M. Pharm. I Semester Main Examinations, 2013
Subject: Pharmaceutical Biotechnology

Section A

1. A) ii) Passive immunity

   B) i) Coiled around a common axis

   C) iii) Anti-sera

   D) ii) A plasmid that contains foreign DNA

   E) iii) Carry foreign DNA into cells

   F) i) IgD

   G) i) Hybridoma

   H) iii) Effluent treatment

   I) iii) Both the above

   J) iii) Priming

   K) i) Low molecular weight

   L) iv) All of the above

Section B

2. Detail proteins and different steps involved in recombinant DNA technology.

Answer:

Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in biological organisms.

- The original recombinant DNA technique proposed in 1974 by Cohen and Boyer.

Proteins/enzymes involved in rDNA technology

- Type II restriction endonuclease: Cleaves DNA at a specific base sequence
- DNA ligase: Binds two DNA molecules or fragments
- DNA polymerase I: Fills single-stranded gaps in duplex. DNA by stepwise addition of nucleotides to 3' ends [removes RNA pirmer]
- Reverse Transcriptase: Makes a DNA copy of an RNA molecule
- Polynycleotide Kinase: Adds a phosephate to the 5'-OH end of a polynucleotide, to label it or permit ligation.
- Terminal transferase: Adds homopolymer tails to the 3'- H ends of a linear duplex
- Exonuclease III: Removes nucleotide residues from the 3' ends of a DNA strand

- Bacteriophage {lamda} exonuclease: Removes nucleotides from the 5' ends of a duplex to expose 3' single-stranded ends
- Alkaline phosphatase: Removes terminal phosphates from the 5' end, the 3' end, or both.

Tools of rDNA technology

1. Restriction enzymes
2. Vectors
3. Host Cell

The steps of rDNA technology are:

- Isolation/selection of gene of Interest: Make or isolate the DNA for the gene of interest
- Cutting and joining: Cut and join DNA (gene) to another type of DNA molecule to prepare it for transfer into the production organism- Formation of rDNA/Chimeric DNA
- Tranformation/transfection: Introduce the joined DNA into the host cell
- Screening: Check the cells for the acquisition of the DNA and expression of the foreign gene
- Culturing: Grow the cells in large amounts for peptide production

Isolation of Gene of Interest

- Genomic Library
- cDNA Library
- Isolation from natural source
- Chemical Synthesis

Amplification of gene

1. PCR
2. Insertion of foreign DNA (Gene of Interest) in to Vector (Amplification of gene of interests)

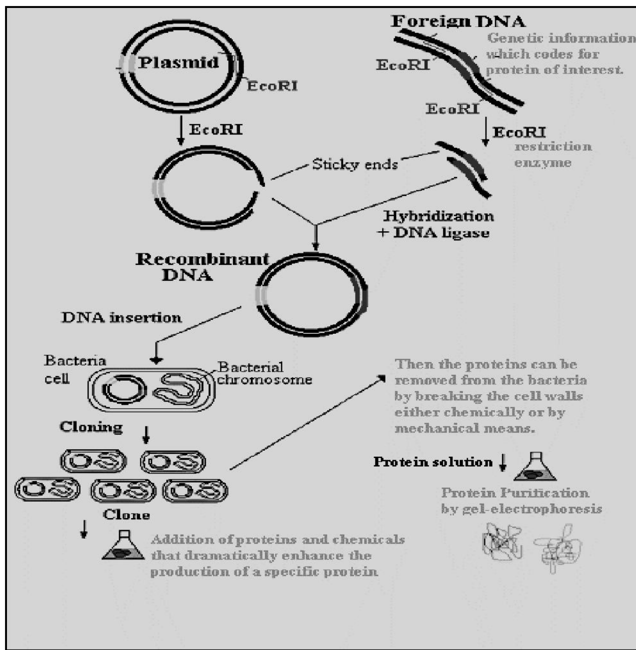
Method of transfer

Transfomration
Transfection

Selection of recombinants

1. Direct Selection using antibiotics resistance gene
2. Blue white Screening
3. Colony Hybridization'
4. Plaque lifting method
5. Immunolgical methoss
6. Blotting

Cloning and culturing (Fermentation) of recombinants
Downstream processing



3. Write a detail account of human gene therapy with special emphasis on single gene disorder.

Answer

Definition: It is an approach to treating disease by either modifying the expressions of an individual's genes or correction of abnormal genes

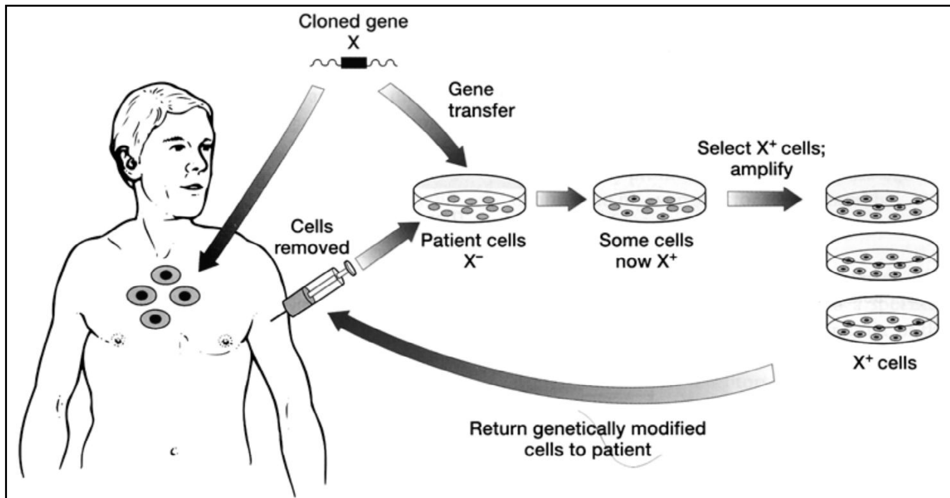
Types of Gene Therapy

- Germline gene therapy
- Somatic gene therapy
  - Gene supplementation
  - Gene replacement
  - Targeted killing of specific cell-types
  - Targeted inhibition of gene expression

Human gene therapy is the replacement of an absent or faulty gene with a functioning gene. As a result, the body is able to produce the correct enzyme or protein, thereby eliminating the cause of the disease.
There are essentially two types of gene therapy:

- Somatic cell therapy and germ line therapy.

  Somatic cell therapy involves treating any cells of the individual, except the gametes, at the cellular level to correct an absent or malfunctioning gene.
  This can be accomplished in three ways:
  - Ex vivo- involves removing cells from the patient, altering the genetic material, and placing them back into the patient.
  - In situ- requires the vector be placed directly into the affected tissues.
  - In vivo gene therapy involves injecting the vector into the bloodstream. The vector then must find the target tissue and deliver the therapeutic genes.
- Germ line gene therapy treats the gametes or an embryo, which would be used in the case of in vitro fertilization. There are some arguments in favour of germ line gene therapy (for example, it would allow the correction of disease-causing mutations that are certain to be passed on) but many more arguments against.



Ex vivo and In vivo Gene therapy

There are several types of gene therapy:

Gene augmentation therapy

This is appropriate for the treatment of inherited disorders caused by the loss of a functional gene product. The aim is to add a functional copy of the lost gene back into the genome and express it at sufficient levels to replace the missing protein. It is only suitable if the pathogenic effects of the disease are reversible.

Gene inhibition therapy

This is suitable for the treatment of infectious diseases, cancer and inherited disorders caused by inappropriate gene activity. The aim is to introduce a gene whose product inhibits the expression of the pathogenic gene or interferes with the activity of its product.

Killing of specific cells

This is suitable for diseases such as cancer that can be cured by eliminating certain populations of cells. The aim is to express within such cells a suicide gene, whose product is toxic. One approach is the expression of an enzyme that converts a harmless prodrug into a highly toxic molecule. Another is the expression of a protein that makes the cells vulnerable to attack by the immune system. It is very important to ensure that suicide genes are appropriately targeted, otherwise the therapy would result in widespread cell death.

The principle hurdles of Human gene therapy are:

– Regulation
– NIH Guidelines
– Human Experimentation
– Ethics
– Eugenics

- Inefficient delivery Systems to Target Cells
- Poor Gene Expression Levels
- Adverse Immune Reactions to Vector
- Insertional Mutagenesis-Causing Other Diseases (e.g., leukemia)
- Human Error-Failure to Adhere To Strict NIH and IRB Procedures (Experimental Therapies).

Gene Delivery vehicle – Vectors

1. Viruses

   Retroviruses
   Adenoviruses

Adeno associated virus
Herpes Simplex Virus
2 Non-viral methods
Injection of Naked DNA
Physical Methods to Enhance Delivery
Electroporation
Gene Gun
Sonoporation
Magnetofection
Chemical Methods to enhance Delivery
Lipoplexes
Polyplexes
Inorganic Nanoparticles
3 Hybrid methods

Single gene disorders

Monogenic diseases are prime targets for gene therapy due to their simple single gene mutations. Their disease causing mechanisms are easier to elucidate which is advantageous for choosing a target for gene therapy. In addition, the execution of therapy is more straightforward, since it is easier to transfer single genes into cells instead of several genes. Other important factors are the location and the type of cell in which the gene has to be transferred. Is the cell reachable with existing delivery systems? Is the cell already differentiated or is it a still dividing stem cell? Does gene transfer need to be repeated or is a one-time transfer sufficient? All these questions have to be considered in order to choose the right target for gene therapy, and it must be noted that not every disease caused by single gene mutations can be targeted.

Examples of successful gene therapy for inherited single gene disorders

Disorder	**Cells altered**	**Gene therapy strategy**
ADA deficiency	T cells and hemopoietic stem cells	*Ex vivo* GAT using recombinant retroviruses containing an *ADA* gene
Cystic fibrosis	Respiratory epithelium	*In vivo* GAT using recombinant adenoviruses or liposomes to deliver the *CFTR* gene
β–thalassemia	hematopoetic stem cells in Bone Marrow	*In vitro GAT using β-globin-expressing lentiviral vector*

GAT, gene augmentation therapy.

4. Write a detail note on production technology of monoclonal antibodies
Answer:
Hybridoma may be defined as — 'the cell produced by the fusion of an antibody-producing cell and a multiple mycloma cell'. Importantly, the 'hybrid cell' is capable of producing a continuous supply of identical antibodies' those bind specifically with the single epitope/antigen.
specific hybridomas are either cultured *in vitro* or made to pass *via* the **mouse peritoneal cavity** to obtain the desired *monoclonal antibodies* (MABs) ; and this sequential procedures encountered is usually termed as **hybridoma technology.**

In 1984, the Nobel Prize for Physiology and Medicine was bagged by Georges Köhler and Cesar Milstein for their remarkable discovery. Olsson and Kaplan in 1980 first time produced Humanized Monoclonal Antibodies.

Immunization

Immunization of a mouse against the antigen followed by repeated booster injections until a suitable serum response has been obtained. The animal is then sacrificed and the spleen removed aseptically.

Generation of Myeloma

Antibody producing hybridomas can be generated either by transformation of the cell line or by fusion of the sensitized B lymphocytes to plasmacytoma or myeloma cell lines.

Cell Fusion

Objective of the fusion process is to produce hybrid cells that incorporate the immortal characteristics of the myeloma cell with the antibody secreting properties of the antigen-sensitized lymphocytes.

Fsogenic agents

Sendai virus
PEG
Electrofusion

Screening Assays

Any fusion will produce a high proportion of clones that do not secrete antibodies of interest. Any cloning of cell cultures will also result in a proportion of previously secreting lines ceasing production. Therefore, it is vital that there are suitable methods available to determine which cells are, and which are not, producing antibodies. Two common assays are used

ELISA
RIA

Cloning

The full potential of the monoclonal antibody technique can be realized only if the cell culture producing the antibody is truly monoclonal, consisting solely of cells derived from a single progenitor. It is necessary to isolate those viable cells still containing functional genes coding for immunoglobulin synthesis. Two methods are commonly used to derive monoclonal populations from heterogeneous cell mixtures.

- Cloning in soft agar.
- Cloning by limiting dilution,

Cloning can also be performed using a FACS. Although a powerful technique, it is available to only a limited number of laboratories because of the cost of the equipment. Antigen coupled to a fluorescent marker is incubated with the cells. Cells with surface immunoglobulins of relevant specificity can then be isolated and sorted into individual tissue culture wells.

Cell Line Characterization

Once a hybridoma has been cloned, it is necessary to characterize the line to determine the conditions for optimal growth and secretion.

After cryostability has been established, growth rate and antibody secretion rates can be examined using defined media.

Antibody Characterization

Initially, the antibody must be tested to establish whether binding occurs with the immunogen, with and without any carrier molecules used in the immunization.

Once the specificity of the antibody has been established, it can be further characterized with respect to it isotype. This may be relevant to its application, but is also important if class switch variants are to be detected.

Humanized Monoclonal Antibodies

The Allergic response of patients to murine MABs is primarily a reaction to the Fc component of the antibody. Early protein engineers fused murine exons coding antibody Fv with human Fc exons to generate chimeric genes. The resulting chimeric MABs preserve the binding characteristics of the mouse antibody but have the effector functions of the human antibody and a reduced potential for antiglobulin reaction. This technique is now well established and several chimeric MABs have been approved by the FDA. This approach can be taken further to produce “humanized” MABs by grafting the CDRs of the murine MAB onto a human antibody framework.

Large Scale Production

Ascites Mice
Mammalian Cell Culture
Bacterial Cell Culture
Transgenic animals
Transgenic Plants

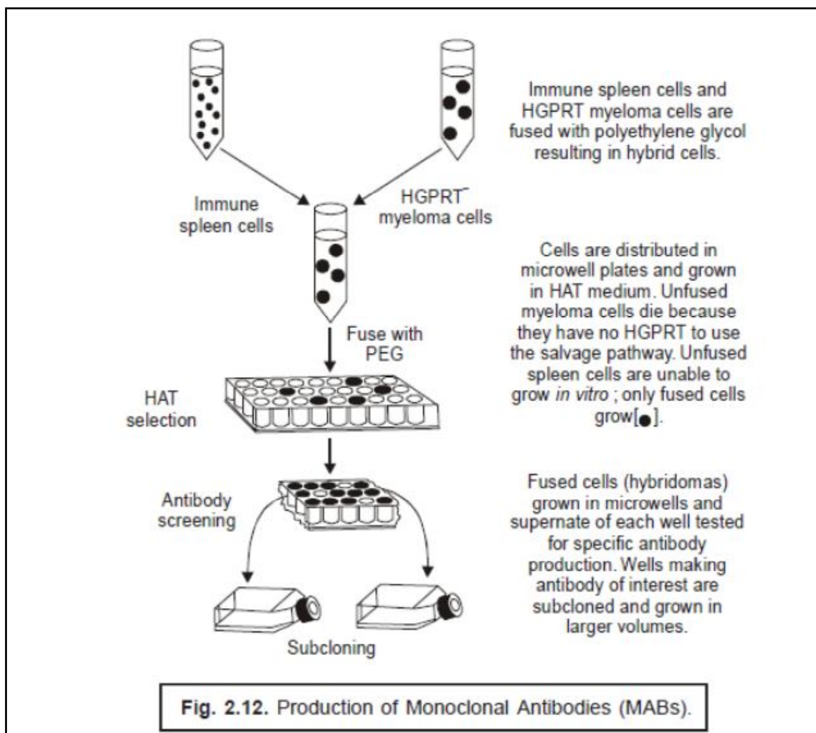


Fig. 2.12. Production of Monoclonal Antibodies (MABs).

5. Write in detail the mechanism and significance of hypersensitivity reactions

Answer:

Hypersensitivity refers to undesirable (damaging, discomfort producing and sometimes fatal) reactions produced by the normal immune system. Hypersensitivity reactions require a pre-sensitized (immune) state of the host. Hypersensitivity reactions can be divided into four types: type I, type II, type III and type IV, based on the mechanisms involved and time taken for the reaction. Frequently, a particular clinical condition (disease) may involve more than one type of reaction.

Type I Hypersensitivity

It is also known as immediate or anaphylactic hypersensitivity. The reaction may involve skin (urticaria and eczema), eyes (conjunctivitis), nasopharynx (rhinorrhea, rhinitis), bronchopulmonary tissues (asthma) and

gastrointestinal tract (gastroenteritis). The reaction may cause from minor inconvenience to death. The reaction takes 15-30 minutes from the time of exposure to the antigen. Sometimes the reaction may have a delayed onset (10-12 hours). Immediate hypersensitivity is mediated by IgE.
The primary cellular component in this hypersensitivity is mast cell or basophil. The reaction is amplified and/or modified by platelets, neutrophils and eosinophils. A biopsy of the reaction site demonstrates mainly mast cells and eosinophils. The mechanism of reaction involves preferential production of IgE, in response to certain antigens, allergens (Figure 1). IgE has very high affinity for its receptor on mast cells and basophils. A subsequent exposure to the same allergen cross links the cell-bound IgE and triggers the release of various pharmacologically active substances.

Type II Hypersensitivity

It is also known as cytotoxic hypersensitivity and may affect a variety of organs and tissues. The antigens are normally endogenous, although exogenous chemicals (haptens) which can attach to cell membranes can also lead to type II hypersensitivity. Drug-induced hemolytic anemia, granulocytopenia and thrombocytopenia are such examples. The reaction time is minutes to hours. It is primarily mediated by antibodies of IgM or IgG class and complement (Figure 2). Phagocytes and K cells may also play a role (ADCC). The lesion contains antibody, complement and neutrophils. Diagnostic tests include detection of circulating antibody against tissues involved and the presence of antibody and complement in the lesion (biopsy) by immunofluorescence. The staining pattern is normally smooth and linear, such as that seen in Goodpasture's nephritis (renal and lung basement membrane) and pemphigus (skin intercellular protein, desmosome). Treatment involves anti-inflammatory and immunosuppressive agents.

Type III Hypersensitivity

It is also known as immune complex hypersensitivity. The reaction may be general (e.g., serum sickness) or may involve individual organs including skin (e.g., systemic lupus erythematosus, Arthus reaction), kidneys (e.g., lupus nephritis), lungs (e.g., aspergillosis), blood vessels (e.g., polyarteritis), joints (e.g., rheumatoid arthritis) or other organs. This reaction may be the pathogenic mechanism of diseases caused by many microorganisms. The reaction may take 3-10 hours after exposure to the antigen (as in Arthus reaction). It is mediated by soluble immune complexes. They are mostly of IgG class, although IgM may also be involved. The antigen may be exogenous (chronic bacterial, viral or parasitic infections), or endogenous (non-organ specific autoimmunity: e.g., systemic lupus eythematosus, SLE). The antigen is soluble and not attached to the organ involved. Primary components are soluble immune complexes and complement (C3a, 4a and 5a). The damage is caused by platelets and neutrophils. The lesion contains primarily neutrophils and deposits of immune complexes and complement. Macrophages infiltrating in later stages may be involved in the healing process.

Type IV Hypersensitivity

It is also known as cell mediated or delayed type hypersensitivity. The classical example of this hypersensitivity is tuberculin (Montoux) reaction which peaks 48 hours after the injection of antigen (PPD or old tuberculin). The lesion is characterized by induration and erythema. Type IV hypersensitivity is involved in the pathogenesis of many autoimmune and infectious diseases (tuberculosis, leprosy, blastomycosis, histoplasmosis, toxoplasmosis, leishmaniasis, etc.) and granulomas due to infections and foreign antigens. Another form of delayed hypersensitivity is contact dermatitis (poison ivy, chemicals, heavy metals, etc.) in which the lesions are more papular. Type IV hypersensitivity can be classified into three categories depending on the time of onset and clinical and histological presentation. Mechanisms of damage in delayed hypersensitivity include T lymphocytes and monocytes and/or macrophages. Cytotoxic T cells (Tc) cause direct damage whereas helper T (TH1) cells secrete cytokines which activate cytotoxic T cells and recruit and activate monocytes and macrophages, which cause the bulk of the damage. The delayed hypersensitivity lesions mainly contain monocytes and a few T cells.

characteristics	type-I (anaphylactic)	type-II (cytotoxic)	type-III (immune complex	type-IV (delayed type)
antibody	IgE	IgG, IgM	IgG, IgM	None
antigen	exogenous	cell surface	soluble	tissues & organs
response time	15-30 minutes	minutes-hours	3-8 hours	48-72 hours
appearance	weal & flare	lysis and necrosis	erythema and edema, necrosis	erythema and induration
histology	basophils and eosinophil	antibody and complement	complement and neutrophils	monocytes and lymphocytes
transferred with	antibody	antibody	antibody	T-cells
examples	allergic asthma, hay fever	erythroblastosis fetalis, Goodpasture's nephritis	SLE, farmer's lung disease	tuberculin test, poison ivy, granuloma

6. What is immobilized enzyme? Enlist various methods applied for immobilization. Write a note on immobilized enzymes for therapeutic uses.

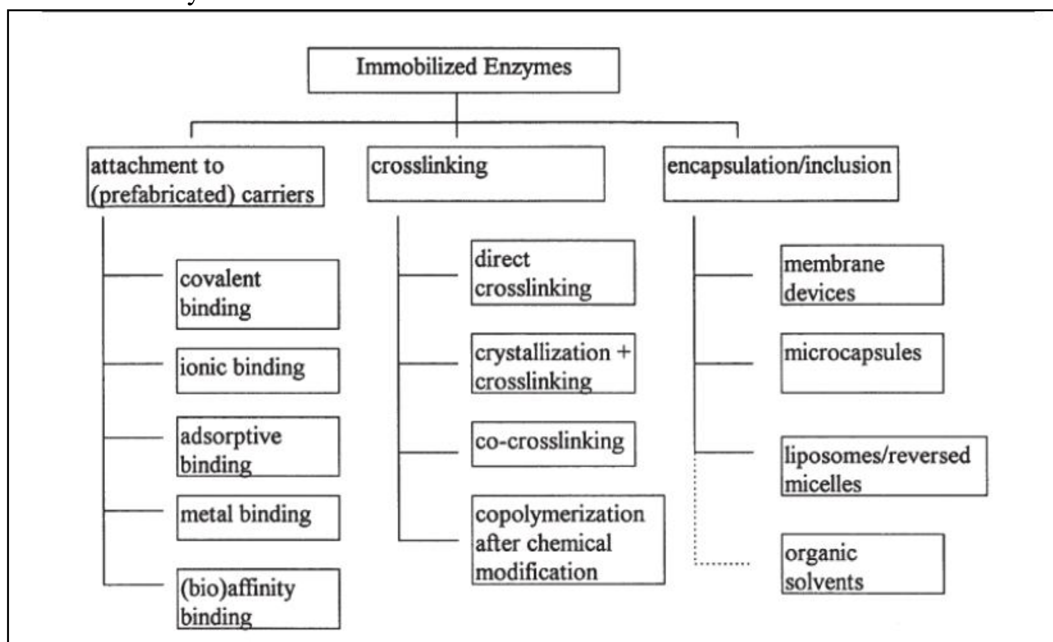
Answer:

Immobilized enzymes can be defined as "Enzymes with restricted mobility using carrier support"

Advantages

- Multipler reuses of enzymes
- Removal of enzyme from reaction mixture is possible
- Stabilization (Process/storage both) of enzyme
- Less effluent disposal problems
- Product contamination due to enzyme is reduced
- Allow development of multiple enzyme systems

Methods of enzyme immobilization



Therapeutic applications of immobilized enzyme

1.Enzyme Delivery
2. Bioreactors for Extracorporeal Enzyme Therapy

1. Enzyme Delivery

THE PRINCIPAL TYPES OF IMMOBILISED THERAPEUTIC ENZYMES

Preparation, possible methods of application (1)	Typical examples (2)	Type of action (3)	Possible methods of immobilisation (4)
Enzymes for replacement therapy in the treatment of digestive organ diseases (implantation, parenteral application)	pepsin chymotrypsin trypsin amylase lipase or their mixtures	local	microcapsulation entrapment into polymeric gels and particles
Antitumour enzymes (implantation; intravascular, parenteral or extracorporeal application)	asparaginase arginase nuclease desoxyribonuclease	local and/or systemic	microencapsulation entrapment into liposomes entrapment into red blood cells ghosts entrapment into gel particles immobilisation on soluble polymers
Enzymes for therapy of inherited lysosomal enzymes insufficiency (intravascular application)	glucosidase glucuronidase galactosidase	local	entrapment into liposomes (with facultative preliminary stabilization by intermolecular cross-linking or immobilisation on soluble polymers)
Enzymes for thrombolytic therapy (intravascular application)	plasmin urokinase streptokinase tissue plasminogen activator	local and/or systemic	immobilisation on soluble polymers entrapment into red blood cells ghosts immobilisation on biodegradable microparticles
Antibacterial, antiviral, antiallergic enzymes (intramuscular or intravascular application)	penicillinase lysozyme nucleases	systemic	immobilisation on soluble polymers entrapment into microcapsules, red blood cells ghosts, liposomes
Hydrolytic enzymes for the action on pathological tissues and anti-inflammatory enzymes (local application)	trypsin chymotrypsin collagenase papain ribonuclease desoxyribonuclease peptidase lysozyme	local	immobilisation on material for dressing, drainages, tampons (films, fibres, etc.) entrapment into microcapsules immobilisation into powdered polymeric gels

Bioreactors for Extracorporeal Enzyme Therapy
Extracorporeal shunts have been proposed for the treatment of several clinical conditions. The most likely applications for enzymatic treatment are the removal of urea during kidney failure, removal of toxins (e.g., paracetamol) during liver failure, or the reduction of key metabolites from the circulation to treat cancer. Urease is one of the most important enzymes in biomedical applications.

1. Urease is an enzyme that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Urea is one of the main metabolic end products, and the removal of its excess has been a major problem for patients suffering from renal failure. Hence, its immobilization by entrapment has been investigated by many workers for applications in biosensors and as artificial kidneys. The most attention has been given to the development of enzyme reactors, where the urea would be removed and the dialysis fluid prepared for further use.

2. L-asparaginase has been used for treating leukemias and disseminating cancers that require asparagines for growth, but this treatment presents several serious side effects. To overcome these problems, immobilized enzyme derivatives have been prepared on various supports for extracorporeal treatment. Blood can be passed over the immobilized enzyme, thus depleting the asparagine supply needed by the cancer cells. The enzyme does not come into direct contact with the organs to which it is toxic, and hypersensitivity reactions do not occur. With this type of treatment, however, the blood plasma must be first separated from the cells to minimize cell damage and then passed through a separate column containing the immobilized enzyme. L-asparaginase covalently attached to nylon tubing may constitute a useful system to be used in clinical applications.
3. The use of a highly specific enzyme Bilirubin oxidase to remove bilirubin from the bloodstream using a small reactor (extracorporeal circuit) containing bilirubin oxidase covalently immobilized in agarose beads.

4. Heparinase, an enzyme that degrades heparin into small polysaccharides, has also been immobilized into an extracorporeal device (artificial kidney bioreactor) to eliminate the anticoagulant properties of heparin (used to prevent clotting in the device) before the blood returns to the patient.

7. Write short note on any two
1) Biotechnology as an interdisciplinary subject
2) Physical and chemical nature of DNA
3) Applications of gene probes

1) **Biotechnology as an interdisciplinary subject**
Biotechnology" term is coined by Hungarian business man "Karl Erkey" in 1919 for mass production of pigs. It can be defined as "The application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services."
In this definition we refer to "scientific and engineering principles". These cover a wide range of disciplines but rely heavily on microbiology, biochemistry, genetics, biochemical and chemical engineering. Therefore, it is unlikely that a biotechnologist will be well versed in all of the disciplines underpinning biotechnology but, following his/her initial training in a particular discipline, will have gained a broader understanding of biotechnology by applying his/her skills to practical problems.
In this definition "biological agents" refers to a wide range of biological catalysts but particularly to microorganisms, enzymes and animal and plant cells. Similarly our concept of "materials" is all– embracing of organic and inorganic materials. In our definition we include not only the actual process in which the biological agent is used but also those processes concerned with its preparation and with the processing of biological materials resulting from its action.
Definition refers to the provision of "goods and services". The former include the products of industries concerned with food, beverages, pharmaceuticals, biochemicals and the winning of metals; the latter is largely concerned with water purification, industrial and domestic waste management.
With regard to the health field, biotechnology is restricted to the production of useful medicines, such as antibiotics, vaccines and antibodies, and does not include their use in medical treatment. Also, it does not cover those areas of medical engineering and technology, often referred to as biomedical engineering (or sometimes

bioengineering)The term “interdisciplinary” is used to describe an approach to learning and knowledge that integrates and benefits from the understanding and application of the approaches of different subjects and disciplines. Biotechnology is an interdisciplinary science including not only biology, but also subjects like mathematics, physics, chemistry and engineering. It is a blend of various technologies applied together to living cells for production of a particular product or for improving upon it. The nature of work of biotechnologists, being interdisciplinary, requires working together of people from different fields such as biology, chemistry, biochemistry, microbiology, molecular biology, Immunology, genetics, engineering, food science, agriculture etc.

2) **Physical and chemical nature of DNA**

Physical Nature of DNA

1. Absorption

The bases in DNA absorb ultraviolet light at the wavelength of 260 nm This absorption can be monitored using a spectrophotometerThis is one method used to f igure the concentration of DNA in solution. The less ordered the bases the more ultraviolet light is absorbed Free bases absorb 1.60 units at 260 nm. Single stranded DNA absorb 1.37 units at 260 nm. Double stranded DNA absorb 1.00 units at 260 nm

2. Density

Density can be measured by CsCl-density ultracentrifugation. CsCl, upon ultracentrifugation, will form a density gradient, with the most dense solution at the bottom Macromolecules, such as DNA, will concentrate in the area of CsCl that has the same density as themselves Hence, more dense DNA will migrate downward and less dense DNA upwards forming bands Density can be used to estimate G+C content. GC base pairs are more dense than AT base pairs Therefore, DNA with more GC base pairs will form bands lower down than an equal number of base pairs with high AT content. Density studies show the existence of satellite DNA

If chromosomal DNA is cut into about equal size pieces and subjected to CsCl-density ultracentrifugation two bands are formed One band contains most of the DNA from the genome. The second band (the satellite) contains about 5% of the DNA from the genome and has a highly repetitive sequence

3. **Denaturation**

DNA is considered denatured when the double stranded DNA molecule is converted into two single stranded molecules This can be monitored by noting the increase in absorption of ultraviolet light. As thermal energy increases, the frequency of hydrogen bonds breaking between the molecules increases.As temperature increases, the two molecules will separate into single-stranded molecules The Tm (melting temperature) of a DNA molecule is the temperature in which half the DNA molecules are denatures. The Tm is used to estimate the G+C content of a DNA molecule G-C base pairs are held together by three hydrogen bonds (A-Ts by two) and it therefore takes more energy (higher temperatures) to separate molecules with high GC contents

4. **Solubility**

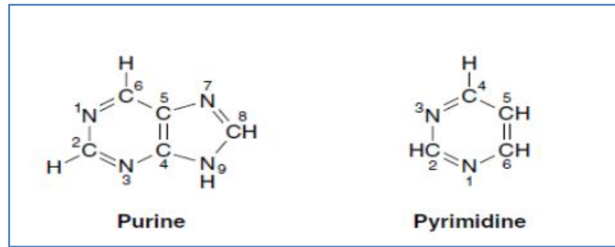
RNA is more soluble in aqueous solutions then DNA Ribose has a 2'-OH group where deoxyribose contains a 2'-H Hydroxyl groups are polar and dissolve in water better C-H is a non-polar bond and is therefore hydrophobic RNA is less stable then DNA The hydroxyl group on the 2' carbon of ribose is more reactive the hydrogen found in deoxyribose

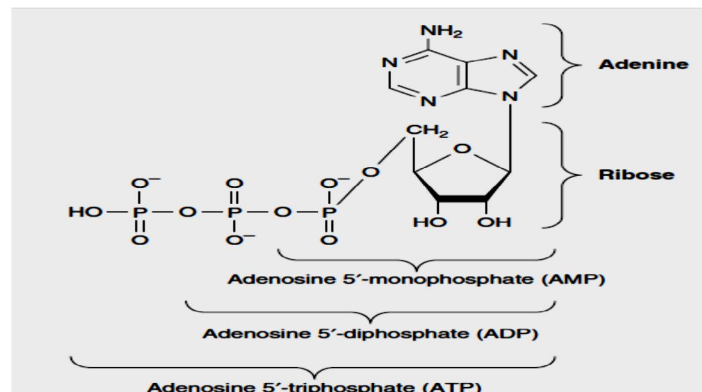
4. **Renaturation studies**

DNA that has been denatured will often come back together when condition are met. This is referred to as renaturation Renaturation occurs because hydrogen bonds of complimentary base pairs reform Slowly lowering the temperature or adding ions to solution may lead to renaturation. Renaturation rates are dependent on DNA concentration. The rate limiting step in renaturation is the collision of complimentary DNA molecules The more molecules of complimentary DNA molecules present, the faster they can find each other and renature DNA molecules in low concentration in solution will take awhile to find a complimentary partner, and will therefore renature slower In eukaryotes, three major drops in absorbance occur in renaturation studies The first drop in absorption is when the highly repetitive DNA sequence renatures. Since these are repeated so often, they are in the highest concentration The second drop in absorption occurs when the moderately repetitive DNA renatures Unique DNA sequences are the last to renature. These are in the lowest concentration and take the longest time to find each other

Chemical nature of DNA

Monomeric deoxynucleotide units of DNA—deoxyadenylate, deoxyguanylate, deoxycytidylate, and thymidylate are held in polymeric form by 3′,5′-phosphodiester bridges constituting a single strand, The informational content of DNA (the genetic code) resides in the sequence in which these monomers—purine and pyrimidine deoxyribonucleotides—are ordered. The polymer as depicted possesses a polarity; one end has a 5′-hydroxyl or phosphate terminal while the other has a 3′-phosphate or hydroxyl terminal. That requirement, together with x-ray diffraction data from the DNA molecule and the observation of Chargaff that in DNA molecules the concentration of deoxyadenosine (A) nucleotides equals that of thymidine (T) nucleotides (A = T), while the concentration of deoxyguanosine (G) nucleotides equals that of deoxycytidine (C) nucleotides (G = C), led Watson, Crick, and Wilkins to propose in the early 1950s a model of a double- stranded DNA molecule.





Base Formula	Base X = H	Nucleoside X = Ribose or Deoxyribose	Nucleotide, Where X = Ribose Phosphate
	Adenine A	Adenosine A	Adenosine monophosphate AMP
	Guanine G	Guanosine G	Guanosine monophosphate GMP
	Cytosine C	Cytidine C	Cytidine monophosphate CMP
	Uracil U	Uridine U	Uridine monophosphate UMP
	Thymine T	Thymidine T	Thymidine monophosphate TMP

3) **Applications of Gene Probes**

- Gene probes are **single-stranded** DNA or RNA.
- Gene probes **hybridize** to a **target** DNA or RNA sequence.
- Probe and target base sequences must be similar to each other but, depending on conditions, do not necessarily have to be exactly identical, *i.e.* their**complementarity** is not always 100%.
- Gene probes must be **labelled** otherwise the hybridization cannot be detected.

Gene probes have 3 basic applications in medicine:

1. Detection of specific nucleic acid sequences

Such sequences may be diagnostic of disease, *e.g.* the detection of a sequence unique to a particular microorganism would demonstrate its presence in a specimen and, perhaps, confirm an infectious disease. This is the principle of probes designed to detect and identify various infectious agents, including bacteria, protozoa and viruses. Probes can be especially useful for detecting microorganisms that grow slowly (*e.g. Mycobacterium tuberculosis*) or which cannot be cultured on artificial growth media (*e.g.* all viruses).

However, they are not usually capable of distinguishing between viable (live) and non-viable (dead) cells, which is an important consideration with, for example, food poisoning organisms - many of which are not harmful unless alive. Another problem is designing a probe to target a unique sequence so that it will only detect the organism of interest. Sometimes an organism may show a unique biochemical characteristic and a probe can be designed to target the gene of the enzyme involved. But it is rarely that easy!

There is more discussion of nucleic-acid based methods for the detection and identification of microorganisms in the PCR section.

2. Detection of changes to nucleic acid sequences

A change to the DNA sequence is a mutation, *e.g.* deletion, insertion, substitution. Changes in certain gene sequences can cause inherited diseases and their detection by probes can be diagnostic. Unfortunately, with some inherited diseases more than one type of mutation can cause the disease. In which case, a probe may have to be used under low stringency (to allow hybridization to a range of sequences) or several probes used (a "battery") to ensure that the target is "hit".

Some examples:

- cystic fibrosis (due to a 3 bp deletion).

3. Detection of tandem repeat sequences

- Tandem repeat sequences are 30-50 bps in length. Their size and distribution are distinctive for an individual.
- They can be detected using probes and PCR. They are the basis of so-called "DNA fingerprinting"
- It is used in forensic science to confirm the identity of a suspect from specimens left at the scene of a crime, *e.g.* any body fluid, skin, hair.
- This technique can also be used for paternity tests, sibling confirmation (or exclusion) and tissue typing.