube divides by a transverse wall to form a terminal cell and a basal cell. The terminal cell develops into filament of variable cells and the basal cell gives rise to the first rhizoid. Further divisions vary in different genera.

In the majority of Marchantiales a germ papilla appears after the rupture of spore coats. The germ cell usually divides to form a germ rhizoid and a germ tube. The germ elongates and divides to form a short filament of cells and from its terminal cell a quadrant is formed. Subsequently, a multi-celled plate of tissue is produced and its apical cell with four cutting faces is organized to yough thallus.

In Jungermannialls Hofmeister (1862) described three different mode of spore germination after rupture of spore walls : (i) formation of globose mass of cells, as in *Frullania* (ii) formation of a filament. as in Jungermannia, and (iii) formation of a disc, as in *Radula*. Subsequent development is highly variable in different members of Jungermanniales and Nehira (1966) recognized as many as 24 sporeling types, 17 of acrogynous and 7 of anacrogynous group.

In Anthocerotales, the spores rupture at the triradiate marks and in most species a germ tube emerges. As in Marchantiales, a multicellular protonema is formed at the apex of the germ tube, but further growth occurs by the activity of a marginal meristem instead of an apical cell. However, in *Megaceros* and some species of *Dendroceros* the spore upon germination develops into a globose, massive protonema but in some species of *Notothylas* germ tube is absent and a cell mass is directly formed from the spore.

In members of sphagnales, the spore coat ruptures and a protuberance is formed usually at the proximal side of the spore. It develops into a short germ-tube which gets transversely divided into two cells, the upper cell subsequently produces a 3 celled protonema.

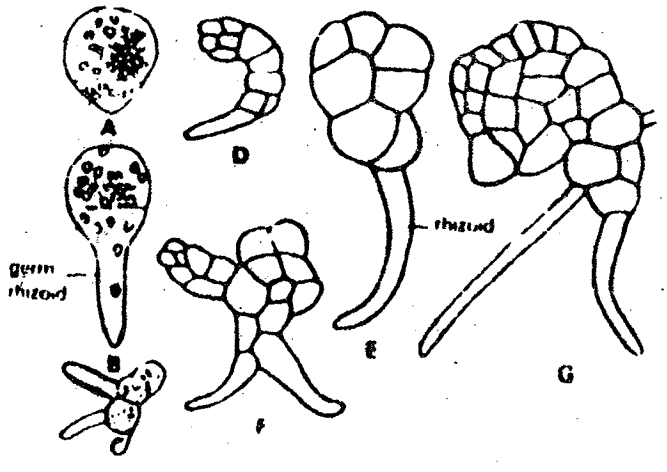

Fig.1. *A-G Stages in the germination of spore in Marchantia sp. A. First division of the spore. B. Germ-rhizoid formation. C-F Early stages of thallus development. G. A row of marginal cells makes its appearance towards the apex*
(A - E after Inoue 1960; F.G. After O'Hanlon, 1926).

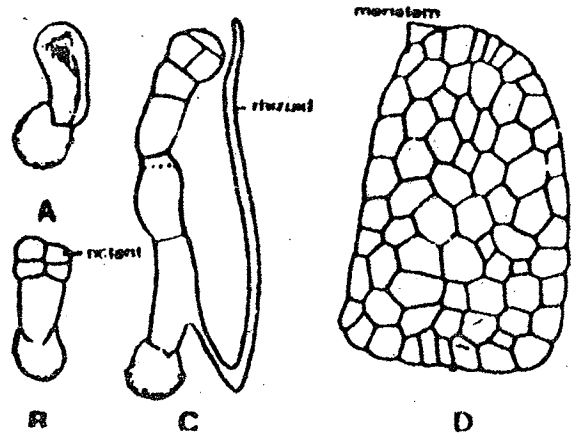

Fig.2 A-D. *Stages in the germination of spore in Anthoceros sp. A. Germtube formation. B. Octant stage. C. Rhizoid formation. D. Organisation of multicellular marginal meristem. (After Mehra & Kachroo, 1926).*

In Andreaeals the spore wall is much thicker showing a very significant exospore. In *Andreaea petrophila* and *A. faurici* the protonema is primarily developed inside the slightly enlarged exospore. After cell becomes several-celled, a protuberance is formed and this consequently develops into a filamentous or thallose protonema.

In Bryales, the spore on germination gives rise to an elongated structure which transversely divides into two cells. Subsequently, a filamentous branched protonema is formed. Here spore germination can be broadly classified under two heads : (i) development of photopositive chloronema and photonegative rhizoids, as in *Funarea hygrometrica* and (ii) development of only chloronemal filaments, as in Dicranella hetromalla

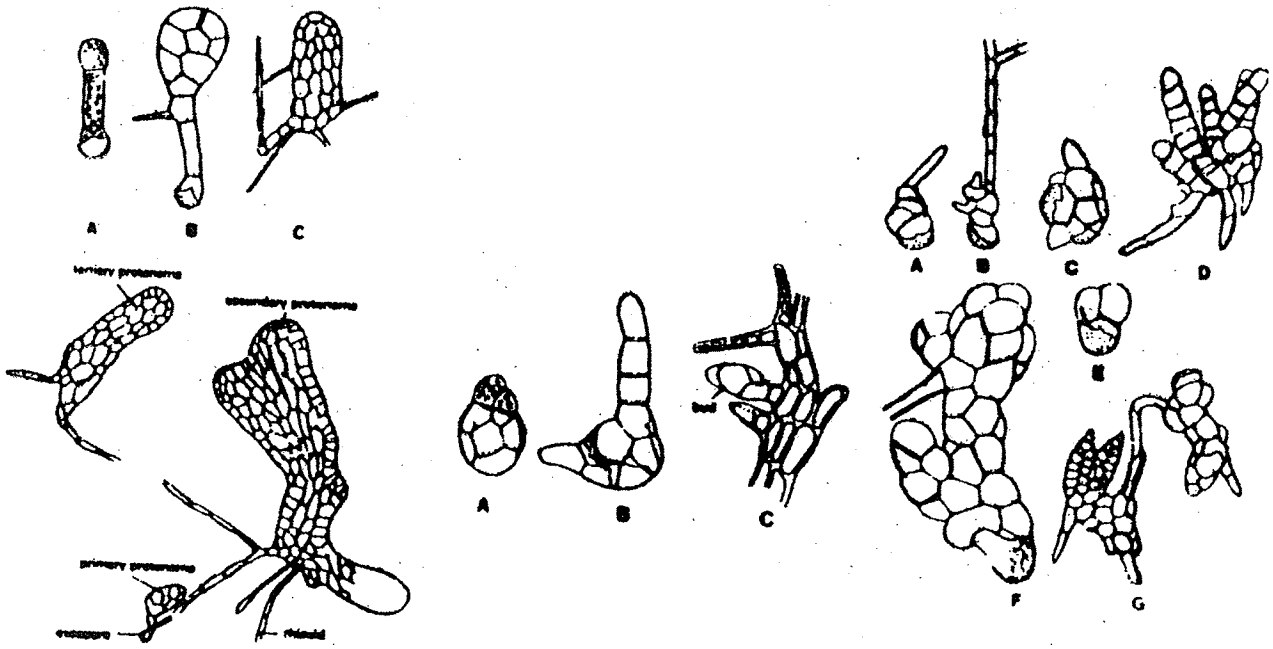

Fig. 3 A-D *Stages in the germination of spore in Sphagnum. A-C. S. imbricatum. A Spore germination, B.C. Thallose protonemata. D. S. girgensohnii. Formation of primary secondary, and tertiary protonemata. (A-C. After Nehira, 1963; D. After Noguchi, 1958).*

Fig. 4. A-C. *Stages in the germination of spore in Andreaea. A, B.A. fauriei, germinating spore and protonema. C.A. petrophila, protonema bearing a leafy bud. (A.B. After Nehira 1963; C. After Waldner 1887).*

Fig.5. A-G. *Sporeling types in Musci. A,B Encalypta rhaptocarpa. Earlier stages of protonema development. The initially formed massive exosporous protonema, comprising a few globose cells, gives rise to a filamentous protonema. C.D Ptychomitrium sinense. C. Spore germination. D. protonema. E.G. Hedwigia ciliata. Massive protonemata developed outside exospore. (A-B. After Nishida & Iwatsuk 1980; E.F. After Nehira 1983; C.D.G. After Nishida 1978).*

On spore germination, the first formed protonema is chloronema which gradually matures into caulonema, and bud primordia normally arise on such caulonemal filaments. These give rise to leafy shoots with rhizoidal systems. Bud induction is regulated by size of protonemal patch, temi of appearance of buds, morphology of gametophers etc.

Factors affecting bud formation :

(i) **Light :** Klebs (1893) emphasized that bud initiation is triggered in presence of adequate light intensity. Cultures of *Funaria* growing in weak light remain indefinitely bud free and attempts to induce buds in dark was found unsuccessful (Robins, 1918). Red light stimulates but formation in *funaria hygrometrica* (Jahn, 1964) and *Pohlia nutans* (Mitra et al., 1959) but blue light inhibits bud formation in both the mosses. A critical balance between red and blue light is required for bud development into leafy shoots. Red light followed by blue light is stimulatory whereas the reverse is inhibitory (Mitra et al., 1965).

(ii) **Temperature :** Buds are formed on protonema in a certain temperature range (12-30°C), and usually low temperature is inhibitory. In some systems but initiation is dependent on the interaction between light and temperature.

(iii) **Growth promoters :** Some growth promoters of auxin, cytokirin and Gibberellin class regulate bud formation. Auxin induced bud formation is concentration dependent. As low concentrations of some auxins are stimulatory but high concentrations are inhibitory. Of the different auxins tested IAA was found to be most effective (Sood and Hackenberg, 1979). Cytokinins are very effective on bud formation and other menphogenetic development in mosses and these are effective over wide range of concentrations. In several mosses kinetic interacts with IAA during bud formation. Gibberellins increase bud formation in some mosses like *Pholia nutans, Funaria hygrometrica, Bryum* sp., *Barbula* sp. etc. However, GA_3 in conjunction with IAA shows better response.

(iv) **Growth inhibitors :** ABA reduces the number of kinetin-induced bud formation (Valadon and Mummery, 1971). There are also reports that ABA accelerates bud formation in *Barbula* sp. and *Bryum* sp. in terms of number and time, the optimum concentration being 10^{-7} and 10^{-6}M.

(v) **pH :** It has been reported that in some species of *Barbula* and *Bryum* very low and very high pH values inhibit bud formation. However, slightly alkaline pH was found conducive for both protonema and bud formation.

(vi) **Sugars :** Sucrose and glucose at moderate concentrations (0.5 to 2%) hasten bud induction and increase their number in *Pohlia nutans* (Mitra and Allsop, 1959a). It has been found that in some mosses like *Bryum* sp., *Leptobryum* sp. and *Barbula* sp. absence of sucrose in the medium delays bud formation.

(vii) **Vitamins :** It has been reported in *Pylaisiella selwynii* that bud initiation is enhanced 3 to 20 fold by vitamin B_{12} at 10^{-5} or by B_{12} coenzymes at 10^{-4}M and the time of appearance of buds is reduced by 6 to 12 days as compared to control plants.

(viii) **Minerals and Chelates :** Macronutrients like K, Ca, Mg, N, P and S are essential for vegetative growth and they indirectly promote bud formation. Chelating agents increase the number of buds in some mosses which normally produce buds in cultures (eg. *Barbula gregaria, Buyum coronatum* etc.). In addition, buds are induced by Fe-EDDHA (Chopra and Rashid, 1969b), on by EDTA (Chopra and Sarla, 1985). The chelates also promote vegetative growth, and buds develop into normal shoots.

(ix) **Adenine and amino acids :** Adeninl increase the number of buds and reduces the time taken for bud induction in Tortella cacspitosa (Gorton and Eakin, 1957), *Bryum coronatum* and *Barbula gregaria* (Kumra, 1981). Among amino acids, lysopine and octopine are stimulatory for bud induction in *Pylaisiella selwynii* (Spicess et al., 1981). Arginine favours Vigorous development of buds and leafy shoots in some mosses (Burkholder, 1959).

4.3 Parthenogenesis

Parthenogenesis is a phenomenon in which a sporophyte is formed from unfertilized egg or ovum by apogamous development. In this case, the ploidy level is, thus, the some in egg and the sporophyte and this is usually a haploid one. This phenomenon is encountered in some members Archegoniatae including bryophytes as a rare event where sexual union of gamets is bypassed. However, the actual mechanism of this phenomenon has not yet been clearly established, although hormonal regulation of parthenogenetic process in many species has been reported.

Parthenogenesis occurs spontaneously in nature but its artificial induction by hormonal manipulative methods is also frequent in a wide range of plants including bryophytes of the different hormonal classes, GA_3 was found to be most effective for its successful induction. Some auxin type (e.g. IAA, IPA, NAA) and even cytokinins play significant role on parthenogenetic development. However, extensive exploration on bryophytic flora has not yet been made for parthenogenetic study, although individual action of a few growth promoters and combined action of a number of growth regulators are reported to be successful.

4.4 Apogany:

De Bary (1978) discovered that production of sporophyte is resulted directly from gametophyte without syngamy or sexual fusion. When apogamy occurs, the sporophyte may be derived from a singly cell of gametophyte, and this may be a vegetative one (Yamanouchi, 1908) or it may be one adjacent to or a component of the archegonium (Stokey, 1918).

Formation of apogamous sporophytes in mosses was first reported by Springer (1935) on the leaf

tips of natural occurring diploid gametophytes of *Phascum cuspidatum*, and it appears to be the only instance of apogamy in vivo. Under drying condition or increased salt concentration in the nutritive medium the leaf tips swell and these swellings developed into apogamous sporangia. Further drying or in reduced hydration of the medium by using 3% agar number of apogamous sporophytes are significantly increased per unit of protonemal patch in *Phascum* (Von Wettstein, 1942). Reports on apogamy in diplophase of mosses like Funarea hygrometrica (Bauer, 1959a), *Grimmia pulvinata* (Hughes, 1969) etc. and in haplophase of mosses like Bryum sp. (Bauer, 1967). Pottia intermedia (Lazarenko, 1963) are available.

Apogamy in bryophytes is regulated by exogenous and endogenous factors which are as follows :

Exogenous factors:

(i) **Light :** Apogamous sporophyte is induced in very low internity of light or in dark. Yellow filtered fluorescent light triggers apogamy in Phascum cuspidatum (Hyghes, 1969).

(ii) **Hydration :** Reduced hydration of the medium favours apogamy in mosses (e.g. *Phascum cuspidatum*).

(iii) **Sugars :** Sucrose and in some extent, glucose influences apogamy in some mosses.

(iv) **Chloral hydrate :** *Splachnum luteum* produces apogamous sporophytes spontaneously but their percentage is very low but when chloral hydrate is added the efficiency is increased upto 80%.

(v) **Growth hormones :** At low concentrations of GA_3, IAA, kinetin and a combination of kinetin and IAA appreciably increase apogamy.

(vi) **Inorganic nutrients :** There have variable influence on the induction of apogamous sporophytes. N_2 promotes apogamy in *Splachnum luteum*.

(vii) **Other growth factors :** Coconut milk stimulates apogamy from the callus of *Physcomitrium coorgense* (Lal, 1961 b) while yeast extract suppress apogamy.

Endogenous factors :

(i) **Sporogons :** Baucher suggested that a sporogon factor emanating from the diploid sporophyte is translocated into the aposporous protonema. The protonema obtained from the hybrid sporogonium (*P. pynformal* X *F. hygromotrica*) produced apogamous sporophytes.

(ii) **Genetic constitutions :** Chromosome number seems to play an important role in apogamy. There are no reports of apogamy in species with monoploid chromosome number and polyploidization is an important factor in apogamy.

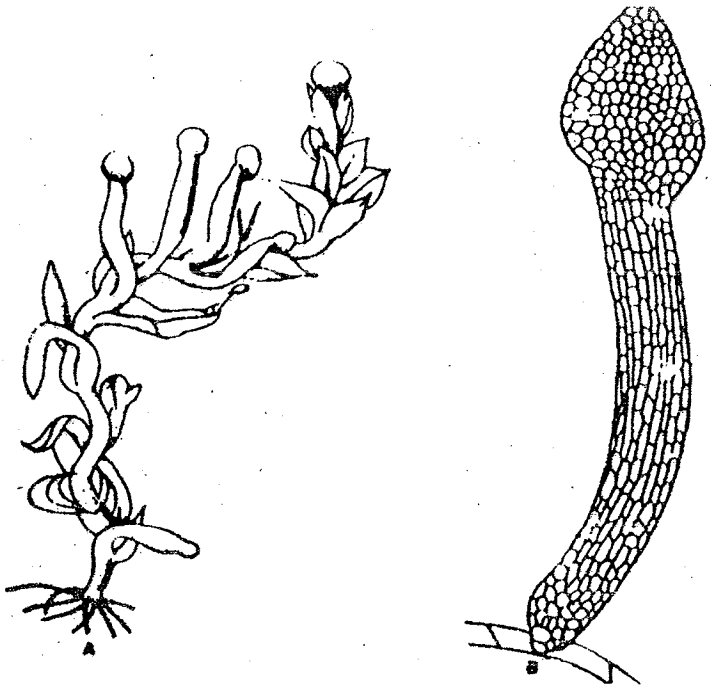

Fig.6. A.B. *Apogamy in diplophase in Desmatodon. A Gametophyte of D. ucrainicus bearing several apogamous sporophytes. B. An enlarged apogamous sporocyte of D. randii (After Lazarenko, 1960).*

4.5 Apospory

Production of gametophyte directly from a sporophyte without spore formation is called apospory. Since the discovery of apospory in Hypnum serpens (Pringsheim, 1876) and Ceratodon purpureus (Stahl, 1876), development of diploid gametophytes from vegetative tissues of sporophyte (i.e. capsule wall, seta etc.) has been demonstrated in some other mosses.

The technique of seta regeneration has been employed to obtain diploid gametophytes for genetic studies in Funarea hygrometrica; aposporous regeneration of gametophyte from capsular wall has also been reported in *Physcomitrium cyathicarpum*.

There are not many reports of apospory in liverworts. Aposporous outgrowths were observed in *Anthoceros* (Rink, 1935) but majority failed to develop into gametophytes. *Marchantia polymorpha* and *Blasia pusilla* also developed diploid thalli aposporously (Burgeff, 1943). Degeneration of tissue is a prerequisite for apospory in *Blasia purilla*. The aposporous plants are all female and they become fertile. Apospory has also been reported in *Pellia epiphylla* and *Pallavicinia lyellii* and some other members of Jungermanniales. Many aposporously produced gametophytes bear antheridia and archegonia that are of normal structure and with functional gametes.

LESSON – 5

5.1 Bryoecology

Ecological study of bryophytes is much interesting because majority of them are highly affected by varied climatic, edaphic and biotic factors, and some members exhibit strong adaptability towards unfavourable environmental conditions. *Hypnum cupressiformae* can withstand pH values 4.6 to 8.6 in the substrate and can grow on varied substratum like wood, stone or soil. *Semibarbula orientalis, Hypophila involucrate, Bryum coronatumi, Tortella* etc. grow on walls, bricks or stone containing some amount of lime and hence they are called calcicolous bryophytes. On the other hand, *Polytrichum, Pogonatum* and *Sphagnum* prefer to grow on acidic soil and hence are called acidophilous plants.*Pogonatum aloides, Funaria hygrometrica, Brachymenium acuminatum, Bryum ghatense* grow on laterite soil and show xerophyte adaptation. The prime ecological factors affecting bryophytean flora are as follows:

Water : Water is a vital criterion for successful completion of life cycle of bryophytes and hence they are amphibians in plant kingdom. Without sufficient water their sexual reproduction is not effected. Many desiccation withstanding members of bryophytes can fully revive and effectively perform sexual reproduction even after extreme drying by a shower of rain water.

Moreover, some members are aquatic and thus grow only in water medium either in floating (e.g. *Riccia fuitans, Riccio carpus natans, Fissidens grandifrons*) on in submerged (e.g. *Riella affinis, R. americana*) condition. *Sphagnum* is aquatic on peat bogs.

Temperature : Many bryophytes are sensitive towards extreme temperatures particularly at higher range beyond 35°C and luxuriant growth is effected only in presence of an optimum temperature levels. As bryophytes prefer shady places their temperature requirement usually ranges from 10 to 25°C. Although some bryophytes can withstand both high and low temperatures, their sexual reproduction is seriously impaired in such extreme temperature levels. In many species spore germination and gametangial induction are adversely affected at supra optimal temperatures specific for a member. As for example in *Riccia crystallina* requires fairly low temperature (8 – 15°C) for spore germination.

Light : In most bryophytes diffuse light favours luxuriant growth and hence they prefer situations of shade with partial exposure of light. In fact, sporophytic development is stimulated when they receive moderate light intensity. Some shade loving species like fissidens nobilis require light for their reproductive growth. However, light is essentially required for spore germination and sporeling formation in many species. In *Plagiochasma* and *Targionia* light of 1300 lux is found optimum and at this level 100% spore germination occurs. In *Riccia crystallina* germ rhizoids develop in 2000 to 2500 lux.

Edaphic factors : There are mainly the conditions of the substrate for which most species have their individual likings. While pH value, chemical and physical constitution of the substrate must be a guiding

principle, it is found that the species are adopted to the particular substrate – terrestrial (on humus, argillaceous or arenaceous soil), rupestrine (it may differ in being silicic, calciferous, granitic etc.) or epixylic. *Sphagnum* grows on bog and plants are interwined to form a substratum called quacking bogs on which their growth is luxuriant. Some Lejeunoid hepateis prefer to grow on leaves of forest plants; some members even grow on sand (e.g. *Fissidens splachnobrycides*), called psammophytic species.

5.2 Bryophytes as pollution indicators and monitoring

Bryophytes can play a significant role as indicators of environmental pollution (Le Blance, 1975). These plants either independently or together with lichens can be used in developing an index of atmospheric purity (IAP) which is based on the number, frequency coverage and resistance factor of species, and this index can provide a fair picture of the long-range effects of pollution in a given area (Rao, 1982). Panda (1978) suggested the use of bryophytes as 'Bryo-meters' – instruments for measuring phytotoxic air pollutants.

Basing on sensitivity of bryophytes towards pollutants they are of two types :

(1) Members which are very sensitive to pollution and show visible symptoms of injury even in presence of minute quantities of pollutants. Such types, therefore, serve as good bioindicators of the degree of pollution and also of the nature of pollutants.

(2) Members which have the capacity to absorb and retain pollutants in quantities much higher than those absorbed by other plant groups growing in the same habitat. Both the types are now-a-days being successfully used as pollution indicators. In the first instance, the nature and degree of pollution-induced injury directly proves the qualitative and quantitative aspects of pollutants. In the second instance, the differential tolerance level of plants against pollutants (say heavy metals) indicates the presence of a pollutant type. Many heavy metals like lead, codmium zinc, mercury, arsenic, chromium etc. are either highly toxic for survival of many bryophytes or some bryophytes are tolerant against these metals. Very little is known about the maximum tolerance levels of bryophytes for individual metals and combination of cations. Many mosses are reported to have a tendency for the accumulation of metals like iron, zinc, lead, nickel from the substrate and atmosphere. Certain mosses like *Weissia sp. Grimmia sp.* and *Rhacomitrium* sp. can successfully grow on soil containing excessive amounts of nickel (4000 ppm) and chromatium (900 ppm) (Shacklettle, 1965b0.

Some species of mosses serve as indicators of high copper concentration in the substrate and are known as 'copper mosses'. The important copper mosses are : (i) *Mielichhoferia elongata* (ii) *M. macrocarpa* (iii) *Merceya ligulata* (iv) *Dryptoion stratus*. Liverworts growing on copper ors are : (i) *Cephaloziella phyllacantha*, (ii) *C. massalongi* (iii) *Gymnocolea acutiloba* and (iv) *G. inflata*. Warnncke (1968) reported

that *Marchantia alpestris* is restricted to copper rich areas in Scandinavia. This, abundance of such members indicates copper pollution and also indicator of copper ores.

Peat mosses have the ability to accumulate heavy metals and serve as indirect indices of air pollution species of *sphagnum* are now being used by most workers in the 'moss bag technique' for measuring the level of heavy metal pollution (Brown, 1984). *Atrichum undulatum* is highly sensitive to air pollution and proves as good bio-indicator.

Brvo-monitoring of pollution is an important field of modern research where bryophyte-induced control or lowering of pollution is dealt. Although very little success is achieved, but some bryophytean species are reported to absorb and retain pollutants and consequently purity atmosphere. Some bryophytes trap and prevent recycling of many pollutants in the ecosystem for different periods of time. It has been reported that in *Grimmia doniana* lead is ionically bound to the cell wall, thus preventing toxic amounts of lead from penetrating the cytoplasm (Brown and Bates, 1972). In *Dicranella varia* lead and zinc are excreted in the form of a power of the metal sulphates from the leaf tips during summer drought. Many mosses can take-up as well as absorb cadmium from the substrate (Chopra and Kumra, 1991). Mosses and lichens tolerate high concentrations of mercury which is collected through rain water or dust particles.

However, the profusely branched and ramifying pleurocarpous as well as densely packed acrocarpous forms are generally more efficient absorbers and entrappers of meal particles than the unbranched and erect acrocarpous forms.

Thus, conclusion is made that bryophytes are reliable indicators of pollution either alone or with some lichens. And monitoring of pollution is also likely to be performed by some mosses which effectively absorb and retain varied pollutants within their body.

5.3 Economic importance of bryophytes:

There are some indirect economic importance of bryophytes. As far as the direct economic importance is concerned, they are of little economic importance except *Sphagnum* which are by far the most important. The importance is enumerated below with special reference to *Sphagnum*:

1. Some mosses provide food for herbivorous mammals, birds and other animals.
2. Mosses usually form dense, extensive mats on the soils and thus prevent soil erosion.
3. Some bryophytes, particularly mosses, absorb and retain pollutants and thus help purifying atmosphere.
4. *Sphagnum* is used as an absorbent in surgical dressings by virtue of its remarkable power to absorb and hold water like a sponge. *S. magenllanieum* is able to retain 24.5 time its dry weight of water.
5. *Sphagnum* is used extensively in seed beds and green house to root cuttings, to hold moisture in dry

soil, to pack bulbs. Cuttings and seedlings for shipment. Flowers packed in moistened *sphagnum* keep fresh for a considerable period.

6. High soil acidity as required by certain plants is effectively maintained by *sphagnum*.
7. *Sphagnum* is also used as stable litter and bedding.
8. *Sphagnum* has some antiseptic property in addition to their superior absorptive power. On account of these, it was used in place of cotton in the Russo-Japanese war and World War I for surgical dressing in hospitals.
9. The peat is used for fuel. It has about half the heating power of good coal and more than twice the heat of wood.

5.4 Xerophilous bryophytes:

Bryophytes are generally poikilohydric i.e. they become dormant in the dry season by losing most of their water. However, they respond to dry environments by developing physiological tolerance to desicceation, a phenomenon called anhydrobiosis (Keilin, 1959).

Xerophilous bryophytes are usually morphologically, anatomically and physiologically adopted for drought tolerance. Some drought enduring species grow in exposed situations such as soil, fence posts, rocks, old stone buildings (*Tortula muralis*), tree trunks (*Hypnum cupressiformae*) and in dry heaths (*Polytrichum junipericum*). *Tortula desertorum* has been reported from the deserts of Transcapsia. Among hepaties, some species of *Plagiochasma, Asterella* and *Targionia* can stand desiccation and are found on the bore rocks with limited rainfall.

In drought tolerant mosses, mRNA is appreciably conserved during drying and polyribosome are reformed on rehydration without the need for new mRNA synthsis. Thus protein synthesis can begin immediately so that repair (Dhindsa and Bewley, 1978).

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Exercises

1. What are the theories on the origin of bryophytes. Write a note on the origin of bryo- tes and put forward your own opinion on this question.
2. What is Spharoriccia? Enumerate the primitive and advanced characters of Bryophyta mentioning the genera where such characters are encountered.
3. How Hepaticopsida is classified. Write down the general characters of Calobryales and name the genera under this order.
4. What Hepaticopsida is classified. Write down the general characters of Calobryales and nam the genera under this order.
5. What are the important factors affecting spore germination in bryophytes? Write a short note on the mode of spore germination in bryophytean taxa:

Write notes on :

(a) Xerophyllous bryophytes

(b) Apospory in bryophytes

(c) Economic importance of bryophytes.

BOTANY AND FORESTRY

Module No. - 11

Part - I, Paper - I (2nd Half)

Gymnosperm

CONTENTS

RECENT TRENDS IN THE CLASSIFICATION OF GYMNOSPERMS IN INDIA.

The Gymnosperms comprise an important group of plants. They arose in the Paleozoic, dominated the world during the Mesozoic, the age of the dinosaurs and the earlier members of the group have become extinct today. Although the Angiosperms form the dominant vegetation today, the gymnosperms are not less important as they form a wide beet of forests in the North Temperate to Subarctic regions. Robert Brown in 1827 first of all recognized the Gymnosperms. Since then attempts have been made by the different workers from time to time to classify the Gymnosperms in different ways.

As early as 1827 Bentham & Hooker placed them in between the Dicotyledons and the Monocotyledons. Bessey (1911) divided the Spermatophytes into three phyla (division) parallel with his three division of pteridophyta. They are *Cycadophyta, Strobilophyta* and *Anthophyta*. Engler in 1936 classified the Gymnosperms in more details.

In Indal Sahni (1920) classified the Gymnosperms in detail in the following manner :-

Gymnosperms

A. **Phyllospermae**

- (i) Pteridospermae
- (ii) Cycadales
- (iii) Bennettitales

B. **Stachospermae**

- (i) Cordaitales
- (ii) Ginkgoales
- (iii) Coniferales
- (iv) Taxales
- (v) Gnetales

The important feature of Sahni's classification is that the laid stress on the origin of ovules (axial or foliar). Sahni's classification of Gymnosperms is to some extent modified from that of Coulter and Chamberlain (1917) who directly divided the gymnosperms into orders.

After Sahni's classification in India we get another classification of Gymnosperms in detail by Pant (1957). The author's classification of Gymnosperms is given below.

Division 1.	Cycadophyta
Class 1	Pteridospermopsida
Orders	Lyginopteridales
	Medullosales
	Glossopteridales
	Peltaspermiales
	Corystospermiales
	Caytoniales
Class 2.	Cycadopsida
Order	Cycadales
Class 3	Pentoxylopsida
Order	Pentoxylales
Class 4	Cycadeoidiopsida
Order	Cycadeoidales
Division2	Chlamydospermophyta
Class 1	Gnetopsida
Orders	Gnetales
	Welwitschiales

Divison 3	Coniferophyta
Class 1	Coniferopsida
Orders	Cordaitales
	Coniferales
	Ginkgoales
Class 2	Ephedropsida
Order	Ephedrales
Class 3	Czekanowskiopsida
Order	Czekanowskiales
Class 4	Taxopsida
Order	Taxales.

The above classification of Pant indicates a tendency to divide the Gymnosperms into three groups, *Cycadophyes, Coniferophytes* and *Chlamydospermophyts* instead of two major groups like *Cycadopytes* and *Coniferophytes*. In fact, Pant's classification was a modification of Arnol's classification where the author (1948) for the first time brought in evidences from fossil records into his system of classification. Sahni and Chamberlain have considered the Gymnosperms as two independent lines of evolution. But. modern concepts of phylogeny show the difficulty in considering only two separate cycadophytic and coniferophytic phyletic groups.

Pant's classification of Gymnosperms was followed by Andrews (1961) who divided gymnosperms into three divisions. Thereafter we get classification of gymnosperms by Sprone (1974), Stewart (1983), Meyen (1984), Gifford & Foster (1989), Kramer & Green (1990) etc.

In summary, it can be said the different classifications of gymnosperms which are in vogue, differ widely in their contents and placement of various orders & families. However, with the progress of scientific knowledge and accumulation of valuable data about the Gymnosperms we are approaching towards the better type of classification of the gymnosperms based on modern concepts of phylogeny where new information has accumulated through the application of modern tools and techniques such as electron microscopy, microspectrofluorometry and computer reconstruction.

Recently, Bhatnagar and Moitra in their book, Gymnosperms have proposed a revised plan a classification of gymnosperms. The plan in their book is as follows :

Gymnosperms

I Class : Progymnospermopsida

Order : Anewrophytales

Family : Aneurophytaceae

Order : Archaeopteridales

Family : Archaepteridaceae

Order : Protopityales

Family : Protopityaceae

II Class

Order : Pteriodspermales

Families : Calamopityaceae, Lyginopteridaceae, Medullosaceae, Callistophytaceae

Order : Cycadales

Family : Cycadaceae

Order : Cycadeoidales (=Benettitales)

Families : Willamsoniaceae, Wielandiellaceae, Cycadeodiceae

Order : Pentoxylales

Family : Pentoxylaceae

III Class : Coniferopsida

Order : Ginkgoales

Family : Ginkgoaceae

Order : Czekanowskiales

Family : Czekanowskiaceae

Order : Cordiatales

	Family	:	Cordaitaceae
Order		:	Volziales
	Family	:	Volziaceae
Order		:	Coniferales
	Families	:	Pinaceae, Taxodiaceae, Cupressaceae, Podocarpaceae, Araucariaceae, Cephalotaxaceae, Taxaceae.
IV Class		:	Gnetopsida
Order		:	Ephedrales
	Family	:	Ephedraceae
Order		:	Gnetales
	Family	:	Gnetaceae
Order		:	Welwitschales
	Family	:	Welwitschaceae.

CYCADALES

The cycadales are the most primitive of cycadopsida. The living cycads form a small natural group comprising only nine genera and about one hundred species, all of which are tropical or subtropical. A complete idea about the order is obtained only by comparing the living cycads.

The cycads are distinguished from other living gymnosperms and the Corditales by the unbranched stem, with a terminal rosette of comparatively few, large, branched leaves, which give rise to columnar habit of the tree ferns or palms.

The Vegetative Organs

The stems are columnar or sometimes tuberous. *Macrozamia* hopci is the tallest, reaching a height of twenty meters. In the tuberous forms the stem is either subterranean or appears more or less above the surface. All stems are usually unbranched but branched individuals are not rare.

The striking feature of the cycad trunk is the investing armor of leaf bases. From the number of leaf bases, the average number of leaves in a crown, and the duration of the crown, the age of a plant can be determined. In the smaller tuberous forms and in some species of cycas, the armor is not so persistent, and may be seen for only a short distance below the crown.

The crown of large leaves at the apex of the stem gives the cycad its palmlike or fernlike appearance. In the seedling time the leaves appear singly at irregular interrvals, the production of a crown of leaves at one time being a feature of the later history of the individual. The leaves are unipinnate in all genera except Bowenia where they are bipinnate. The venation is various, with or without a midrib.

Circinate vernation, a fernlike feature is a common in many genera. The leaflets are tough and leathery. The first leaves of the seedling are always scale leaves, one or more of which form a protection for the first foliage leaf.

The primary root usually continaes as a taproot. The tap root bears numerous small secondary roots. The soil about the root is full of algal & fungal flora. Some of the secondary roots become infected and become negatively geotropic. Near the surface of the ground such roots are dichotomously branched, forming the so-called "root tubercles". Which occur in coralloid masses just above or little below the surface. There are lenticels on the surface of these roots which get infected by bacteria. The bacterial growth is followed by incursion of the alga Anabena which multiply rapidly forming a blue green zone lying between the vascular cylinder and outer cortex.

ANATOMY

A transverse section of the stem of a cycad shows a large pith, a thin vascular cylinder of collateral endarch bundles and a very thick cortex having numerous conspicuous girdles, the leaf traces. The cortex and the pith show numerous mucilage canals. The primary cambium is short-lived or persistent, as in Dioon. There are no annual rings. When the cambium is short lived, a succession of secondary cambiums in the cortex produce cortical cylinders. Protoxylem of seedlings shows spiral tracheids and those of older plants scalariform tracheids. Secondary xylem shows rows of multiseriate bordered pitted tracheids with rows of thin walled cells in between. A pecularity of cycas, an allied genera is the girdling of leaf traces. A leaf trace formed of scalariform tracheids in the xylem, after arising from the stelar cylinder, usually does not pass into the nearest leaf directly but turns a semicircle or 'girdles' the stem before passing to the next higher leaf.

The leaflets are tough and leathery due to thick-walled hypodermal cells. The epidermis is strongly cutinised, as in conifers. Except Bowenia, stomata are on the under surface and are deeply sunken. In most forms the mesophyll is differentiated into palisade and spongy parenchyma. In some cases, as in cycas

colorless cells, elongated parallel with leaf surface lie between the palisade and the spongy parenchyma. The vascular bundle of the median vein has a mesarch xylem on the top with a full metaxylem above and metaxylem patches below the protoxylem. The phloem is placed below the xylem. Thebundle is encircled by a sclerenchymatous sheath.

The roots in cycads are usually tetrach. Secondary growth takes place but secondary xylem formation is irregular.

REPRODUCTIVE ORGANS

The spore-producing members :

The Microsporangium

In fact, all the living cycads are dioecious. The staminate strobili are formed and usually occur singly in the centre of the crown. The length of the cone varies from 2 c.m. in some species of 2 amia to more than 50 c.m. as in the case of Encephalartos and cycas. The sporophylls are arranged spirally in a compact strobilus. The sporophylls are sterile at the apex and base of the cone and the remaining sporoplylls bearabaxial sporangia in two more or less distinct groups. In all the genera sporangia occur in sori-Each microsporangium is unilocular and develops in the eusporangiate way.

There is a multilayered jacket with thickened epidermis. Next to it tapetum encloses micropore mother cells which on reduction division give rise micropore or pollen grains. At maturity the sporangium splits by a slit at the top.

The Megasporangium

In all the genera except cycas the megasporophylls are in compact strobili. The first strobilas is terminal upon the primary axis but all others terminate secondary axes, except in *Encephalartos, Macrozamia* and *Bowenia,* where all strobili are axillary. In cycas the sporophylls bear resemblance to the foliage leaves. This condition resembles that of the cycadofilicales. In *Cycas revoluta* the megasporophyls are like the vegetative leaves. *C.circinalis* and *C. normanbyana* show more redution. In cycas revoluta several ovules are borne on the margins of the sporophyll. The number of ovules becomes reduced to two, asin *C.normanbyana* and *C. siamensis.* The two ovules are characteristic of all the other genera. The size, color and surface of the ovules differ in the various genera. The largest ovule is found in *cycas circinalis.*

The megasporangium or the ovule is orthotropous and becomes considerably large even before fertilization. There is a single integument which is free at the top from the nucleus. The integument is three layered. The middle layer is stony, the soft outer layer is soft and pulpy. The soft inner layer is used up and becomes papery before maturity. The vascular supply from the base of the ovule divides into three strands the middle one of which supplies the base of the sporangium and the two side ones pass to the integument. A single megaspore mother cell within the nucellus undergoes reduction division forming a linear tetred of megaspores. Usually the lowest one becomes function and she others degene rates.

The Gametophytes

The Female Gametophyte:

The megaspore is the first cell of the female gametophyte. It germinates immediately, a prolonged period of free nuclear division being accompanied by much increase in the size of embryo sac than in quantity of cytoplasm resulting in the formation of a large central vacuole. Cell formation begins at the periphery of the embryo sac and advances towards the centre. Even after the gametophyte has become cellular throughout, sugar is the principal food content of the cell. The cells of the outermost layer are sharply differentiated from the rest by their smaller size and also by their almost lack of starch. The layer next within is also differentated, its cells being smaller than those of the ordinary endosperm cells of the remaining vegetative portion of the gametophyte.

The development of the archegoniam has studied for *cycas revoluta* by IKENO and for *Dioonectly* by chamberlain. In *Dioon edule* the archegonium initials are usually four in number. The initial soon divides, giving rise to a primary neck cell and a central cell. The neck initial divides immediately to form two neckcells while the central cell increases in size. The nucleus of the central cell divides to form a ventral canal nucleus (eventually disintegrates) and an egg nucleus. There is no wall between two nuclei. In the mature female gametophyte the archegokia are found below a depression very often called archegonial chamber formed by the surrounding tissue. The membrane of the gametophyte and the top nuclear tissue are destroyed at this region so that archegouial chamber fuses with the pollen chamber. The gametophytic tissue below the archegonia is full of starch grains, serving for the nutrifi on of the archegonia and then of the growing embryo. This is the endosperm which is still a gametophytic tissue.

The Male Gametophyte

The microspore is the first cell of the male gametophyte. The microspore has two distinct coats, the exine and the intine. The exine is thickest in the basal region and becomes very thin towards the apex. The intine is thinnest in the basal region. The microspore germinates while remaining in the microsporangium.

The division of the nucleus gives rise two nuequal cells, a small persistent prothalial cell and a larger cell, the anther idial initial. The nucleus of the larger cell divides to form two unequal cells, one a small cell closely applied to the prothalial cell and the other the tube cell. The small has been called generative cell. Thus the pollen grain becomes a three nucleate cell.

Cycas is wind pollinated. The air-borne pollens are deposited on the top of the ovule and are drawn through the micropyle by a drying speak of mucilage. They are then deposited within the pollen chamber where the pollen germinates here and the tube cell of the gemetophyte comes out through the thinner end of the pollen forming pollen tube. The pollen tube penetrates into the tissue of the nucellus. It actually acts as haustorium, a feature unlike that of Angiosperimic siphonogamy. They haustorial pollen tube branches like a root. It sucks in food material from the tissue it penetrates while its growth pushes down the base of the pollen to near the archegonium. In the base of the pollen tube the generative cell divides into a stack cell & a body cell. Just before fertilization the body cell enlarges and two blepharoplasts appear at the two poles. Soon the body cell divides into two sperms to each of which a blepharoplasts gets attached forming a spiral bond with numerous cilia on it.

The sperms of cycads are remarkably large as are the eggs. They are even visible to the naked eye. The discharge of fluid of the pollen tube causes the sperms to be forced into the egg cell. The sperm nucleus fuses with the egg while the cilia and cytoplams are left behind on the top of the egg cell.

Inter-relationship

The cycadales are connected with the Bennettitales in origin. In fact, no two gymnosperm group have so many features in common. The transition form the trunks of cycadeodisa having numerous lateral strobili, through such a form as *William sonia gigas*, with its several strobili appearing from the center of a crown of leaves, to the cycads with a usually solitary and terminal strobilus, seems natural and suggests that the cycadales are an offshoot from the Bennettitales. The striking differences in the strobili may suggest independent origin from the cycadofilicales.

The cycadales, taking *cycas* and *Pseudocycas* as its oldest known representatives, suggest an origin from groups of cycadofilicales bearing ovules as did pecopteris pluckentii. This in dicates a separate origin.

The cycadales are remarkable in the retention of more primitive characters than are possessed by any living group of gymnosperms. The swimming nature of sperms are shared by Ginkgoales. The structure of the ovules goes with them. The vegetative structures are more fernlike than in arry ofter living group. In this background the cycadales can be considered as the most primitive of living gymnosperms.

GINKGOALES

The order Ginkgoales is reduced to a single family Ginkgoaceae having the single genus Ginkgo with the solitary species called *Ginkgobiloba*. The species is very often called 'maiden-hair tree' for its leaves resembling the 'maiden-hair ferm (Adiantum). It is almost unknown in the wild state. It was sometimes known as *Salisburia adiantifolia* sm. The species has probably escaped extinction by cultivation. Its extensive cultivation first in china and Japan, and later in Europe and in the United States has made it accessible. Such a history has made *ginkoa* living fossil. Now it is cultivated throughout the world in temperate and subtropical regions. Some plants are known to grown at Darjeeling, Dehradun and Mussoorie and more are now being grown elsewhere in India.

The Vegetative Organs

Ginkgo plant has the general habit of a conifers but branching becomes irregular with age. The trunk attains a height of 30 metres and a girth of 1 metre. I usually bears two kinds of branches – the long branches of indefinife growth bearing short branches or dwarf shoots of limited growth. The dwarf shoot elongates slightly every year, bearing a terminal group of leaves. The older portion is covered with leaf scares of previous years. A dwarf shoot may become a long shoot-after several years.

A great variation is noticed in the size and in the lobing of the leaves of Ginkgo. The blades are often deeply cut ad with more than two lobes. The leaves of mesozoic species were much more divided. In the living genus Ginkgo, the lobed condition is always found in the leaves of seedling Sand usually on the long shoots. The leaves at the top are particularly deeply cleft. On the other hand the leaves on the dwarf shoots are usually nearly entire. The deep lobing is a juvenile character and many authors it should be regarded as an illustration of recapitulation. The deciduous habit of ginkgo is being shared by only some coniferous genera like*Larix, Taxodium & Glyptostrobus.*

ANATOMY

The transverse section of the stem of Ginkgo shows the general features of Coniferales. It shows a comparatively narrow cortical zone, a thick and compact cylinder of secondary wood formed by a persistent primary cambium and a relatively small pith. In dwarf shoot the pith is large and the zone of wood narrow. The primary xylem is endarch and there is not distinct mesarch structure except in the bundles of the cotyledons. The leaf trace is double in conformity with more primitive gymnosperms, each of the strands forking at the base of the bundle. In protoxylem tracheids are spiral whereas the tracheids of the secondary wood are with bordered pits arranged in one or two rows showing 'bars of Sanio'. There are no parenchyama cells in the wood excepting the medullary rays. Annual rings are feeble. Periderm formation is distinct.

In case of seedling, the cotyledons show mesarch bundle, a cycadean character. This character can be obtained in the leaves which show two exarch, collateral vascular bundles covered by a sheath. The spur

leaves do not contain palisade cells but those on the long shoots have them. The upper epidermis is continuous and the stomata are present in the lower surface.

The lower mesophyll parenchyma cells are placed parallel to the leaf surface forming a transfusion tissue. The vascular bundles are closed collatural with the xylem on top. The mesophyll also shows prominent mucilage canals.

The root is diarch with radial xylem and phloem. The stele is surrounded by a pericyle, and endodermis and a large pith. The mesophyll also shows prominent mucilage canals.

REPRODUCTIVE ORGANS

The spore-producing members:

The Microsporangium

The male or staminate strobili consists of numerous microsporophylls borne in loose catkinslike clusters from the axils of the scale of the scale leaves developed at the top of dwarf shoot. The general structure of the strobilus resembles the staminate strobilus of cordaitales if the intermixed sterile sporophylls and bracts of the latter are eliminated.

The development of the sporophylls is a cropetal. The mature sporophyll has a long stack which terminates in a knoblike enlargement. Two pendent sporangia are borne beneath one side of this knob. The more or less expanded knob at the tip of the stack may be regarded as reduced lamina bearing sporangia pendent from its abaxial surface. The knob or hump contains a large mucilage cavity. The structure is considered by some as a sterile third microsporangium capable of developing a fertile one. Each microspongium develops in the eusporangiate way. An archesporial cell gives rise to a sporongenous tissue. The spore mother cells give rise to the microspores or pollen on reduction division. The microsporangium ruptures by a longitudinal split.

The Megasporangium

The ovulate strobili are very much reduced and borne in groups at the top of the dwarf shoot. Each strobilus consists of a long stack arising from the axil of a bract and bearing at its apex mostly two ovales. Of the two, one usually aborts but in some cases both are fully developed. Occasionally more than two ovules also appear and this indicates that strobilus of Ginkgo has been derived from one bearing several ovules.

About the base of the ovule there is or "Collar" which is prominent in the young stages of the ovule.

This collar has become a matter of prolific discussion. According to van Tieghem (1869) the stack is interpreted as a petiole; the two ovules as the two characteristic lobes of the blade; the collar as a reduced aril. The whole structure thus represents a single megasporophyll (carpel). Strasburger (1872) suggested that stack is a shoot; that the collar is the rudiment of the first pair of leaves of a secondary shoot. The whole structure is therefore an inflorescence of two flowers in which the carpel is suppressed. Eichler (1873) interpreted the collar as the outer integument of the ovule. Celakovsky (1890) concluded that the stack is a shoot usually bearing two rudimentary carpels or megasporophylls which are represented by the two collars.

The vascular anatomy dissents from the current view that the collar is a much reduced megasporophyll. The bundles of the collar show inverse orientation. A comparison with Lagenostoma suggests that the collar of Ginkgo is a vestige of the cupule that invested seeds of the Lagenostoma type.

The vidence suggests the structure under discussion is a strobilus bearing two or more megasporophylls which are usually reduced to the so called "collars", but which sometimes resume original leaflike feature.

The ovule generally resembles cycas. The well marked nucellus has a large pollen chamber surmounted by a beak which becomes hard. The nucellas is free from the integumenta at the top. The single contains an outer green fleshy layer, a thin hard middle part and a thin fleshy inner part which ultimately becomes papery. The two vascular strands supplying the base of the integument to not branch. On the top of the nucellus there is micropylar opening. Although one or two megaspore mother cells are formed but one of them undergoes meiosis forming a linear tetrad of megaspores of only one becomes functional.

The Male Gametophyte

The microspore is the first cell of the male gametophyte. It germinates while still within the microsporangium. It divides to form a small first prothalial cell and a large antheridial initial cell. The antheridial initial cuts off a second prothalial cell which persists while the first prothalial cell disintegrates early. The antheridial cell remaining in contact with the second prothalial cell divides to for a generative cell and a tube cell. It is at this 4-celled stage the microsporangium dehisces and the pollens are shed. The pollens are carried by wind and get deposited on the micropyle of the ovule where they are sucked down along with drying up of a drop of liquid, the pollination drop Ultimately they are deposited in the pollen chamber. Here the exines bulges and protrudes as a pollen tube and becomes anchored in the tissue of the mucellus. As in the case of cycas, it acts as a haustorium. The spermatogenous end of the pollen tube advances towards the female gametyphyte breaking down and absorbing the tissue that comes on the way. Finally the pollen chamber is enlarged so that nothing remains between the pollen tube and the female

gametophyte. Now, the generative cell divides into a stack cell and a body cell. Next, the body cell divides to form two multiciliated sperms just as in cycas. The blepharoplasts also behave in the same way. The sperms are slightly more elongated than in cycas. The spirally arranged cilia are more restricted to the apical region.

The Female Gametophyte

The female gametophyte develops as a result of germination of only functional megaspore. The megaspore enlarges and its nucleus divides by rapid free nuclear division. Through free nuclear division over 256 nuclei may formed when the ovule and the embryo sac go on enlarging and the megaspore membrane is becoming thicker.

Prior to wall formation, a delicate membrane appears on the outer surface of the protoplast of the embryo sac which is separabley from the megaspore membrane and called the endosperm membrane. Cell wall formation then begins at the periphery and gradually proceeds towards the centre. As in cycas a spongy nutritive tissue is formed round the growing gametophyte. The gametophyte is soon filled up with an endosperm tissue which develops chlorophyll t a later stage as the integument is more of less transparent. The endosperm is entirely free from the megaspore membrane which later on differentiated into two layers.

The archegonium initials, usually two in number but rarely three, appear very early in the history of the gametophyte. The development of the archegonium si very much similar to that in the cycadales. At first a two-celled neck & rapidly increasing central cell is formed. The central cell nucleus divides to form a ventral canal nucleus and an eggnucleus and a definite wall is seen between the two. In this respect Ginkgo can be considered to be more primitive than cycas where no such partition wall is formed. Pinus shows an ephemeral partition wall. Again, unlike the cup-shaped archegonial chamber of the cycads, ginkgo has a somewhat cylindrical chamber surrounding a mass of solid gametophyitc tissue upon which the nucellus wall rests like a tent on a pole.

Inter –relationship

The mesozoic Ginkgoales seem to hold the same relation to the paleozoic cordaitales that the mesozoic Bennettitales hold to the paleozoic cycadofilicales. The connections between the former pair seem coser and more definite than those between the latter pair. Just as the whole cycadophyte retained characters of seed structure and swimming sperms that belonged to the ancient cycadofilicales, so the Ginkgoales have retained the primitive characters of the cordaitales. Scott opines tha Ginkgo is "the one surviving member of an ancient stock, derived from the same cycle of a affinity as the paleozoic cordaiteae".

Among living gymnosperms, Ginkgo can be compared with cycads and conifers. It resembles the cycads in the structure of the ovule, the seed and in the details of fertilizatin, while in habit, stem structure and vascular anatomy it resembles conifers. Attempt has been made to make connections between Ginkgo and the taxaceae, in which Ginkgo was placed by some taxonomists as a n aberrant member. The characters in common with the cycads are primitive and these are shared with cycad of ilicales and cordaitales.

The characters in common with the conifers are said to be advanced. At the same time, the characters of the foliage and structure of the strobili are peculiar to Ginkgo. The combination of peculiar characters with others belonging to Cycadales and Coniferales justifies the separation of Ginkgoales as a coordinate group. The Ginkgoales can be nicely considered to represent a phylum that has retained certain primitive features in common with the cycaetophytes and certain advanced features common with the coniferales and over and above has developed certain peculiarities of its own.

CONIFERALES

The Coniferales occupy the most prominent group in the present gymnospermous flora. They have about 40 genera and approximately 350 species distributed in the temperate regions of both northern and southern hemispheres. The conifers contribute the world's most extensive forest belt. Some members of *Sequoia* are deemed to be 4000 years old, attaining a height of about 300ft. and drunk of 30ft. in diameter.

The Conferales, here do not include the Taxales for the reasons which have been mentioned in connection with the latter. The existence of the coniferales dates back to carboniferous. They were predominant in the Mesozoic and now form an constituent of gymnospermous flora.

Excluding the fossil families the order includes 6 families such as Pinaceae, taodiaceae, Cupressaceae, Podocarpaceae, Araucariaceae and Cephalotaxaceae. The families are based on such features as the arrangement of foliar leaves on the shoots, nature of fusiion of bract and ovuliferous scales and their arrangement on the cone axis microsporophyll arrangement along with the microsporangia, the number of ovules etc.

Althoug 6 families have been mentioned in the order but here our discussion will be confined to Pinaceae with special reference to Pinus which is the most well known representative of Pinaceae. The genus pinus is very often called "Pine" with 80 to 90 species spread over the temperate to subalpine regions of Northern Hemisphere. The following six species of Pinus occur in India.

1. *Pinus Wallichiana* A.B. Jacks — Distributed in the Himalayas
2. *P. roxburghii* Sarg. — Distributed in the Himalayas
3. *P. insularis* Endl. — Distributed in the Himalayas

4.	*P. gerardiona* Wall. Ex Lamb.	Distributed in the Himalayas
5.	*P. armandii* Franch	Found in Arunachal Pradesh
6.	*P. merkusii* Jungh and de Vries	Found in Andaman and Nicobar islands

The Vegetative Organs

Pinus is a nice tree and beautiful to look at. The tree is provided with horizontal branches in whorls thus giving a pyramidal appearance. The young plant exhibits a strong tap rot which may persist or may be replaced by stronger adventitious roots. Primarily single needle leaves are arranged spirally. Very soon the nature of the leaves changes. Small scale leaves appear on the stem. These are brown, thin & scaly. From the axils of these arise the dwarf shoots which are of limited growth. A dwarf shoot is small piece of stem having scale leaves and from its apex comes out a fascicle of two or more narrow, long, green needle leaves. The needle leaves form the main photosynthetic organs of the plant. The branches are almost in a whorl but only a few of them survive.

During the spring, at the end of every year, new branches appear from the branch of that year's growth. The regular branching offers an excurrent shape of the growing tree. The tree are evergreen but the dwarf shoots are shed every two or three years leaving scars on the stem. The full grown tree has a single, stout, cyclindrical stem covered by a scaly bark and lateral branches of unlimited growth popularly known as long shoots. Long shoots exhibit leaf scars and scale leaves only. The younger branches show the dwarf shoots of definite limited growth. Thus, there are two types of shoots. Leaves again are of two kinds such as scale leaves on the long and dwarf shoots and needle leaves on the dwarf shoots.

ANATOMY

The T.S. of the stem shows an endarch siphonostele broken up by leaf gaps. The stem is externally bounded by a single layered epidermis which is cutinised on the outer face. The epidermis may be followed by a sclerenchymatous hypodermis. Next to hypodermis there is parenchymatous cortex having resin canals or ducts. The innermost layer of the cortex forms the endodermis which surrounds the stele. The stele shows the presence of pith at the centre and the pith is surrounded by a ring of conjoint, collateral, open bundles showing xylem, cambium and phloem. The individual bundles are separated by primary medullary rays.

In the xylem true vessels are absent. The protoxylem is made of annular and spiral tracheids, while the metaxylem is formed of bordered pitted trachids. Resin canals are present in the primary wood. The phloem shows sieve cells and phlem parenchyma with no companion cells.

Secondary growth is similar as in dicots. It is both intrrastelar by the formation of a cambium ring and extrastelar by a phellogen giving rise to a periderm under the epidermis. The wood shows the annual rings along with the presence of resin ducts. Ther wood of pine shows some difference with that of the Angiosperms. The tracheids possess one or two rows of bordered pits along the radial walls only. The summer trachids are narrower and much thinner than the spring tracheids. The walls of tracheids have thin. tranverse, unthickened lines on the walls the borders of which are thickened and are known as bars or rims of Sanio. The secondary medullary rays as well as resin ducts and canals form an anastomosing system in the secondary wood. The rays are narrow and fewcells in depth. In the extrastelar region, the phellogen is replaced by successive tanguential layers of phellogens deeper & deeper into the cortex giving rise ot strips of scaly bark.

The root structure anatomically resembles the dicots. There are 2 to 4 exarch bundles (diarch, trarch or tetrarch). The bundles appear as 'Y' shaped structures. Secondary growth occurs showing the compact xylem & phloem. Phellogen develops outside the pericycle giving rise to a periderm. Pinus root shows mycorrhizal association. Leaf anatomy shows xerophytic structure. The needles are triangular in cross section. The epidermal layer is cutinised with very deeply sunk haplocheilic stomata. Below the epidermis lies the hypodermis of 2 or 3 layers of very thickwalled cells. Internal to this is the mesophyll composed of large, thinwalled and lobed chlorophyllose cells. Resin canal are present in the mesophyll and the hypodermis. The mesophyll is internally lined by an endodermis having casparian strips. There are two vascular bundles internal to the endodermis. Vascular bundles are surrounded by a broad pericyle which bears the special name of transfusion tissue which shows longitudinally elongated, bordered pitted, tracheids in the xylem and albuminous cells, with proteoplasm containing starch and protein. The xylem lies in the upper angular surface of the leaf the phloem lies below. The leaf shows the presence of one vascular bundle at the base which divides into two parallel vascular bundles within the common sheath. This is a case of double leaf trace, a situation observed in primitive gymnosperms.

REPRODUCTIVE ORGANS

The Spore – producing Members

The Microsporangium

The staminate cones are clustured on the base of the young shoot during the spring. The microsporophylls are arranged spirally on an axis. The male cone here can act as a flower and not an inflorescence. On the under surface of the scale-like micro-sporophyll there are two microsporangia containing numerous microspores or pollen grains. The mature sporangium dehices by longitudinal slit. The development of the sporangium is eusporangiate. It shows sporangium wall tapetum and microspore

mother cells which are numerous in number. After reductiion division the microspore mother cells give rise to numerous microspores. Each pollen is provided with an exine and an intine. Prior to the separation. the exine is inflated into two wings resulting to wind pollination.

The Megasporangium

The female cone is not a simple structure like the male cone. The cone is about a cm. long and red in colour but gradually turning green. The cone has an axis which bears a number of bract scales, on each of which originating from the axil is an ovuliferous scale. Each ovuliferous scale bears on its surfac two ovules or megasporaniga. Separate vascular traces supply the bract scle & the ovuliferous scale. Each ovule has the main tissue as the nucellus covered by an integument growing from its base. The integument is open at the top forming the micropyle which is free from the ovule. The integument is differentiated into three layers, an outer fleshy, the middle stony and the inner fleshy. The pair of ovules is associated with two scales and the two scales with the ovules are considered to form a separate shoot. In this regard, the female strobilus is not simple but rather compound and homologous with an infloresecence.

The morphology of the ovuliferous structure is strongly debatable. Different views have been put forward from time to time to explain the morphological nature of the ovuliferous scale. The views are given below.

1. robert Brown (1827) considered the ovuliferous scale as an open foliar 'carpel' naked ovules on it. The carpel arises in the axil of the bract.
2. Schleiden (1829) explained that the ovuliferous scale was not a leafy carpel but a flattened 'axis' forming the placentum.
3. A. Brown (1842) considered the ovuliferous scale to represent the first two bares of an axillary shoot which had become fused.
4. Dickson (1860), Goebel and others concluded that the bract was to be considered as megasporophyll or carpel bearing at its axil a shoot which was the ovuliferous scale.
5. Sachs (1868) being supported by Eichler concluded that the bract was carpel and the ovuliferous scale was a ligular on its surface.
6. Van Tieghem (1869) on the basis of anatomical studies believed that the ovuiferous scale was the first and the only leaf of a suppressed shoot in the axil of the bract.
7. Celakovsky (1879) considered the ovuliferous scale as the fused outer integuments.
8. Chamberlain (1934) considered the bract to be a megasporophyil bearing in its axil a modified

shoot (ovuliferous scale) which is the sporophyll.

9. On the basis of a comparative study of fossil conifers like *Lebachia, Ulmannia* and *Voltzia Florin* (1951) pointed out that in *Pinus* the inflorescence bears the bracts (brat scales) in the axils of which are the very much reduced dwarf shoots or "flowers". The ovuliferous scale represents a compound structure formed by what were originally two megasporophylls and a few sterile scales.

The archesporial tissue appears in the spring and gives rise to sporogenous tissue which is deep seated in the nucellus. A single megaspore mother cell is formed and it is surrounded by a sponsy nutritive tissue. The megaspore mother cell on reduction division forms a linear tetrad of four megaspores as in Angiosperms. The lowest one is functional.

The Male Gametophyte

The microspore is the first cell of the male gametophyte. The pollen germinates while remaining within the microsporangium a month before pollinatiion actually takes place during March-April. The nucleus divides to form a small prothalial cell which remains pressed against the intine at the top. A second prothalial cell is cut off from the other cell and is similarly pressed against the first prothalial cell. The two prothalial cells slowly disorganise. The remaining cell is the antheridial cell which next divides to form a generative cell and a tube nucleus in the remaining cell. The smaller generative cell remains attached to the top intine below the degenerating prothalial cells. At this stage the pollen are shed.

The pollination is anemophilous. During March-April in the Eastern Himalayas the pollens are profusely liberated by the Pine forests so that the air gets saturated with them. This situation makes the forest floor yellow. This phenomenon is popularly known as "Sulphar shower". As a result, most of the pollens are wasted and only a small amount reach the female cones. These now pollinate the female cones of the last year as the current year's cones are not yet ready. The scales of the last year's female cones slightly spread apart at this stage allowing the wind-carried pollens to reach the micropyles of the ovules. Now, the 'pollination drop' appears on the top of the micropyle and the pollens are drawn to the top of the nucellus with the pollination drop. After the pollination is over the scales are again closed.

Post-pollination changes in the male and female gametophytes are slow and take another year for them to complete fertilization.

In conformity with the formation of ventral canal cell and the egg cell in the archegonium further development of the male gametophyte takes place. After being lodged on top of the nucellus its intine develops a pollen tube corrodes and grows down the nucellus. The pollen tubes are full of starch grains & shows a tendency to branching. Now, the generative cell divides to form an upper stack cell and a lower body cell. The stack cell soon breaks to liberate its nucleus and loosing the body cell. Just before fertilization

the body cell divides into two naked cell or gametes having a nucleus with some cytoplasm round it. The male gametes are therefore, very simple and at the some time the most advanced as the tendency among the Spermatophytes is towards reduction. On the contrary, in the female gametophyte a ventral canal cell with a distinct wall is found in Pinaceae. This character is said to be primitive. So, the gametophytes of Pinaceae show both advanced and primitive characters.

The Female Gametophyte

The development of the female gametophyte starts with the division of megaspore nucleus. Series of simultaneous nuclear division follow till the number of free nuclei is very indefinite. Along with this the megaspore rapidly increases in size. A large central vacuole filled with transparent fluid presses the protoplasm with the free nuclei on the wall so that the latter form a lining. The megaspore membrane is yet thin. This stage of development carries the female cones from one spring to another spring and they become ready for pollination.

Post-pollination changes in the female gametophyte are slow and take another year to complete fertilization. The megaspore membrane now becomes thicker and more prominent. Next, wall formation starts. Cell wall formation proceeds inwards ultimately removing the vacuole and filling up the whole of it with the cells. Now, at this stage a few archegonia develop near the sumit of the female gametophyte. The cells lying below will form the nutritive endosperm. The archegonial initial is a superficial cell near the apex. The initial cell divides transversely to form upper neckinitial and a lower central cell. The neckinitial through divisions form 8 cells in two tiers of four cells each. Neck coanal cells are absent. An archegonium jacket is organised around it. The nucleus of the central cell divides just before fertilisation forming a distinct cell wall between the daughter nuclei. This gives rise to a small ventral canal cell above and the lower cell is the egg cell. The ventral canal cell eventually degenerates and the nucleus of the egg cell becomes enlarged and descends to a central position in the egg.

Inter – relationship

The Coniferales known since the Carboniferous were predominant in the Mosozoic and form an important constituent of the flora today. It is opined that by most of the workers that coniferales began their extensive differentiation during the Mesozoic period which has resulted in six recognized tribes in our present flora. Among these tribes, the earliest to be recognized are the Abietineae and the Araucarineae and their very early separation is evident to raise the question whether they may not be independent in origin. The other tribes recognized were of later origin. The Taxodineae and the Cupressineae might have originated from the mesozoic abietineae and the Podocarpineae possibility arising from the mesozoic Araucarineae.

That the Coniferales evolved from the Cordiatales is now accepted by most of the workers. According to Florin (1951) the female strobili of Pinaceae has evolved from the Cordiatales through Lebachiaceae and Voltziaceae, Araucariaceae closely resembles the Cordaitales in its secondary wood and foliage. Jeffery (1971) considers the wood of Pinaceae more directly related to the cordaitales and suggests that the similarity with Araucariaceae is a later development. He supposes that the Araucariaceae evolved out of Pinaceae at an early stage.

The affinities of the coniferales with the cycadofilicales seem to be very remote. The coniferales are related to Ginkgoales because of habit of the tree, similar type of wood & the gametophytoc structures.

The coniferales show affinity with the Ephedrales in having *haplocheilic* type of stomata.

The coniferales are also related to Ephedrales in respect of the structure of the archegonium & similarity of embryogeny.

The Coniferales exhibit many features shared by the cycads, namely endosporic and reduced male gametophyte; archegonia with neck cells and evanescent ventral canal cell.

TAXALES

The family Taxaceae has been placed in a separate order, Taxales by many workers including Sahni. Many workers consider Taxaceae to represent a phylogenetic line quite different from that of the general coniferales. They do not form a female 'strobilus' and the solitary ovules and placed on the tips of side dwarf shoots. The fossil history of the order is known upto Upper Triassic but there is no indication of their origin from any strobilate plant.

Sahni (1920) expressed the opinion that Taxaceae should be separated from the Coniferales. Florin (1938-1941) also considered the 'Taxales' as separate from the coniferales. Podocarpaceae and Cephalotxaceae are sometimes included within the Taxales by various authors. But, there is discrepancy of opinion as to their nonstrobilate nature and at any rate, arguments for placing them in a separate order is not as unanimous as for Taxaceae. Only Taxaceae is, therefore, placed here within the Taxales.

Indian Species of Taxaceae

Taxus baccata L:

Spreading from England & Europe over to temperate Himalayas, Khasia Hills, Naga Hills, Manipur and Upper Burma. Upto 50 ft. tall and often very old. Hard timber.

The Vegetative Organs

Taxus is an evergreen, densely branched tree of medium height. It is long living. Some of the British yew trees are claimed to 2000 years old. The stem is short, thick and is covered by a reddish brown bark that pells off easily. It bears spreading branches which are surrounded by scales at the base. The shoot bear green foliage leaves and are arranged in close spiral. But the leaves become spread in two ranks by the twisting and curving of the petioles. The lamina is entire, dark green, glossy with slightly recurved margin and pointed horny tip. On the upper side, the median vein is marked as narrow and sharp keel in a shallow longitudinal furrow. There is a long tap root system without any mycorrhizal association.

The tree is strictly dioecious and the young male and female trees are morphologically alike and can not be distinguished till the appearance of reproductive structure.

ANATOMY

T.S. of the young stem shows the presence of parenchymatous & cuticularised epidermis which is followed by broad parenchymatous cortex. Resin canals or resin parenchyma are absent from cortex & wood. The cortex encircles a single layered endodermis and pericycle. The vascular bundles are conjoint, collateral, endarch & open.

The secondary growth takes place at an early stage. The annual rings are distinct. Bordered pits occur on the radial walls of thetracheids. The vascular rays are uniseriate, 1 to 5 cells high. Secondary phoem consists of sieve elements, phloem parenchyma and phloem fibres arranged in tangential bands.

The leaf is dorsiventral with distinct upper and lower epidermis of rectangular parenchymatous cell. Stomata are present on the lower epidermis only. They are sunken and haplocheilic in origin. The mesophyll is composed of palisade and spongy parenchyma. Foliar sclereids are also in record. The single vascular bundle in the midrib region is covered by a prominent bundle sheath. The transfusion tissue, at the sides of the bundle is distinct.

REPRODUCTIVE ORGANS

The Spore-producing Members

The Microsporangium:

The male strobilus or flower is a solitary, stacked, subglobose, short catkin of 6 to 10 microsporophylls borne on an axis. Scaly bracts are present at the base. Each microsporophyll is peltate with 5 to 9 microsporangia hanging below the peltate disc. Each microsporangium has a wall which encloses a tapetum.

Inside the tapetum there are microspore mother cells which result into numerous microspores or pollen grains which are not winged. The microsporangia split and curl out when ripe to liberate the pollens. Pollination is anemophilous. The wingless pollens are shed in the uninucleate stage.

The Megasporangium:

The female strobilus arises in the axil of a leaf on the previous year's branch as small bud. It bears a number of closely overlapping scale leaves and a growing apex on the axis soon ceases to function after the development of a secondary lateral shoot in the axil of one of its top scales. The secondary shoot may be called the female as it bears a few scale leaves and ends in a single terminal ovule. The structure of the ovule is as in Pinus excepting the presence of the aril. The archesporial cells seem to be hypodermal in origin but the megaspore mother cells are found deep within the nucellus. Usually, megaspore mother cell gives rise to a linear tetrad of megaspores of which the lowest one is functional.

The Male Gametophyte:

The wingless pollens begins germination after they are lodged on the tip of the megasporangium nucellus. The first division of its nucleus results in a generative cell and a tube cell so that the prothallial cells are completely eliminated. Next, it develops as in Pinus. The two male gametes are always of unequal in size. The pollen tube reaches the female gametophyte top piercing through the nucellus very quickly, usually two months before the archegonia are formed. The actual brusting of the pollen tube and fusion of the male gamete and the egg take place later.

The Female Gametophyte:

In Taxus, there are cases where different megaspore mother cells have given rise to more than one female gametophyte. But in such cses only one gametophyte survives to the last. The development of the female gametophyte in general is similar to that of Pinus and similar to the situation of Pinus several archegonia are formed. An important point of difference is that no cell wall develops between the *ventral canal nucleus* and the *egg nucleus* before the former disintegrates so that there is no ventra canal 'cell' in Taxus.

Inter -- relationship:

The Taxales present greater difficulties, for their resemblances are not so definite and their

combination of so-called primitive and advnaced characters is more perplexing. The Taxales were formerly included within coniferales but the order shows no sign of a strobiloid ancestor although it may be clearly traced to the upper Triassic and probably even earlier. According to Jeffery as well as Takhtajan the order might have developed from podocarpaceae stock. Sahni suggested the separation of the Taxales from the coniferales.

It would seem impossible to connect the Taxales, which have no history earlier than the cretaceous, directly with the palozoic cordaitales. In fact, the opinions that have been expressed in reference to the relationship of the Taxales are so diverse that it is evident the order presents a pecularly difficult combination of resemblances. They have been called the oldest living conifers and the most recent. They have been connected with *Ginkgo* through *Cephalotaxus* and with the Cordaitales on account of the structure of the ovuliferous shoot of *Taxus*. At the present state, it is better to consider the Taxales as a separate but problematic order.

GNETALES

The Gnetales contains only one family Gnetaceae which in turn consists of only one genus Gnetum. The genus Gnetum consists of thirty species distributed in the tropical and subtropical zones of the world. Five species are known occur in India (Bharadwaja, 1957), Maheshwari and Vasil 1961). Of these *G ula* is the most widely distributed species, extending from Western Ghats, Kanara to evergreen forest of Coorg, Kerala, Nilgiris, Godawari district of Andhra Pradesh and some parts of Orissa.

The Vegetative Organs:

Gnetum resembles more an angiosperm than a gymnosperm. Most of the species are climbers but *G. gnemon* is a tall, stout tree. It exhibits two types of branches viz. Branches of limited growth and branches of unlimited growth but this distinction is absent in the shrub or tree types of Gnetum. Some species of Gnetum have articulate stems. The branches of unlimited growth bear short 'dwarf shoots' of limited growth. Leaves are borne on the dwarf shoots. The leaves are large, oval, entire, leathery and pinnate reticulate veined like dieot leaves and thus a plant not in flower may be considered as an Angiosperm. These leaves are borne in opposite decussate pairs without stipule. In the long shoots of climbers the leaves are reduced to scales.

Gnetum differs from the true Gymnosperms in many characters. Its similarities with the Cycadales have been stressed by Thoday and Takhtajan has supposed its origin from the cycadopsida. On the whole the origin of Gnetales still remains unsolved.

The most interesting question in connection with the relationship of Gnetales is their possible relationship to angiosperms. It was natural at first to regard them as the immediate progenitors of angiosperms, for their structures seemed to point irresistibly to that conclusion. When transition groups were in vogue, there was no better illustration of one than the Gnetales. But those who are now claiming a phylogenetic connection between Gnetales and angiosperms do not claim it in the sense that the former is a transition group. After a critical cytological investigation of the female gametophyte and of fertilization in Gnetum, Strasburger has concluded that it is impossible to derive the angiospermous embryo sac from that of gnetum, and that such resemblance's as do exist are illustrations of phylogenetic parallels.

Evolution of archegonia in Gymnosperms

Archegonia can be had from the level of Bryophyta. They are found also in Pteridophyta and gymnosperms. In Gymnosperms the archegonia are formed in the female gametophyte. The female gametophyte of gymnosperms exhibits a progressive series of changes leading towards the angiosperm condition. The first phase of development of the gymnospermous female gametophyte is characterised by an extensive series of free nuclear divisions. Ultimately wall formation begins at the periphery and proceeds centripetally until the entire gametophyte consists of cells containing reserve food materials. Throughout the antogeny of the gametophyte this structure is encased by well defined megaspore wall. According to Schnarf (1937) the presence of a conspicuous megaspore wall surrounding the female gametophyte is one of the most important and definitive features common to gymnospermous seeds.

During the process of wall formation, certain individual superficial cells of the gametophyte, usually those near the micropylar end give rise to archegonia. The number of archegonia varies considerably, ranging from usually two in Ginkgo to many as in some of the conifers. All living gymnosperms, with the exception of Gnetum and Welwitschia are archegoniate plants.

By and large, the general tendency of the archegonia among gymnosperms is to eliminate the ventral canal cell. The gymnosperms differ from the pteridophytes by the complete elimination of neck canal cells and this tendency of suppressing all of the axial row except the egg continues among gymnosperms. Among the living members a walled ventral canal cell is retained only among the Abietinae and Ginkgo, but it seems clear that it was present among the ancient gymnosperms. In other living groups the wall has disappeared. The ventral canal cell is represented by a free nucleus. In certain forms even this nucleus is lacking when the archegonia are eliminated.

An entirely different type of male gametophyte in *G.ula* was reported by Swamy (1974). According to him the microspore nucleus divides into a small cell and a large cell. The small cell has its own sheath of cytoplasm & his free in the large cell. It moves into the pollen tube and gives rise to two male gametes. The nucleus of the large cell divides into two daughter nuclei. The pollen grains wall consists of an outer thick exine with minute spines and inner intine. Like Ephedra and Welwitschia, pollenkitt is lacking in Gnetum (Hesse, 1980).

The Female Gametophyte

The development of the female gametophyte is tetrasporic. As the female gametophyte develops, a large vacuole appears in the centre and the nuclei in the peripheral cytoplasm undergo repeated divisions. Later, as divisions continue, the gametophyte in the upper part widens and contains a vacuole. In the lower part the gametophyte shows accumulation of cytoplasm. With further growth, the gametophyte becomes elongated and acquires the shape of an inverted flask. Wall formation starts from the chalazal end, slowly proceeds upwards and is not complete at the micropylar end even at the time of fertilization. The tissue of the nucellas immediately below the gametophyte serves for its nutrition and becomes especially organised to form the 'pavement tissue'.

No archegonium initial is formed in case of Gnetum and thus it shows maximum reduction in respect of archegonium formation ataining the Angiosperm level. One or more nuclei out of the free nuclei on the top of the gametophyte become a little large, accumulate some cytoplasm round them and are then identified as eggs in *G. gnemon.* In *G.ula* a group of cells are formed on the top, a few of which act is eggs.

Inter – relationship:

The origin of Gnetales is very obscure and in absence of history any statement in reference to it must be regarded as a guess. The three orders, Ephedrales, Welwitschiales and Gnetales represent the most advanced gymnosperms. Gnetales are related to Ephedrales and Welwitschiales on the basis of the following common features.

1. The compound male & female strobili representing inflorescence of flowers.
2. The two apparent integuments of which the inner forms a long micropylar tube.
3. The opposite decussate leaves.
4. The apparent vessels in the stems.
5. The dicotyledonous embryos.

and a sheathing perianth which may be apparently formed by the fusion of two bracteoles. In the young stage flowers remain hidden under the rim of the connate bracts. The perianths only peepout. Finally, the flowers open by a quick elongation of the stalk which pierce the perianth. In the microsporangium the tapetum is formed from the sporogenous tissue and not from the wall cells. The microspores develops by the reduction division of spore mother ells. At maturity all cells between the microspores and the epidermis disintegrates. The pollen are released by longitudinal slits of the anther.

The Megasporangium

The female strobili or spike also develop from positions as the male and also have the same outward appearance. In the young condition the flowers remain hidden under the connate bracts. At maturity the flowers, placed in one ring only, peepout. The structure of the female flower of Gnetum is very problematic. There are three investments round the nucellus of the ovule instead of two as in Ephedra. There are separate vascular supplies for each of the three investments.

According to Chamberlain and Beccari the outermost investment should be considered as the perianth and the two inner ones as the outer and inner integuments, a condition as in Angiosperms. The inner integument shows a long micropylar tube as in Ephedra. The inner integument is reduced to papery layer while the outer integument is differentiated into stony layer while its outside becomes fleshy, green in young stage, red when ripe and often edibel. Thus, there are three coats of the seed covering as in *cycas*.

There are several archesporial cells developing from the hypdermis and the sporogenous cells become deep-seated. Thompson found that reduction of one or more megaspore mother cells gave rise to linear tetrads of four megaspores of which the lowest became functional. According to Maheswari and Vasil in Gnetum the four megaspores do not have partition walls between them and all the megasporic nuclei take part in formation of female gametophyte. In this regard, the female gametophyte is tetrasporic. There is no nucellar beak in the female gametophyte and the tip is slightly disorganised to form a rather shallow pollen chamber.

The Male Gametophyte

The microspore nucleus divides to form a small lens-shaped prothallial cell and a large antheridial initial. The prothallial cell rounds up and does not undergo any further division. The antherdial initial divides forming a antheridial cell and a tube cell. Since a stalk cell is not formed in Gnetum, te antheridial cell directly functions as spermatogenous cell. At this three-celled stage, the pollen are shed. Whereas the tube nucleus is hyaline with a large nucleolus, the spermatogenous cell nucleus is rich in chromatin with a cytoplasmic sheath. The prothallial cell eventually degenerates.

ANATOMY

The transverse section of the stem resembles a dicot. It shows an endarch siphonostele made of 20 to 24 open, conjoint, collateral bundles arranged in a ring. The stem bounded by externally by epidermis with thck cuticle & sunken stomata. Next to epidermis there is a collenchymatous hypodermis which is then followed by parenchymatous cortex which develops subcrised fibres in irregular patches. The endodermis and pericycle are not distinct. The primary vascular bundles are separated by side primary medullary rays. Just outside the phloem is a patch of spicular sclerotic cells. The phloem proper is very regular in G ula showing sieve cells and the so called 'companion cells' in radial rows. The 'companion cells' or different from those in Angiosperms as they are cut off independently from the cambium. The cambium is not distinct. The xylem is endarch and the protoxylem is very small. The secondary xylem. in addition to normal tracheids show a number of vessels of different sizes. Such members are with multiseriate pits but endwalls show large single pits formed by the fusion of enlargement of circular pits and ultimate disappearance of endwalls – a condition more advanced than that in Ephedra but different from that of Angiosperms where vessels are scalariform. Secondary growth is normal in the arborescent species like *Gnemon.* In case of lianous members several successive rings of cambia develop one after another giving rise to polycyclic rings of anomalous secondary growth. Periderm is thin, irregular and the lenticels are absent.

The antomy of leaf recalls the characters of a dorsiventral dicotyledonous leaf. Florin and others have noted the presence of syndetocheilic stomata which are on the lower epidermis only. Mesophyll shows palisade and spongy layers but spicular sclerotic cells are mixed with the latter.

REPRODUCTIVE ORGANS

The Spore – producing members

The Microsporangium

Gnetum is usually dioecious member but bisexual inflorescence are also known in a few cases. As in Ephedra, both male and female flowers are borne in compound strobili or inflorescence. The inflorescence is usually a panicle & grows in the axils of leaves or in terminal positions on dwarf shoots.

Male strobilus develops at the axil of a bract or apically on a dwarf shoot. It shows a slender axis with a pair of concrescent bracts at its base and higher up each node of the axis bears opposite decussate bracts which are connate throughout their whole length making them look like cups. In the axils of these connate bracts (very often called collars), all the way around the axis, the staminate flowers are borne in several rings. In some cases male strobili are found with female flowers in the upper rings as in *G. gnemon* but the latter usually soon abort. Each male blower has a stalk with two unilocular anthers or microsporangia

The distribution and position of archegonia are also taken into consideration. The position of archegonia is related to the position of archegonia is related to the position of the pollen tube which sometimes reaches the embryo sac before the archegonium initials are selected. When the pollen tube is lateral in position in reference to the gametophyte as in *Sequoia* and *Widdringtonia*, the latter responds by the selection of numerous deep-seated and laterally placed archegonium initials. The usual micropylar position of archegonia is due to the usual micropylar position of the tip of the pollen tube. It is assumed that numerous scattered and indefinitely placed archegonia were a feature of ancient gymnosperms. On the contrary the paleozoic ovules that reveal archegonia and also the archegonia of heterosporous pteridophytes suggest the opposite conclusion. However, the archegonia tend to become definite in number and are organized in two ways: either as individual archegonia, each with its own jacket and chamber; or as an archegonial complex with a common jacket and chamber. The latter is exhibited chiefly by cupressineae, but it also seems to be natural condition from which to derive the free eggs of *Welwitschia* and *Gnetum* when archegonia are eliminated.

Evolution of Gymnosperms

The evolution of Gymnosperms can be presented group by group but they should be viewed as a whole. The gymnosperms comprise a class which includes extremely ancient lines of seed bearing plants. Their long evolutionary history contains many examples of organisms which flourished and finally became extinct. One of the most important and interesting of these extinct gymnospermous lines was an assemblage of plants having fern-like in foliage and general appearance but which possessed a primitive type of seed. For a long time, the leaves of these plants were classified as parts of fossil ferns. It was not until seeds were found attached to the pinnatified fronds that their unique nature could be fully understood. This group has been Well-mamed seed ferns or cycadofilicales. Their former existence is evidence of the origin of the gymnosperms from an ancient stock of fern-like pteropsid plants.

It is strongly opined that paleozoic groups cycadofilicales and cordaitales represent the historical background of gymnosperms. The two groups are of equal egg so far records are available. The cycadofilicales are Very much fernlike in every feature except their seeds, that their derivation from some ancient fern stock is or certain as to make phylogenetic connection. Two alternatives have been suggested for the origin of cordaitales. They might have originated from the same ancient fern stock or they could arise from the cycadofilicales very early. Of the two views latter seems to be much more tenable, because the cordaitales are much more removed from the ferns than are the cycadofilicales.

If this conclusion be accepted, it follows that gymnosperms began with cycadofilicales more ancient than any yet known. The cordaitales branched off from cycadofilicales earlier than our present records. It is more tenable that the two groups made the extensive gymnosperm flora of the carboniferous.

This paleozoic gymnosperms were succceeded by the mesozoic members in which four groups are recognized. The cycadofilicales gave rise to mesozoic Cennettiales and she Cycadales. The mesozoic Ginkgoales and Coniferales took their origin from Cordaitales. The Bennettitales have been traced almost to the Paleozoic. The relation of the Bennettitales to the Cycadales is not so clear. The two groups were either differentiated from a common stock that arose from the cycadofilicales and continued into the Mesozoic, or the Cycadales were differentiated early from the Bennettitales. The available records indicate that the Cycadales are much younger than the Bennettitales. They were scantly represented during the Mesozoic. The only question is whether the gymnosperms stock which came from the cycadofilicales into the Mesozoic is to be called Bennettitales or a Bennettitales – Cycadales plexus. Among the gymnosperms, the Cycadales, although relatively a modern group, are the nearest representatives of the paleozoic cycadofilicales.

The Ginkgoales and Coniferales are deemed to be connected independently with the cordaitales in the paleozoic period and they were well displayed in the Mesozoic. It is also thought that Ginkgoales were not a large group although such members were well represented in the Mesozoic. The group has continued as a single line into the present flora bearing certain features of the cordaitles which the coniferales have lost. On the other hand, the coniferales began that extensive differentiatiion during the Mesozoic & thus giving rise to six recognized tribes in the present flora. Among the six tribes, the Abietineal and the Araucarineae said to be earliest in their origin. The other tribes of our present flora were of later origin. The Cupressineae, the Taxodineae and possibily the Taxineae might have originated from Abietineae. The Podocarpinear possibily evolved from the mesozoic Araucarineae.

So far the Gnetales are concerned, their connections with the others are said to be obscure and the opinions regarding their origin should be considered as very tentative. Although proper fossil records are not available in the group still their widely scattered geographical distribution can indicate a considerable history. However, the present evidences go to indicate that Gnetales may have been derived from the cupressineae. According to Eames (1952) the cordaitales of the Paleozoic have been regarded as the ancestral stock from which the Gnetales may have originated. The Gnetales are said to have many puzzling features in their method of reproduction and because their evolutionary history is obscure they constitute a some What is olaned & poorly understood group of seed bearing plants.

Economic Importance of Gymnosperm

The gymnosperms are an economically important group of plants. They are spread all over the globe, with special reference to the temperate regions and higher elevations in the tropical regions. They are used economically in a variety of ways. A brief discussion on the economic uses of gymnosperms is given below

Wood : Wood is a very important economical product mainly obtained both from the gymnosperms and the antiosperms. The gymnospermous wood consists of tracheids, xylem parenchyma and xylem rays. Such wood lacks xylem fibres but has more cellulose. There is not differentiation between the heartwood and the sapwood unlike that of angiospermous wood. The gymnospermous wood finds great use where strength and durability are not required. It is much used for cabinet and furniture making, joinery work and interrior deoration.

The wood of *Pinus palustris* (America) is hard, heavy, strong and course-grained and is mainly employed in bridges and ship-building. The wood of *pinus contorta* is very knotty but moderately strong and straight grained. It is used for log houses, interior finish, box making etc. The wood if *Pinus densiflora* is moderately hard, strong and resinous. It is used for general construction work and indoor finish of houses. The wood of *Pinus monticola*, *Pinus strobus* and *Pinus radiata* (merica) is straight grained, easily worked, compact and soft, but less durable. It is used for boxes, doors, frames and in door finish. *P. sylvestris* offers an excellent quality wood that is moderately strong and resinous. It is used extensively in house building, furniture, indoor finish, telegraph poles etc. *P. roxburghii*, chir pine, is one of the most widely used commercial timbers of India. It offers a resinous, non-durable and light wood much used for packing cases, construction work, poles, furniture, truck and buschassis etc. The wood of *P. wallichiana* is known as blue pine and used similarly as in the case of *P. roxburghii*.

The wood of *Abies alba*, an important timber tree of Europe is used in general carpentary. *Abies balesamea* is distributed mainly in North America and Canada. The wood obtained is light, weak and often used for ordinary buildings and box making. A *amabilies*. *A. grandis* (Canada), *A. concolor* (Western N America) and *A. procera* (America) offer wood for interior furnishing, boxmaking and general carpentary.

Agathis australis, 'kauri pine' is the chief timber tree of New Zealand. It is one of the largest timber producing trees of the world. The wood is useful in building construction, boats and wooden machiner.

Araucaria angustifolia is found mainly in Brazil. The wood is sueful in making doors, bus chassis and plywood. A. araucana produces pale brown wood for use in general building, carpentary etc.

Cedrus deodara and C. libani are much valued among the conifers. The wood of *cedrs* is highly durable, oily, scented and generally without resin ducts. *C. deodara* is one of the most important timbers of North India. The wood is as strong as teak. The wood is used for making doors, furniture, beams, wood carving etc.

Cryptomeria japonica yields strong, fragrant and durable wood. It is an important timber of Japan. The wood is used for building construction, furniture etc.

Cupressus torulosa is found to grow in North Himalayas. The wood is valued for building & furniture purposes.

Juniperus macropoda is the Himalayan species. It yields wood suitable for making pencils, wall plates and beams.

The wood obtained from the different species of Pinus like *P. palustris, P. sylvestris, P.densiflora, P. khasya, P. wallichiana, P.roxburgii* is used commercially for different purposes, such as in making packing boxes, construction work, furniture poles etc.

The wood of *Podocarpus nerifolius* is used for paddles, boat hooks, spars and masts.

Taxodium distichum has been introduced in Himachal Pradesh. The wood is dark coloured, dense and durable and mainly used for building construction, posts fencing, panelling etc.

The wood of *Thuja plicata* is one of the most durable ones for the presence of certain antibiotics. Because of its weather resistant properties, it is useful for glass house construction, lumber and outhouses.

Resins : Resins are said to plant exudates which is insoluble in water but soluble in organic solvents. It is mainly obtained from the Conifers. Resins are used in varnishes, enamels, plasters, medicine and ointments.

Copai: The term copals refers to hard resins which contain little essential oil. Copal is obtained both from living and fossil trees like *Agathes australis, Agathis alba.* The copal resin is used for interior work in preparation of spirit varnishes, printing ink, linobum etc.

Canada balsam: The resin is obtained from *Abies balsamea.* It has a very high refractive index nearing that of glass and for this reason it is highly preferred as mounting medium for microscopic objects and a cement for lenses in optical work.

Amber : It is a fossil resin mostly obtained from extinct pine. It is collected through mining and used in medicine and X-ray therapy.

Essential oils : Such oils are obtained from almost all those conifers that yield resins. The oil extracted from cedrasdeo dara is used in perfumery and scenting soaps. It is recommended for clearing tissued in histological work and also for use with oil immersion lens of the microscope.

The cedarwood oil obtained from *Juniper us mexicans* is used in scenting soaps, deodorants, insecticides etc.

Turpentine oil is obtained from a large number of species of *Pinus.* The turpentine oil obtained by distillation of oboresin is used as solvent, paint and thinner etc.

Fatty oils : A large number of gymnosperms contain fatty oils in the seeds. Such seeds are mainly eaten raw as nuts. The fatty oil obtained from the seeds of *cephalotaxas drupacea* is used as an illuminant in Japan, whereas the oil from *Pinus cembra* seed is used for food and paint.

Paper : Conifers are preferred throughout the would as source of pulp. Newsprint industry is largely based on conifer pulp. Writing and printing paper is manufactured from the wood of *Picea, Tsuga* and *Abies*. In India, *abies pindrow, Pinus roxburghii, Picea smithiana* provide excellent pulp. The wood of *cryptomeria japonica* is used for getting kraft paper.

Food : Starchy food is obtained from the seed kernels or from stem pith from a good number of cycads. The stem starch popularly known as 'Sago' is obtained from *cycas circinalis, cycas rumphii, C. revoluta* and also from Zamia and Macrozamia.

The young unfolded leaves of *cycas circinalis C. Pectinata* and *C. rumphii* are eaten in the Malay Peninsula.

Pine seeds have been used as a food item for a long time. The seeds of *Pinus gerardiana* have long been marketed in India.

The seeds of *cycas & Dioon edule* are a rich source of starch. Cycas seeds are made into a paste and eaten as cake in Nicobar Islands.

Tannins : Tannin is obtained from the bark of *Tsuga canadensis, Larix decidua, Picea alba, Aranceria* etc. Tannin is mainly used in leather and petroleum industry.

Drugs : Ephedrine is an important alkaloid obtained from green branches of *Ephedra sinica, E.equisetina* and *E.genardiana*. Ephedrine is an important component in the cough mixture. It is also used in nasal drops an dinhalents and in the treatment of asthma. The extract of leaves of *Ginkgo biloba* is useful in the treatment of cerebral insufficiency. The leaves of *Taxus baccata* are used in asthma, *bronchitis*, epilepsy and for indigestion. Taxol obtained from *Taxus brevifolia* has been found to be effective against ovarian cancer, breast cancer. In India male cone scales of *C.rumphii* and *C. cirinalis* are sold as an anodyne to lessen pain.

Decoration: By and large all the conifers beautify the hills. They always attract the minds of garden levers. Gymnosperms offer a good source material for making 'Bonsi' plants which are very much attractive to many people. A good number of species like *Juniperus chinensis, Pinus rox burghii, P. densiflora, P. nigra, P. parviflora, P. mugo* are used for 'Bonsai'.

The cycads are very often used as outdoor plantation for their graceful appearance.

Thuja, Juniperus, Araucaria and Pinus are also used as

Questions

1. Write a brief essay on the economic uses of Gymnosperms.
2. Give an account of evolution of Gymnosperms.

3. Describe the female strobilus of Pinaceal. Discuss the different views regarding the ovuliferous scale of Pines.

4. Enumerate the Indian species of Ephedraceae. Describe the salient anatomical features of Ephedra. Discuss the inter-relationship of Ephedrales.

5. Why is Ginkgo called a "living fossil"? Describe the ovulate strobilus of Ginkgo with reference to "Collar" morphology. Discuss the inter-relationship of Ginkgoales.

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BOTANY AND FORESTRY
Module No. - 11
Part - I, Paper - I (2nd Half)

Paleobotany

CONTENTS

AIMS AND OBJECTIVES OF PALAEOBOTANY

Palaeobotany is an interdisciplinary science which encompasses Botany, Geology, Physics and Chemistry. Palaeobotany is the study of fossil plants, as the derivation of the word itself suggest (Botan - herb, Paldegic - earth).

OBJECTIVES :

The objectives of Palaeobotanical studies include a) Botanical, b) Geological & Chemical and c) Applied aspects.

(a) BOTANICAL ASPECT :

i) Paleobotany can be approached from the view point of Botany when emphasis is placed upon the plant and from the geological angle in which rock containing the fossil primarily concerned. ii) It deals with the mysteries of plant life, its origin and future to depict the picture the picture of evolutionary trend. iii) It deals with the knowledge of plant life of the past (i.e. about the extinct plants), their morphology, structure which are studied from the fossil remains preserved in stratified rocks under the discipline of Palaeobotany. From the botanical standpoint Palaeobotany pertains to the interpretation of the following aspects :

1) Evidence of the early plant life.

2) Origin of land plant and their Antiquity.

3) Evidence of regressive Evelution.

4) Phylogeny of Gymnosperms.

5) Antiquity of Angiosperm.

6) Palaeoclimate.

7) Palaeography.

8) Palaeophytic flora.

(b) GEOLOGICAL ASPECT :

i) The geological objectives to the subject of Palaeobotany is mainly from the standpoint of the correlation of rocks. ii) It helps in the biostratigraphical Zonation. iii) It deals with index fossil, palaeopalynology. Paleopalynology helps as the stratigraphic markers in location of strata in relation to excavation and exploration of oil bearing strata and also coal bearing strata.

In addition to these, with micro-palaeobotanical data the riddles of a) Palaeo-phytogeography, b) Palaeoclimatology, c) Plantevolution d) Stratigraphical correlation, e) dating of ages etc. can be established, depending on the preservation of microfossils.

c) APPLIED ASPECTS :

Palaeobotanical study serves an important clue in the i) exploration of Hydrocarbon deposits and ii) other deposits like limestones etc. iii) Plants' microorganisms play a vital role in the formation of a number or rocks and minerals, all of which have a great economic value in the formation of lime stone, phosphorite, stromatolite, magnesite, salt, iron ores, bauxite deposit, manganese mineral, sulphur, copper ore, Diatomite, Anber and Coral reefs.

AIMS AND APPLICATION:

i) It aims at to know the evidence of earliest occurrence of life on earth; evolution of free O_2 in atmosphere; evolution of O_3.

ii) It helps to know how life began (origin of life) origin of prokaryote-metaphytes-metazoans.

iii) It helps to know Extinctions of plants and animal, earliest biosphere and ecosystem, change and diversification of ecosystem.

iv) It aims at to know aquatic to terrestrial life adoptation data base analysis to trace the pathway of origin of species. Data base analysis to trace the origin of diverse ecological riches.

v) It aims at to know distribution of life in time and space, biostratigraphy, distribution of continents, oceans, latitudes, continental drift, plate tectonic, Earth expansion theory, Palaeobiogeography, Palaeogeography, correlation of sediments parts of globe of small tectonically disturbed area geological napping, exploration, exploitation of other organic sediments.

vi) It helps in knowing Geotechnical property analysis of subsurface sediments in relation to heavy contrustraction.

vii) It has application in industries as follows :

* Palaeobiology of last 10,000 years to trace the sea level fluctuation pattern related to climatic

changes and global warming in recent past.

* Coastal area development and Management.

To trace guidance in modern biotechnology from Nature the best biotechnologist.

DATING OF SEDIMENTARY ROCKS

The geological ages of fossils are determined by two methods. The age of the rock which bears a fossil is determined and it is taken as the age of that fossil. The age of a fossil is determined directly using that fossil itself. Fossil bearing rocks usually can be dated by relative means, whereas non-fossilferous rocks are easily dated by calender system. This is called absolute dating.

The discovery of radioactive elements helped a lot in the estimation of the age of the rock. All radioactive elements are subject to disintegration. The time interval in which half of the atoms of a radioactive element disintegrate is called half-life. The half–life of different radioactive elements ranges from a fraction of a second to billions of years. The rate of disintegration is not influenced by external terrestrial conditions. The radioactive transformation that are most valuable in determining geological ages are uranium to lead, thorium to lead, rubidium to strontium, potassium to argon and carbon to nitrogen. This is commonly called the'radioactive clock method.

Uranium ^{238} is a radioactive element. Spontaneously, it undergoes radioactive decay emitting helium ions. During this process of radioactive decay of uranium ^{238} (Ur^{238}), fourteen new isotopes are formed. The fourteenth of which is the lead (Pb^{206}) which is a stable isotope. Emition of helium ions continues until lead is formed. This conversion of Ur^{238} to Pb^{206} can be summarized as

Uranium ..... lead + helium + energy.

The half life of Uranium^{238} is roughly about 4.5 billion years. Half of the Uranium ^{238} present in a rock will get transformed into lead^{206} by 4.5 billion years. By finding the ratio of Uranium^{238} to lead 206, of a given rock sample, it is possible to find out the age of the rock sample. Uranium ^{238} is accompanied by a small quantity of Uranium^{235}. This produces lead^{207} on radioactive disintegration. Thorium like Uranium also undergoes radioactive disintegration and produces lead. The simplified transformations are,

Uranium – 238 ... lead 206 (half – life 4510 million years)

Uranium – 235 ... lead 207 (half-life 7100 million years)

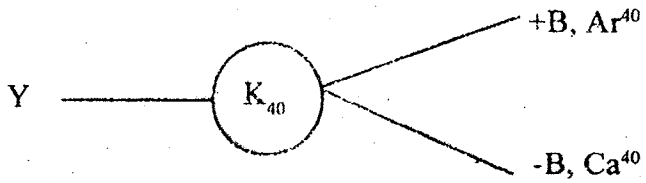
Thorium – 232 ... lead 208 (half – life 13,900 million years)

A fourth kind of lead, called natural lead or lead-204 does not appear to be derived from ratioactive sources and is constant in amount since the creation of the element. If the three dates obtained (Uranium

and Thorium) were similar then the results were termed concordant. If different dates were found they were called discordant.

THE POTASSIUM ARGON TRANSFORMATION

Potassium-40(K^{40}) can decay by emitting or capturing an electron. If it sheds a beta particle it becomes calcium-40 (Ca^{40}). If it picks up an electron, it becomes argon-40 (Ar^{40}). About one atom of potassium in seven becomes argon. Potassium mineral is common in both sedimentary and igneous rocks



Potassium^{40}.... Argon^{40} (half-life 1350 years)

The ratio of K^{49} to Ar^{40} is also found to be fairly dependable measure of age.

Rubidium-Storontium Method

Rudidium is generally dispersed in potassium bearing minerals. Rubidium-strontium method and potassium argon method give the same result.

Rubidium-87 ... Strontium 87 (half -life billion years)

ORIGIN AND EARLY EVOLUTION OF LIFE

The origin of life on earth is still an enigma. However, scientists could search out the factors and situations in the primitive earth leading to the evolution of organic molecules and origin of life. Theory of chemical evolution prior to origin of life is the most sensational hypothesis of early twentieth century propagated by the Russian physicist A.I. Oparin (1938-1922) and renowned biologist J.B.S. Haldane (1929) which is widely accepted by the scientists till to day. Stanely Miller (1953) demonstrated in his famous laboratory experiment the maximum possibility of the synthesis of amino acids in atmospheric condition of primitive earth rich in either CH_4, NH_3, H_2, H_2O or CO_2, CH_4, NH_3, H_2 and H_2O. These gases emitted due to volcanic erruptions in the primitive earth chemically reacted by the frequent electric discharges. According Oparin (1922) these reactions' started in the atmosphere and culminated in the sea'where the primitive life originated through the "coacervate" droplets formed in the "hot dilute primordial soup".

It is suggested that –

i) Life initiated and thrived t least at 30ft. (9.18m.) depth under water for a very long time in the primitive earth as because the lethal UV rays of sun do not penetrate water beyond this depth.

ii) Although ozone was present in the early atmosphere, the O_3 layer which is protecting the biosphere of the earth today was absent at the early phase of evoiûtion of earh; this layer started to accumulate slowly when O_2 liberating photosynthetic algae was growing abundantly during Proterozoic and gradually diversified since Cambrian; a portion of this O_2 with the effect of UV rays turned to O_3.

iii) The concentration of free O_2 in the atmosphere could reach only 0.01% of PAL by the end of Pre Cambran and was not sufficient for the terrestrial biosphere.

iv) Global climate is fluctuating between Glacial and Interglacial phases since Pre Cambrian.

Palaeobiological records of organic substances as Kerogen, aminoacids, in the Precambrian rocks are thus considered with enormous importance in the study of origin of life on earth and a separate branch of science.

The primitive organisms could develop the mechanism to assimilate directly the simpler compounds. The discovery of highly productive population of sulphur oxidizing bacterium and the very interesting new benthonic community including vestimentiferan tubeworms in the deep ocean (2.5 km. depth) hydrothermal vents at about 23°C and supported by chemosynthesis (Vincent 1984) indicate the possibility of occurrence of living organisms in such unfavourable condition.

Eventually, the mechanism to prepare food developed by the primitive organisms with the help of the energy source from the abundant supply of Sun rays led to the formation of Earliest Biosphere (Schopf. 1983) on earth. This is "the event for making inn earth a living planet. Because in this mechanism of utilising Sun rays for synthesis of food by the organisms with the help of a special ingredient of the cell, the pigments (blue green in the primitive stage), oxygen (O_2) is a by'product, Presence of Oxygen (O_2) at optimum percentage is essential for the survival of living organisms on earth which is about 21% in the Present Atmospheric level (PAL), abundant Sunlight acelerated the autotrophic mechanism of photosynthesis liberating more and more free O_2 while the bacteria of anoxic atmosphere took shelter in the anoxic mud and stagnant water as is found to day.

A portion of the free O_2 produced through the mechanism of photosynthesis was transformed into O_3 by the effect of UV rays of particular wavelengths and gradually accumulated around the earth's surface. This O_3 layer prevents the UV rays lethal for the living organisms to reach the earth's surface. In fact, availability of free O_2 in the atmosphere as well as formation of O_3 layer around the globe are equally significant phenomena in turning the darth to a living planet.

Thus the oxygen producing photosynthetic prokaryotes (non-nucleated) bloomed abundantly in the Pre Cambrian rocks dated between 3200-2000 m.a. through out the world is evidenced from widespread records of Stromatolites. Palaeobiological records of eukaryotes starts from late Precambrian (1500 m.a.) at least after 2000 millions of years of appearance of prokaryotes and strongly suggest prokaryotic origin of the eukaryotes. Recent experimental data on nuclear genes also indicate origin of the land plant nuclear gene from the prokaryotes which appeared 3500 million years ago.

A GENERAL ACCOUNT OF PLANT LIFE THROUGH THE INDIAN GONDWANA

The fossil floras of India revealed that the Gondwana flora is the richest, highly diversified and thoroughly investigated. The Gondwana land has been named after the Gond tribe of Madhya Pradesh ruled by Rani Durgabati during the region f Akbar. The term Gondwana was introduced by H.B. Medilicott in 1872. The term Gondwana system was published by Ottokar Feistmantel rocks with coal seams and fossil plants and animals. Sastry et al. (1977) assigned the geolocal time span from the Early Permian to the Early Cretaceous for the Gondwana sequence. However, recent work has indicated a span of 225 m.y., i.e. from the Upper Carboniferous to the Lower Cretaceous for the entire Gondwana system.

CLASSIFICATION OF THE GONDWANAS

Classification of the Gondwanas in India has been an issue of debate. Fiestmantel divided Indian Gondwanas into three divisions. According to the bipartite division, the Indian Gondwana deposits were divided into two. Feistmantel (1882) introduced a new division the Middle Gondwanas in which he placed all the localities containing Lower Gondwanas fossils but bearing the lighological aspect of the Upper Gondwanas. He included, Panchet and the transitional beds of Parsora in the Middle Gondwana. According to the period of dominance of these three floras, palaeobotanically the Gondwanas could be classified into

1) Lower, with dominance of the Glossopteris flora (Upper Carboniferious to permian) in which the Talchir Stage, Rikba stage, Kartharbari stage, Barren Measures stage and Raiganj stage are included.

2) Middle, from the arrival of Dicroidium with declining Glossopteris flora up to the dominance of Dicroidium flora (Triasic period) (Panchet, Mahadeva).

3) Upper, from the first arrival of Ptilophyllum flora to the end of the Gondwana era (Jurassic and Lower Cretaceous) (Rajmahal, Kota, Jabalpur, Umia).

LOWER GONDWANA FLORA

Lower Gondwana flora was predominated by gymnosperms, particularly members of the order Pteriodospermales. The flora is believed to have emerged either during the extensive Permocarboniferous glaciation or soon thereafter. The most predominant plant was Glossopteris, represented by numerous species of foliage, fructifications and stem-root axes. Hence some paleobotanists refer to Lower Gondwana flora as Glossopteris flora".

In addition to several species of Glossopteris and allied plants, a few blonging to Lycopsida and Filicopsida have also been reported from various Lower Gondwana localities in India.

List of Plants representing different groups

1. Pteriodophytes

Equisetales	:	Schizoneura, Phyllotheca, Stellotheca
Sphenophyllales	:	Sphenophyllum, Ranigantia, Trizygia
Lycopodiales	:	Cyclodendron.
Filicales	:	Alethopteris, Gondwanidium, Merianopteris, Pecopteris, Ptychocarpus, Sphenopteris, Angiopteridium, Cyathea, Callipteridium, Belemnopteris.

2. Gymnosperms : Gangamopteris, Glossopteris, Rubidgea, Rhabdotaenia

Glossopteridales	:	Taeniopteris, Macrotaeniopteris, Vertebraria, Cisteila, Lanceolatus, Dictyopteridiums, Scutum Ottokaria, Lidgettonia, Glossotheca.
Cycadales	:	Pseudoctenis.
Cordaitales	:	Noeggerathiopsis, Euryphyllum, Cordaicarpus, Samaraopsis
Ginkgoales	:	Ginkgophyton, Psygmophyllum.
Coniferales	:	Buriadia, Walkomiella, Damudoxylon, Barakaria, Moranocladus, Indoxylon, Barakaryoxylon, Dadoxylon, Kaokoxylon.
		Megaporoxylon, Trigonomyelon, Prototaxopitys, Araucarioxylon, Agathoxylon, Prototaxoxylon.

MIDDLE GONWANA FLORA

The flora combines some elements of the Lower and Upper Gondwana floras but is dominated by Dicroidium, Some of the other floral elements are : Glossopteris, Vertebraria, Schizoneura, Pecopteris, Cyclopteris, Sphenopteris, Noeggerathippsis and Cladophlebis.

UPPER GONDWANA FLORA

The fossil flora preserved in the Rajmahal hills, Bihar is the most highly diversified and most fascinating.

RAJMAHAL HILL FLORA:

The plant fossils described from the Rajmahal Hills are listed below :

The conifers in this flora includes members of Podocarpaceae, Araucariaceae and Taxaceae.

Podocarpaceae is represented by the following genera :-

1) Podosporites tripakshi Rao (1943a). with three ventral inflated wings similar to the microspores (pollen) of modern Podocarpus dacrydioides. 2) Podostobus sahnil (Vishnu-Mittre) Rao and Bose, a microsporangiate strobilus and 3) Elatocladus conferate foliage.

Araucariaceae is represented by : Petrified woods with araucarian type of pitting : Dadoxy amraparense, D.mandronense. Foliage : Brachyphy Hum florini, Pagiophyllum araucarioides, Dadoxylon agathiodes Krausel and Jain.

Taxales

Taxaceae : Petrified woods : Taxaceoxylon sp. cf. Rajamahalense, Torreyites constricta (Feist) Seward and Sahni. Taxoxylon rajmahalense and Taxites lanceolata.

Pentoxyleae : B.P. Srivastav described the first fossilmember of this group in 1946, a petrified stem which he named Pentoxylon sahnii. Recent work on Pentoxylon (Bose, Pal and Harris, 1985) has indicated that in addition to the main long there are three other types of short soots:1) slender parenchymatous short shoots; ii) thick woody short shoots, each with a broadly concical head and iii) slightly thicker parenchymatous short shoot, culminating in Sahnia flower.

Bennettiales:

The family Williamsoniaceae is known from the following assemblage preserved in the for of impressions, compressions, casts and petrifaction's.

Stem	:	Bucklandia, Sahnioxlon, (Homoxylon) and Williamsonia, Sewardiana
Fructifications		Willamsonia and Weltrichia.
Foliage	:	Ptilophyllum, Dictyozamites, Otozamites, Zamites and Pterophyllum.
Cycadales		
Stem	:	Sewardioxylon
Leaves	:	Nilssonia, Taeniopteris and Pseudoctenis.

AN OUTLINE IDEA OF CONTINENTAL DRIFT HYPOTHESES

Palaeobiological evidences in time and space have provided sufficient convincing data base in understanding the successive phases of Geological – Geographical evolution of the earth. The geographical positions of the continents and oceans are constantly changing as a result of the continuous geological (tectonic) activities.

EDWARD SUESS HYPOTHESIS

In 1858 there appeared a work titled La creation et res mysteries Devoiles, A suider, postulated that before the time of noah and the biblical flood there existed a great cracks encrusted with volcanoes. and during the great Deluge, a portion separated at a North - South and crack and drifted West word. Thus North America came into existence. Near the close of the 19th century the Australian Scientist Edward Suess became particularly intrigued by the many geological similarities shared by India, Africa and South America. He formulated a more complete theory of supercontinent that drifted apart following fragmentation success called that great land mass Gondwanaland and Gondwana, a geologic province in east central India.

ALFRED WEGNER'S HYPOTHESIS :

Another pioneering worker in this field is Alfred, Wegner, an energetic German materiologist. The primary concept of palaeogeography, the movements of continents and oceans through geological time was postulated by him (Wegner 1915-28 from Biram, 1966) supported by Du Toit (1937) on the basis of sedimentological evidences of glacial activities in the rocks of same age, the closer geographical fits of the continents now widely separated from each other and paldeomagnetic data in addition to the close correlation of the carboniferous – permian bioprovinces. His book. Die Eustenung der kontinents and Ocean. is considered a milestone in the historical development of the concept of continental drift. Wegner's hypothesis was quite straight forward. Building on the earlier notions of Edward Suess, he argued again for the existence in the part of a super continent that has dubbed Pandgoea. That portion of pangaea that was to separate and from North America and Eurasia come to be known as Lauresia. whereas the southern portion retained the earlier designation of Gondwana. According to Wegner's perception, Panagoea was surrounded by a universal ocean named Panthalassa, which open to receive the shifting continents when they began to split apart some 200 million years ago. The fragments of Panagoea drifted along like great stony rafts on the denser material below. In Wegner's view, the buldozing forward edge of the slab might be expected to produce mountain ranges.

PRESERVATION OF PLANTS/PLANT AS A FOSSIL

Fossil is any evidence of prehistoric life. It may be direct or any indirect evidence of life. It includes the remains of organisms or of parts of it and also anything connected with an organism proving its existence. Examples a) the impression of foot prints of dinosaurs, b) coprolites, c) gastroliths all are considered fossils.

TYPES OF FOSSILS

A. According to the nature of fossilisation fossils are of the following types.

1. Petrifaction or cellular permineralization
2. Coalified compression
3. Cast formation or Authigenic preservation
4. Impression

B. Indirect evidence of organism's presence is substances having definite organic present in the sediments.

1. Coal
2. Petroleum
3. Graphite
4. Algal lime stones and dolomite
5. Diatomite or diatomaceous earth
6. Amber (Fossilised resin of some ancient conifers). All these come under "Chemical fossil" category.

C. SUB-FOSSILS

These are prehstoric remains of plants and animals preserved in absolutely unaltered form by certain protective environment eliminating decomposition.

Eg. Refrigerated mammots of Siberia of Late Pleistocene age, and large tree trunks found at the base of the cretaceous oil sands of Northern Alberta.

1. PETRIFACTION OF PERMINERALIZATION

Permineralization occurs when the soluble silicates, carbonates, iron compounds and soon, infiltrate cells and the spaces between them. The precipitation of these compounds forms a rock matrix supporting the plant tissues. A possible mode of precipitation may be the interaction between "humic acids" with the soluble compounds. The humic acid may result from partial decomposition of some organic material. A few examples of slicified permineralizations are Devonian Rhynie cherts, Triassic woods from the petrified

forest of Arizona Tertiary Sequota trunks in yellow stone park vegetative and reproductive remains of flowering plants, conifers, ferns, bryophytes and fungi, have been found in a silicified condition from the Tertiary Deccan Intertraphion series of India.

Petrifactions are considered to be best preservation for study of external form as well as internal structure two types of petrifactions are mostly used for the purpose are silicified and calcified petrifaction, from carboniferous rocks popularly known as "Coal Balls". Besides these a few other types of petrifactions are a) Pyritic, b) Limonitic acid c) Phosphatic specimens.

COAL BALL: These are mostly ovoid or spherical, sometimes irregular shaped lime stone rocks found associated with seams of bituminous coal. They are generally of localized occurrence, and considered to be important sources of plant fossil in coal.

2. COALIFIED COMPRESSION

Following deposition in the sedimentary environment, the cell walls of the plant parts soften and then collapse. After a loss of gas, moisture and other soluble materials, because of pressure exerted by accumulated sediments and water, the residues are altered and consolidated to form a black, coaly deposit, when the sediments have become lithified, splitting of the stralified rock reveals the coalified compression on one surface and its impression or counterpart on the opposite face. They are wide spread in sedimentary rocks and are frequently found in shales above coal seams.

The vast majority of megafossils found preserved as coalified compressions are remains of leaves and branches, although the occassional flower, fruit, seed or cone is found. Microfossils include spores, pollen grains, and fragments of leaf cuticle.

Paleobotanists rely on coalified compressions to learn about leaf form and venation patterns, as well as details of epidermal cells and stomatal characteristics. These data are used on problems of stratigraphy and in addition we can learn more about paleoclimates and other aspects of physical and biological environments, as well as changing patterns in plant distributions.

3. CAST FORMATON or Authigenic preservation:-

This process is distinct from petrifaction and compression that leads to fossilization of plants. One explanation of the process is that the plant material develops an electric charge as it starts to decay in the depositional environment. Here is attracts colloidal and other small ionized particles of sediments that have an opposite charge. In this way sediments usually composed of iron and carbonate minerals accumulate and become cemented around the plant part. During the process internal structure "usually decomposed or lost and can be completely replaced by surrounding sediments. After lithification the external surface the plant is faithfully reproduced as a mold of rock, while the replaced internal structure of the plants form a cast. Probably the best known example of authigenic preservation is the iron stone concretion.

On rare occassions some infiltration and petrifaction can occur, thus preserving the internal structure as external features Other exceptional specimens combine authigenic preservation to form an internal cast covered by a qualified compression of the external tissues.

4. Some plants, notably certain algae, precipitate hard coats of limestone or silica. This revitant parts are preserved a without being changed by oxidation or physical factors. Such "hard part" preservation is called duripartic. So-called coralline algae belonging to the red and green algal groups, as well as some blue-green algae, are examples of plants whose calcareous hard parts are preserved. Diatoms with their indestructible silicified cell walls provide us with another example of duripartic preservation.

METHODS OF FOSSIL STUDY

Extraction of information from fossils has been done by the following methods of study

GROUND THIN SECTIONS

The credit for invention of this method goes to William Nicol.

METHOD

The specimen is cut to a convenient size and the surfacement for study is smoothed with # 400 or #600 carborundum on a grinding lap or sheet of plate of glass. The smooth surface is then mounted to a glass slide. After warning both the specimen and the slide smooth side of the specimen is put with melted resin. Subsequent cooling until resin hardens fixes the specimen with the glass slide. Synthetic resins specially prepared for this purpose, are recommended. The fastened specimen is cut as close to the glass as possible to obtain thin slice. The thin slice, thus, procured is ground on revolving #100 carborundum lap until it gets thin enough to permit light to pass through it. A thin section is obtained by washing and finishing the section with #400 carborundum. The specimen tends to chip away during grinding because of its fragile nature. This may be prevented by pouring peel solution on etching lightly along the smoothed surface. The peel solution forms a thin cellulose film between the specimen and slide. Such a film also helps in changing slides by soacking it in resin solvent and remounting it on the other slide. The section is then mounted, using a liquid resin mounting medium under a coverslip

PEEL TECHNIQUE

Koopmans and Walton developed peel technique. This method yields remarkable results on compressions of partially mineralized nature, like anthracite coasl; fine grained sediments, calcite and bituminous. The process involves two aspects 1) Selective maceration or ething of the surfaces ment of peling and 2) exact transfer of the structure of fossil.

Hydrofluric acid is essential for etching silicified specimens. A mixture of Nitrocellulose 115 gms. Butyl acetate 1000ml, Amyl alcohol 200ml Toluol 10ml and dehydrated castor oil 5ml, kept of two weeks before use is well suited for etching. The specimen is ground and etched with suitable mineral acid. Etched surface is washed with running water and dried. Then etched surface is covered with nitrocellulose and the film thus formed is allowed to dry for nearly 8 hours. Finally film is pelled from matrix.

ASHBY CELLULOSE FILM TRANSFER METHOD

This method was proposed by Lang. Plant fragments adjoining the rock surface coated with two coasts of peel solution. When peel solution film gets dried rock matrix removed by cutting it away. The material is then immersed with 25% hydrofluric acid to dissolve away the rock matrix until it is entirely removed.

MACERATION

Maceration may be employed in the study of compression fragments, where fragments are tough for removal without a transfer medium. Pollen and spore analysis has been done by the use of maceration technique. Coals are rich in spores, pollen, cuticle, mycelia and other tissue.

HARRIS METHOD : Harris (1926)

Material desired for maceration is immersed for several days in a mixture of strong nitric acid and 5% potassium chlorate. Acid is then washed out with water. Material is then placed in dilute sodium hydroxide. Break down of matrix results into a fine mud and plant fragments comprising cuticularized parts, which may be cleaned with 25% hydrofluric acid.

HYDROFLURIC ACID METHOD

Removal of intact plant materials before oxidation is sometimes advantageous. Fossiliferious Rocks are immersed in 25% hydrofluric acid for several hours until the mineral matrix breaks down. The plant fragments which settle at the bottom are repeatedly washed with water. Finally fragments are screened, separated, and subjected to Nitric acid, potassium chlorate and sodium hydroxide, as described by Harris.

'X' RAY RADIOGRAPHY

During cutting or peeling critical structures are either destroyed or by passed. X-ray radography overcomes this difficulty. Structure of calcified seeds are obtained from coal ball by this method.

Position of seed, spore, collapsed and boundaries between the tissues can be studied by this method.

BALSMA COATING METHOD

[Back (1955)] A thin slice of petrification is boiled gently on hot plate, in solution of one part of canada balsam and 3 parts of toluene. till toluence evoprates. Enough boiling results in firmness and prevents resin from getting extremely brittle on cooling. Surfaces are ground with carborundum, and chromium oxide in succession. Section is then washed and dipped in xylol or toluene. Both surfaces of section are examined under reflected light on mounting the section with a coverslip.

ACETOLYSIS METHOD

Acetolysis method is a modified method of Erdtman used in Palymology. It is used to study the wall sculpture of spores and pollens. Acetolysed, stained, and bleached specimens reveal internal structures.

ACETOLYSIS TECHNIQUE

Acetolysis Techniques was first introduced by Eerdtman in the study of Pollen grains. This method is of special significance to study wall sculpture of spores and pollens. The main drawback of this method is the expansion in the size of the material acetolysed. Acetolysed preparations should be employed in the study of wall structure alone and size-determination should be made on unacetolysed specimens. The acetolysis technique is as follows :-

1. Collect material (antherlobs for pollen study) and keep it in 70 percent alcohol for an hour.
2. Transfer material with the alcohol to a polythene centrifuge tube. Crush the material by a glass rod.
3. Pass the dispersion through a metal sieve (48 divisions per sq. cm.) and collect it in a glass centrifuge tube.
4. Centrifuge the content and decant off the alcohol.
5. Wash the sediment with glacial & acetic acid. Centrifuge and decant the acid.
6. Add about 5 ml of freshly prepared "acetolysis mixture" (a mixture of 9 parts acetic anhydride and 1 part con. sulphuric acid) into tube (the mixture when made, reaches a temperature of about 70°C).
7. Place the tube with acetolysis mixture in a water bath and boil the water to boiling point. Stop the flame when the water boils but leave the tube in hot water for 3-5 minutes (till the time a medium

of brown colouration is attained by the acetolysis mixuture).

8. Centrifuge and decant of superantant.

9. Add glacial acetic acid over the sediment. Cenrifuge and decant the acid.

10. Wash the sediment with distilled water, centrifuge and decent the water. This may be repeated once or twice. The acetolysed pollens or spores are brown in colur.

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3. Principles of Physical Geology, A. Holmes.

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SET OF QUESTIONS

1. Discuss the aim and obfectives of Palaeobotany.

2. What are the different forms of evidence of earliest life recorded in the pre-cambrian rocks? Describe the diverse type of life form during the time of early evolution of life.

3. Who postulated the theory of continental drift? Discuss the theory which supports continental drift.

4. Discuss briefly the fossil flora of Indian Gondwana.

5. Discuss the Tertiary flora of India.

"Learner's Feed-back"

After going through the Modules / Units please answer the following questionnaire.
Cut the portion and send the same to the Directorate.

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1. The modules are : (give ✓ in appropriate box)
   ☐ easily understandable; ☐ very hard; ☐ partially understandable.

2. Write the number of the Modules/Units which are very difficult to understand :
   .....
   .....
   .....

3. Write the number of Modules / Units which according to you should be re-written :
   .....
   .....
   .....

4. Which portion / page is not understandable to you? (mention the page no. and portion)
   .....
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5. Write a short comment about the study material as a learner.
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(Full Signature of the Learner)

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