SYLLABUS

ZOOLOGY

Molecular Biology, Bioinstrumentation & Biotechniques

UNIT-I **Protein Synthesis-I : Process of Transcription :** Fine structure of gene, RNA polymerases, Transcription factors and machinery, Formation of initiation complex, Initiation, elongation and termination of transcription in prokaryotes and eukaryotes.

UNIT-II **Protein Synthesis-II : Process of Translation :** The Genetic code, Ribosome, Factors involved in translation, Aminoacylation of tRNA, tRNA-identity, aminoacyl-tRNA-synthetase, Initiation, elongation and termination of translation in prokaryotes and eukaryotes.

UNIT-III **Regulation of Gene Expression-I :** Regulation of gene expression in prokaryotes : lac and trp operons in E. coli, Regulation of gene expression in eukaryotes : Role of chromatin in gene expression, Regulation at transcriptional level, Post-transcriptional modifications : Capping, Splicing, Polyadenylation, RNA editing.

UNIT-IV **Regulation of Gene Expression-II :** Regulation of gene expression in eukaryotes, Regulation at translational level, Post-translational modification etc., Intracellular protein degradation, Genes silencing, RNA interference (RNAi).

UNIT-V **Principle and Types of Microscopes :** Principle of Microscopy and Applications, Types of Microscopes : light microscopy, dark field microscopy, phase-contrast microscopy, Fluorescence microscopy, confocal microscopy, electron microscopy.

UNIT-VI **Centrifugation and Chromatography :** Principle of Centrifugation, Types of Centrifuges : high speed and ultracentrifuge, types of rotors : Vertical, Swing-out, Fixed-angle etc., Principle and Types of Chromatography : Paper, ion-exchange, gel filtration, HPLC, affinity.

UNIT-VII **Spectrophotometry and Biochemical Techniques :** Biochemical techniques : Measurement of pH, Preparation of buffers and solutions, Principle of Colorimetry/ Spectrophotometry : Beer-Lambert law, Measurement, applications and safety measures of radio-tracer techniques.

UNIT-VIII **Molecular Techniques :** Detection of nucleic acid by gel electrophoresis, DNA sequencing; DNA fingerprinting, RFLP, Polymerase Chain Reaction (PCR), Detection of proteins, PAGE, ELISA, Western blotting.

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CONTENTS

UNIT-I	Protein Synthesis-I : Process of Transcription	...3
UNIT-II	Protein Synthesis-II : Process of Transcription	...20
UNIT-III	Regulation of Gene Expression-I	...42
UNIT-IV	Regulation of Gene Expression-II	...57
UNIT-V	Principle and Types of Microscopes	...70
UNIT-VI	Centrifugation and Chromatography	...87
UNIT-VII	Spectrophotometry and Biochemical Techniques	...107
UNIT-VIII	Molecular Techniques	...127
	Model Paper	...152

UNIT-I

Protein Synthesis-I : Process of Transcription

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. What is gene in DNA?

Ans. A gene is the basic physical and functional unit of heredity. Genes are made up of DNA. Some genes act as instructions to make molecules called **proteins**.

Q.2. What is RNA polymerase and what is its function?

Ans. RNA polymerase synthesizes RNA by following a strand of DNA. RNA polymerase is an enzyme that is responsible for copying a DNA sequence into an RNA sequence, during the process of transcription.

Q.3. What did Archibald Garrod discover about DNA?

Ans. In 1908 British physician Archibald Garrod proposed the important idea that the human disease alkaptonuria and certain other hereditary diseases were caused by inborn errors of metabolism, suggesting for the first time that linked genes had molecular action at the cellular level.

Q.4. What is the difference between DNA and gene?

Ans. DNA is the genetic material, which is involved in carrying the hereditary information, replication process, mutations and also in the equal distribution of DNA during the cell division.

Gene are the DNA stretches which encode for specific proteins that regulates the traits of an organism.

Q.5. How are DNA and proteins packaged to form a chromosome?

Ans. In the nucleus of each cell, the DNA molecule is packaged into thread-like structures called chromosome is made up of DNA tightly coiled many times around proteins called histones that support its structure.

Q.6. Where are the genes located?

Ans. Generally, we say that the genes are present on chromosome. This gives a false impression that genes are some round ball like structures, which are present on chromosome. We know that genes are made up of DNA nucleotides. They are specific portions of DNA. A DNA molecule may contains few to several hundread genes with specific function. So, when DNA molecule undergoes supercoiling and packaging (condensation) to in division phase (metaphase), the genes becomes integral part of this condensed structures, which also known as chromosomes.

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Q.7. Is RNA polymerase used in DNA replication?

Ans. RNA polymerase does not play a role in DNA replication, it plays a role in DNA transcription. RNA polymerase makes *m*RNA from DNA.

Q.8. Which enzyme produces RNA polymerase?

Ans. Primase is an enzyme that synthesizes short RNA sequences called **primers**. These primers serve as a starting point for DNA synthesis. Since primase produces RNA molecules, the enzyme is a type of RNA polymerase.

Q.9. Is a promoter a transcription factor?

Ans. Promoters contain specific DNA sequences such as response elements that provide a secure initial binding site for RNA polymerase and for proteins called transcription factors that recruit RNA polymerase.

Q.10. Is the TATA box a promotor?

Ans. A TATA box is a DNA sequence that indicates where a genetic sequence can be read and decoded. It is a type of promoter sequence, which specifies to other molecules where transcription begins.

Q.11. Which enzyme produces *m*RNA?

Ans. During transcription, the DNA of a gene serves as a template for complementary base-pairing and an enzyme called RNA polymerase-II catalyzes the formation of a pre-*m*RNA molecule, which is then processed to form nature *m*RNA.

Q.12. What removes introns from RNA?

Ans. Introns are removed from primary transcripts by cleavage at conserved sequences called splice sites. These sites are found at the 5' and 3' ends of introns. Most commonly, the RNA sequences that is removed begins with the dinucleotide GU at its 5' end, and ends with AG at its 3' end.

Q.13. How many exons are there in a gene?

Ans. One average, there are 8.8 exons and 7.8 introns per gene.

Q.14. Why prokaryotes do not have introns?

Ans. After DNA is transcribed into RNA in a eukaryotic cells, its further edited in the nucleus, part of the RNA is cut out, this part cut out is the introns. Its absent in prokaryotes because there's no nucleus.

Q.15. Why is RNA splicing important?

Ans. It assists in the evolution process by forming different combinations of exons and thereby making new and improved proteins. New exons can be inserted into the introns to create new proteins without discrupting the functionality of the original gene.

Q.16. Why is *m*RNA capped and tailed?

Ans. *m*RNA capped and tailed both protect the transcript and help it get exported from the nucleus and translated on the ribosomes (protein-making 'machines' found in the cytosol first start super script, first end superscript. The 5' cap is added to the first nucleotide in the transcript during transcription.

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Q.17. Who first discovered introns?

Ans. Richard Roberts and Phil Sharp's labs showed that eukaryotic genes contain many interruptions called interons.

Q.18. How is DNA packaged into cells?

Ans. To packaged DNA inside the nucleus, cell wrap their DNA strands around scaffolding proteins to form a coiled condensed structure called chromatin. Chromatin is further folded into higher orders of structure that form the characteristic shape of chromosomes.

Q.19. How is the packaging of DNA different in eukaryotes and prokaryotes?

Ans. Prokaryotes contain a single, double-stranded circular chromosome. Eukaryotes contain double-stranded linear DNA molecules palkeged into chromosomes. The DNA helix is wrapped around proteins to form nucleosomes.

SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. What do you understand by gene?

Ans.

Gene

The definition of a gene changes with respect to different aspects of heredity. Classical concept considered gene as a unit of inheritance. Which explained phenotypic and genotypic similarities and differences, between parents and offspring. When the chemical nature was not known, a gene was defined as "the unit of heredity" or as an inherited factor that determines a characteristic.

After knowing about the chemical structure of DNA and the process of transcription we are now in a position to understand the exact concept and fine structure of gene.

We know that the genetic information is encoded in the base sequence of DNA. So, a gene consists of a set of DNA nucleotides. But how many nucleotides are encompassed in a gene and how is the information in these nucleotides organized? **Archibald Edward Garrod**, in 1902, suggested, that genes encode proteins. All proteins in a cell come from the genetic information, packed in the sequences of several nucleotides. So, in this light we can say that a gene is a segment of DNA consisting of several nucleotides that specify the amino acids of a protein.

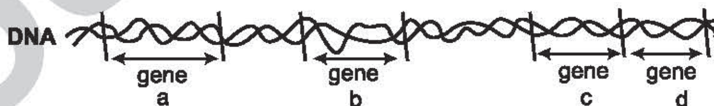

Fig. : Arrangement of Genes in a DNA

Q.2. What do you mean by RNA polymerase?

Ans.

RNA Polymerase

The primary step in the process of protein synthesis is the transfer of genetic information from DNA to *m*RNA. This process is called transcription, which is mediated by an important holoenzyme, called **RNA polymerase**. RNA polymerase is also required for the synthesis of tRNA, rRNA and other types of RNAs.

RNA polymerase is a DNA-directed or DNA dependent polymerase, which can initiate and synthesise RNA molecule taking genetic information from a DNA molecule.

It is often abbreviated as RNAp or RNA pol.

RNA polymerase has an inbuilt helicase activity which is required to cut the hydrogen bonds between two strands of DNA. During transcription, the RNAp locally opens the double-stranded DNA (using helicase activity), so that one strand of the exposed nucleotides can be used as a template for the synthesis of RNA. RNAp can initiate transcription at specific DNA sequences known as promoters. It then produces an RNA chain, which is complementary to the template DNA strand. The process of adding nucleotides to the RNA strand is known as elongation.

In eukaryotes, RNAp will preferentially release its RNA transcript at specific DNA sequences encoded at the end of genes, which are known as **terminators**.

RNAp are very specific and efficient enzymes and can build RNA chains as long as 2.4 million nucleotides (the full length of the dystrophin gene, the longest human gene).

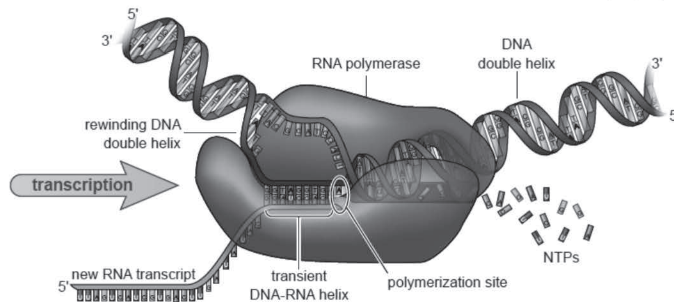

Fig. : An ultrastructure of RNA polymerase

The RNA polymerase is a big enzyme and its action is enhanced by a number of accessory proteins that join and leave the polymerase at different stages of the transcription process.

In prokaryotes (bacteria), only one type of RNA polymerase is present which can synthesise all type of RNA molecules including *m*RNA, *t*RNA and *r*RNAs.

Q.3. What is the differences between RNA polymeras and DNA polymerase?

Ans.

Differences between RNA and DNA polymerase

S.No.	Comparison	RNA Polymerase	DNA Polymerase
1.	Function	Transcription of DNA.	Replication of DNA.
2.	Purpose	To make RNA copies of genes.	To copy the entire genome.
3.	Time of Occurrence	Used in transcription during G phase(s).	Used in replication during S phase.
4.	Primer	Not required for transcription.	Required for initiation of replication.
5.	Base pairs used to synthesize product	Adenine, Guanine, Cytosine and Uracil.	Adenine, Guanine, Cytosine and Thymine.
6.	Resulting product	Single-stranded RNAs (*e.g.*, *m*RNA).	Double-stranded DNAs.

Q.4. Write about the eukaryotic RNA polymerase.

Ans.

Eukaryotic RNA Polymerases

All eukaryotic polymerases are large, multimeric enzymes, typically consisting of more than a dozen subunits. Some subunits are common to all RNA polymerases, whereas others are limited to one of the polymerases.

In eukaryotes, a large number of accessory proteins bind to the core enzyme and affect its function.

Most eukaryotic cells possess three distinct types of RNA polymerase, each of which is responsible for transcribing a different class of RNA. RNA polymerase I is located in the nucleolus, a distinct region of the nucleus where *r*RNAs are synthesized and combined with ribosomal proteins. RNA polymerase I catalyses the synthesis of all ribosomal RNAs except the small 5S *r*RNA.

RNA polymerase II stranscribes nuclear genes that encode proteins and gives rise to *hn*RNA (or *m*RNA). RNA polymerase III catalyses the synthesis of the transfer RNA molecules, the 5S *r*RNA molecules, and small nuclear RNAs.

Characteristics of the RNA Polymerases of Eukaryotes are as follows :

S.No.	Enzyme	Location	Products
1.	RNA polymerase I	Nucleolus	Ribosomal RNAs, excluding 5S *r*RNA.
2.	RNA polymerase II	Nucleus	Nuclear pre-*m*RNAs.
3.	RNA polymerase III	Nucleus	*t*RNA, 5S *r*RNA, and other small nuclear RNAs.
4.	RNA polymerase IV	Nucleus (plant)	Small interfering RNAs (*si*RNAs).
5.	RNA polymerase V	Nucleus (plant)	Some *si*RNA plus noncoding (antisense transcripts of *si*RNA target genes.

In present time, RNA polymerases IV and V have been identified only in plants.

Q.5. Write about the transcription unit with diagram.

Ans.

Transcription Unit

A transcription unit is a stretch of DNA that encodes an RNA molecule and the sequences necessary for its transcription. A transcription unit consists of three critical regions : a **promoter**, an **RNA-coding sequence**, and a **terminator**.

1. **Promoter** is a small DNA sequence which is present upstream. It is recognized by the RNA polymerase enzyme and it indicates which of the two DNA strands is to be read as the template and signals for the direction of transcription. The promoter also determines the transcription start site, the first nucleotide that will be transcribed into RNA.
2. **Structural gene** (RNA-coding region) is a sequence of DNA nucleotides that is copied into an RNA molecule. It actually contains the information for protein to be synthesized.

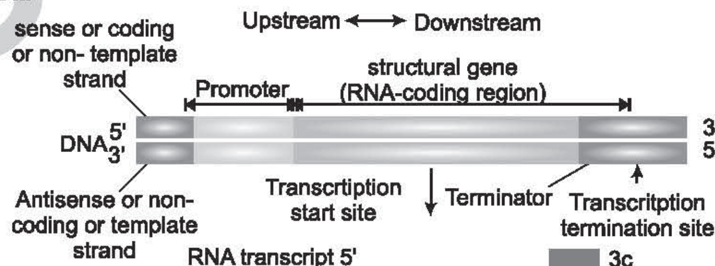

Fig. : A part of DNA, showing a transcriptional unit

3. **Terminator** is a sequence of nucleotides that is present downstream and it signals where transcription is to end. Terminators are usually part of the coding sequence, so transcription stops only after the terminator has been copied into RNA.

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Q.6. Differentiate between prokaryotic and eukaryotic transcription.

Ans.

Differences between Prokaryotic and Eukaryotic Transcription

S.No.	Prokaryotic Transcription	Eukaryotic Transcription
1.	Transcription takes place in cytoplasm.	Transcription takes place inside nucleus.
2.	Some RNA polymerase can synthesise all kinds of RNAs.	Each type of RNA is synthesised by a different RNA polymerase. *m*RNA is synthesized by RNA polymerase II.
3.	Transcription is immediately followed by translation because transcription and translation sites are the same.	Transcription is not immediately followed by translation, because transcription and translation sites are different.
4.	*m*RNA does not contain any non-coding regions (introns) so it is not heterogenous.	Newly formed *m*RNA contains many non-coding regions (introns), so it is heterogenous (*hn*RNA).
5.	*m*RNA is stable and therefore no RNA processing is required.	*m*RNA is unstable so RNA processing capping, polyadenylation and splicing are required.

Q.7. What are the functions of genes?

Ans. Functions of genes are as follows :

1. Genes control the morphology or phenotype of individuals.
2. Genes carry the hereditary information from one generation to the next generation.
3. Replication of genes is essential for cell division.
4. They control the structure and metabolism of the body.
5. Genes undergo mutations and change their expression.
6. Genes changes their expression due to position effect and transposons.
7. Reshuffling of genes at the time of sexual reproduction produces variations.
8. Development or production of different stages in the life history is controlled by gene.

SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. What is collinearity principle of gene? Why is this not applicable in case of eukaryotes?

Ans.

Fine Structure of Gene (*Gene Organization*)

Francis Crick in 1958, on the basis of examination of mutations in bacteria and viruses, proposed that genes and proteins are **collinear**, which means that there is a direct correspondence between the nucleotide sequence of DNA and the amino acids sequence of a protein.

The Concept of Collinearity : The concept of collinearity clearly revealed out the fact that the number of nucleotides in a gene are proportional to the number of amino acids in the protein encoded by that gene. In simple words, this concept is true for genes found in some bacterial cells and many viruses. However, in majority of the cells, the genes are slightly longer than expected if collinearity is strictly applied. This is because the *m*RNAs encoded by the genes

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contain some sequences (UTRs or un-translated regions) at their ends that do not specify amino acids.

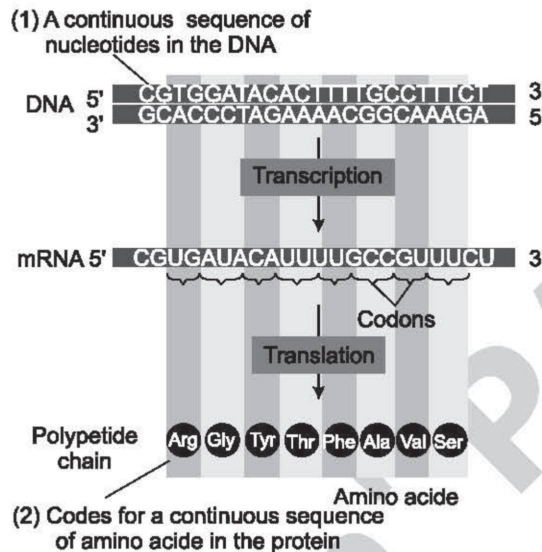

Fig. : Concept of Colinearity : With colinearity, the number of nucleotides in the gene is proportional to the number of amino acids in the protein

Eukaryotic gene structure is fundamentally different. Eukaryotic cells contain far more DNA than is required to encode proteins. Many large RNA molecules which are observed in the nucleus are absent from the cytoplasm. This suggests that nuclear RNAs undergo some type of change (RNA processing) before they are exported to the cytoplasm.

On the basis of modern findings a gene can be defined as any part or segment of DNA that is assigned a particular function. This function may be the synthesis of protein, recombination or even mutation. **Seymour Benzer** (1957) has coined different terms for different nature of gene and genetic material in relation to the chromosome on the basis of genetic phenomena to which they involve. He postulated three fundamental units namely recon, muton and cistron.

1. **Genes as unit of transmission or Cistron :** The part of DNA specifying a single polypeptide chain is termed as cistron. Cistron is a segment of DNA which represents unit of function. A cistron can have 100 nucleotide pairs in length to 30,000 nucleotide pairs. It transmits characters from one generation to other as unit of transmission. Cistron of *E.coli* may contain up to 1500 base pairs, some cistrons are made up of large number of nucleotides. Beginning of each cistron is marked by initiated codon and each cistron ends with termination codon. Each cistron can be transcribed to form a functional *m*RNA.

2. **Gene as unit of mutation or Muton :** The shortest chromosomal unit capable of undergoing mutation has been called the muton. It is the smallest unit of mutation. A change in muton is sufficient enough to cause a change in phenotype. It means that muton is that segment of genetic material (DNA) which can undergo mutation and lead to a mutant phenotype. Thus muton is delimited to a single nucleotide. The muton consists of one or many pairs of nucleotides within the DNA molecule.

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3. **Genes as unit of recombination or Recon :** The smallest segment of DNA capable of being separated and exchange with other chromosome is called recon. Recon has been recognized as smallest unit of recombination. It is a unit of genetic material which can be separated from other units (recon) by means of genetic recombination. A recon consists of not more than two pairs of nucleotides. Thus, each recon is generally made up of one to two nucleotides.

Split Genes in Eukaryotes

Prokaryotic genes are relatively simple and often follow the principle of collinearity. But Eukaryotic genes are very large compared to the protein that they encode. They do not follow the collinearity principle. Such genes contains many large intervening sequences of nucleotides that do not code of any amino acid. As a result the gene contains many small segments, which are of two types :

1. **Exons :** These segments are also called **coding sequences** *i.e.,* they contain genetic information and code for amino acids.
2. **Introns :** These segments are also called **non-coding sequences** *i.e.,* they do not have any genetic information and therefore do not code for any amino acid. They have only structural value.

Genes containing these intervening sequences are called split genes. Such genes are strictly found in eukaryotes. So, eukaryotic genes consist of stretches of coding and non-coding nucleotides. For example, the ovalbumin gene has 8 exons and 7 introns; the gene for cytochrome *'b'* has 5 exons and 4 introns. All the introns and the exons are initially transcribed into RNA but, after transcription, theintrons are removed by splicing and the exons are joined to yield the mature RNA.

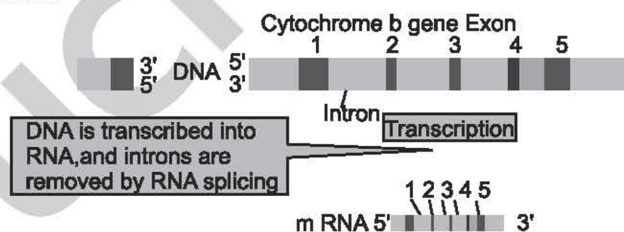

Fig. : Split genes and splicing

The number of introns in eukaryotic genes is variable. Similarly, the size of intron (*i.e.,* the length of an intron) also varies from fewer than 200 nucleotides to more than 50,000.

Q.2. What are DNA packaging? What is their role in eukaryotic genome packaging?

Ans.

DNA Packaging and Chromosome

The packaging of large amounts of genetic information into the small space within a cell demands special storage and DNA packaging. Consider the chromosome of the bacterium *E. coli*, a single molecule of DNA with approximately 4.6 million base pairs. If we stretched out straight, this DNA would be about 1000 times as long as the cell within which it resides. Human cells contain more than 6 billion base pairs of DNA, which would measure some 1.8 meters stretched end to end.

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This clearly suggests that the DNA molecules must be tightly packed to fit into such small spaces. The structure of DNA can be considered at three hierarchical levels :

1. The primary structure of DNA, which is its nucleotide sequence;
2. The secondary structure is the double-strandedhelix;
3. The tertiary structure, that refers to higher-order folding that allows DNA to be packed into the form of chromosomes.

Eukaryotic DNA is closely associated with his tone proteins, creating chromatin. The two basic types of chromatin are **euchromatin**, which undergoes the normal process of condensation and decondensation in the cell cycle, and heterochromatin, which remains in a highly condensed state throughout the cell cycle, even during **interphase**.

The most abundant proteins in chromatin are the histones, which are of five major types : **H1, H2A, H2B, H3**, and **H4**. All histones have a high percentage of arginine and lysine, positively charged basic amino acids that give the histones a net positive charge. The positive charges attract the negative charges on the phosphates of DNA; this attraction holds the DNA in contact with the histones. These proteins together with DNA, form small rounded bodies, called **nucleosomes**.

Except H1, all other proteins (*i.e.*, H2A, H2B, H3 and H4) are highly conserved proteins among different organisms. H1 proteins is variable in different organisms. It serves to seal the nucleosome structure, with DNA. Due to this reason, it is also called as sealing protein.

Table : Important Properties of Histone Proteins

Histone	Mol. wt.	Amino acid composition	Variation in different species	Molecules per 200 base pairs of DNA
H1	20,000	Lysine rich	Highly variable	1
H2A	13,700	Moderately lysine rich	Farily well conserved	2
H2B	13,700	Moderately lysine rich	Fairly well conserved	2
H3	15,700	Arginine rich	Highly conserved	2
H4	11,200	Arginine rich	Highly conserved	2

Other proteins which are associated with DNA and chromosomes, are called **non-histone proteins**. They help in high level packing and folding of chromosome. They also help in making up the **kinetochore**, cap the chromosome ends (telomeres) by attaching to telomeres and also help in the movement of chromosomes in mitosis and meiosis.

Nucleosome (Structural Units of Chromatin)

The nucleosome is a core particle consisting of DNA wrapped about two times around an octamer of 8 histone proteins, much like thread wound around a spool.

1. In interphase the chromatin shows a **'beads on a string'** appearance. The beads are called nucleosomes. These are the structural units of chromatin.
2. These are about 10 nm in diameter and are connected by the DNA threads.
3. In a nucleosome, there is a histone octamer (formed by 8 molecules, 2 of each H2A, H2B, H3 and H4). DNA gets turned over the histone octamer taking 1.65 turns (roughly 2 turns).
4. The DNA in direct contact with the histone octamer is between 145 and 147 bp in length.

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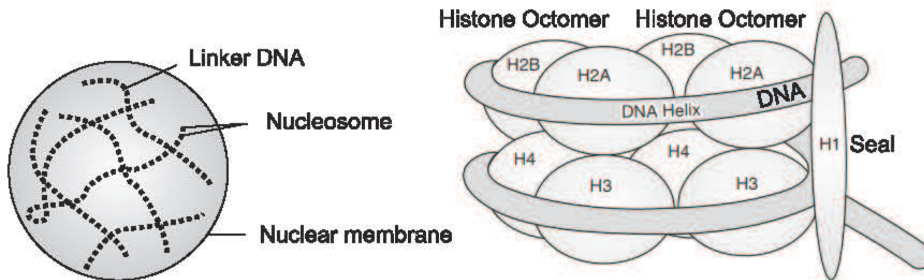

Fig. : (a) Interface nucleus showing chromatin threads (b) Composition of the Nucleosome

5. H1 does not take part in the formation of histone octamer.

H1 binds to 20 to 22 bp of DNA where the DNA joins and leaves the octamer and helps to lock the DNA into place, acting as a clamp around the nucleosomeoctamer. It therefore seals and stabilizes the nucleosome. Hence, It acts as a sealing protein.

After leaving a gap of about 40-60 base pairs, DNA again turns over a new octamer. In this way, a single DNA may be associated with thousands of histone octamers. The DNA between two successive nucleosomes is called **linker DNA.**

This structure (octamer and wrapped DNA) is called a nucleosome (bead). Along with H1 protein the structure is called **chromatosome.**

Each chromatosome consists of about 167 bp of DNA. Chromatosomes are located at regular intervals along the DNA molecule and are separated from one another by linker DNA, which varies in size in different cells.

Non-histone chromosomal proteins may be associated with this linker DNA, and a few also appear to bind directly to the core protein.

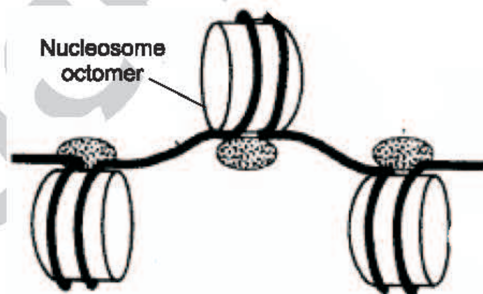

Fig. : Small part of chromatin thread, showing nucleusomes

DNA Packing and Formation of Chromosomes

When chromatin is in a condensed form, the nucleosomes fold on themselves to form a dense, tightly packed structure that makes up a fiber with a diameter of about 30 nm. In this structure, the nucleosomes are arranged in a *zig-zag* ribbon that twists or supercoils (helix model).

Further condensation forms a series of loops of 30-nm fibers. Each loop contains about 20,000 to 100,000 bp of DNA and about 300 nm in length. The 300-nm loops are packed and folded to produce thick fibre of diameter 250-nm. Further helical coiling of the 250-nm fiber, finally produce the most compact structure that appears in a chromatid of metaphase chromosome.

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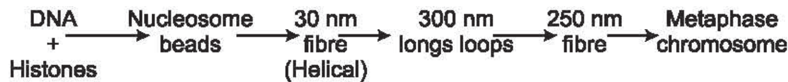

Q.3. Define the basic concept of transcription. Also mention its factors.

Ans.

Transcription

The formation of RNA from DNA is called **transcription**. It is the first step in the process of protein synthesis. By this process, DNA transcripts (provides) all the required information (for protein synthesis) to *m*RNA molecule.

The *m*RNA molecule, therefore has the perfect complementary base sequence. The process of transcription in eukaryotes takes place in **nucleus** but in prokaryotes it occurs in **cytoplasm**.

Basic Concepts : The enzyme, which is responsible for transcription (*i.e.*, the synthesis of *m*RNA from DNA) is called **RNA polymerase** (or RNAP or RNAp). It is a DNA dependent enzyme, which acts on a single DNA template and polymerises, RNA nucleotide to form *m*RNA.

An important distinction must be made here. One of the DNA strands in the double helix holds the genetic information used for protein synthesis. This is called the **Sense Strand** or **Coding Strand**, or information stand. The complementary strand that binds to the sense strand is called the **Antisense Strand** or **Template Strand**, and it serves as a template for generating a *m*RNA molecule.

We must remember that only template strand is transcribed (not the coding strand). Template strand delivers a copy of the sense (coding) strand to a ribosome. (so the strand, which is transcribed is the anti-sense or template strand. The *m*RNA which is synthesised holds the information exactly similar to that, present in coding-strand).

Transcription Factors

Transcription factors are vital for the normal development of an organism, as well as for routine cellular functions and response to disease.

Transcription factors are protein molecules that bind to DNA sequences, near the 5' end (upstream) region of target genes. They regulate and modulate the rate of gene transcription. This may result in increased or decreased gene transcription, protein synthesis and subsequent altered cellular function.

Transcription factors are a very diverse family of proteins and generally function in multi-subunit protein complexes. They may bind directly to special "promoter" regions of DNA, which lie upstream of the coding region in a gene, or directly to the RNA polymerase molecule.

Transcription factors can activate or repress the transcription of a gene, which is generally a key determinant in whether the gene functions at a given time.

Transcription factors are necessary for RNA polymerase to function at a site of transcription. They are considered the most basic set of proteins needed to activate gene transcription, and they include a number of proteins, such as TF-IIA (transcription factor II A) and TF-IIB (transcription factor II B).

Several families of transcription factors exist and members of each family may share structural characteristics. These families include are :

1. helix-turn-helix (*e.g.*, Oct-1),
2. helix-loop-helix (*e.g.*, E2A),

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3. zinc finger (*e.g.*, glucocorticoid receptors, GATA proteins),
4. basic protein-leucine zipper (cyclic AMP response element-binding factor (CREB), activator protein-1 (AP-1)],
5. β-sheet motifs [*e.g.*, nuclear factor-kB (NF-kB)].

Many transcription factors are common to majority of the cell types, such as AP-1 and NF-kB, and may play a general role in the regulation of inflammatory genes.

Other transcription factors are cell-specific and may determine the phenotypic characteristics of a cell. Transcription factor activation is complex and may involve multiple intracellular signal transduction pathways, including the kinases PKA, MAPKS, JAKs, and PKCs, stimulated by cell-surface receptors.

Transcription factors may also be directly activated by ligands such as glucocorticoids and vitamins A and D.

During embryogenesis, the transcription factors are responsible for dictating the fate of individual cells. For example, homeotic genes which control the pattern of body formation, encode transcription factors that direct cells to form various parts of the body. These factors can activate one gene but repress another, producing effects that are complementary and necessary for the ordered development of an organism. If a mutation occurs in any of such factors; the organism will not develop correctly. For example, in fruit flies (*Drosophila*), mutation of a particular homeotic gene results in altered transcription, leading to the growth of legs on the head instead of antenna; this is known as the antennapedia mutation.

Transcription factors are a common way in which cells respond to extracellular information, such as environmental stimuli and signals from other cells. Transcription factors can have important roles in cancer, if they influence the activity of genes involved in the cell cycle (or cell division cycle).

In addition, transcription factors can be the products of oncogenes (genes that are capable of causing cancer) or tumour suppressor genes (genes that keep cancer in check).

Role of Promoters and Terminators in Transcription

Promoters in Prokaryotes : There is large number of promoters in different species and for different genes. However, in prokaryotes (bacteria), two important sequences (one at –10 bp upstream and other at –35 bp upstream) have been recognized to be common in all species. Thus they are called the –10 sequence and the –35 sequence, respectively. Such promoters are called **conserved sequences.**

1. –10 consensus sequence (Pribnow box) : It is TATAAT in the non-template strand (5' to 3').
2. –35 consensus sequence : It is TTGACA in the non-template strand (5' to 3').

Promoters in Eukaryotes : Promoters for RNA polymerase-II, in eukaryotes are highly varied, species to species and gene to gene.

However, two important conserved sequences have been recognized in eukaryotes also. These are TATA box and CAAT box.

1. **TATA box** (also called **Hogness Goldberg box**) : This is 25 to 35 bp upstream (5' to 3' on non-template strand) sequence, which has consensus TATAAT in majority of these cases. It resembles the Pribnow box of the prokaryotes. Its function is also similar to Pribnow box, i.e., to recognize RNA polymerase. Besides this, the initiation of transcription at promoter also requires the presence of many general transcription

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factors (also called TF II, *i.e.,* transcription factors for RNA polymerase II). One very important of these factors is TFIID, which is specific to TATA box.

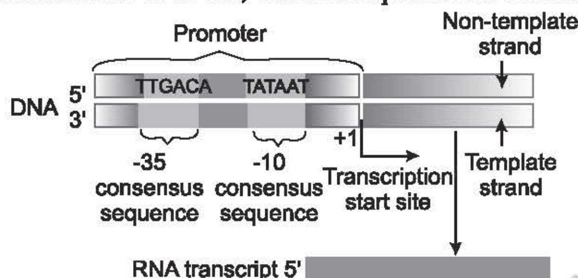

Fig. : Transcriptional unit, showing promoters

2. **CAAT box :** It usually occurs near position – 80 (5' to 3' on non-template strand) and has the consensus sequence GGCCAATCT. This sequence determines the efficiency of the transcription.

Besides these two, some more conserved sequences such as GC box and octamer box etc. have also been noted in eukaryotes.

Terminator : Transcription ends after RNA polymerase transcribes a terminator. Transcription therefore does not suddenly stop when polymerase reaches a terminator. Rather, transcription stops after the terminator has been transcribed, like a car that stops only after running over a speed bump. Bacterial cells possess two types of terminator :

1. A *rho*-independent terminator, which RNA polymerase can recognize by itself; and
2. A *rho*-dependent terminator, which RNA polymerase can recognize only with the help of *rho*-protein.

Q.4. Describe the mechanism of transcription with the help of suitable diagram.

Ans.

Mechanism of Transcription

RNA synthesis occurs by a mechanism that is similar to that of DNA synthesis except that :

1. the precursors are ribonucleoside triphosphates rather than deoxy-ribonucleoside triphosphates,
2. only one strand of DNA is used as a template for the synthesis of a complementary RNA chain in any given region, and
3. RNA chains can be initiated *de novo*, without any requirement for a pre-existing primer strand. The RNA molecule produced will be complementary and antiparallel to the DNA template strand and identical, except that uridine residues replace thymidines, to the DNA non-template strand.

The synthesis of RNA chains, like DNA chains, occurs in the $5 \rightarrow 3$ direction, with the addition of ribonucleotides to the 3-hydroxyl group at the end of the chain.

The reaction involves a nucleophilic attack by the 3—OH on the nucleotidyl (interior) phosphorus atom of the ribonucleoside triphosphate precursor with the elimination of pyrophosphate, just as in DNA synthesis. This reaction is catalysed by enzymes called **RNA polymerases**. The overall reaction is as follows :

$$n(\text{RNP}) \xrightarrow[\text{RNA polymerase}]{\text{DNA template}} (\text{RMP})_n + n\,(\text{PP})$$

Where, *n* is the number of moles of ribonucleotide triphosphate (RTP) consumed, ribonucleotide monophosphate (RMP) incorporated into RNA, and pyrophosphate (PP) produced.

During transcription process. RNA polymerase moves over the template strand in 3' to 5' direction and adds new ribonucleotides in 5' to 3' direction.

The synthesis of *m*RNA therefore occurs always in 5' to 3' direction. As a result of this, the starting point of transcription corresponds to the 5' end of the *m*RNA and termination point to the 3' end.

Transcription process can be details as follows :

1. **Initiation of Transcription :** Initiation of Transcription involves three steps :
   - (i) binding of the RNA polymerase holoenzyme to a promoter region in DNA,
   - (ii) the localized unwinding of the two strands of DNA by RNA polymerase (helicase activity), providing a template strand free to base-pair with incoming ribonucleotides, and
   - (iii) the formation of phosphodiester bonds between the first few ribonucleotides in the nascent RNA chain.

   The **holoenzyme** (Core enzyme + Sigma factor) remains bound at the promoter region during the synthesis of the first eight or nine bonds; then the sigma factor is released, and the **core enzyme** begins the elongation phase of RNA synthesis.

   The sigma subunit of RNA polymerase mediates its binding to promoters in DNA. Hundreds of *E. coli* promoters have been sequenced. Two short sequences within these promoters are sufficiently conserved (fixed). The mid points of these two conserved sequences occur at about –10 and –35 nucleotide pairs, respectively, before the transcription initiation site. So, they are called the **10 sequence** and the **35 sequence**, respectively. The nucleotide sequences that are present in such conserved genetic elements most often are called **consensus sequences**.

   The –10 consensus sequence in the non-template strand is TATAAT; the –35 consensus sequence is TTGACA. The sigma subunit initially recognizes and binds to the –35 sequence. So, this sequence is sometimes called the **recognition sequence**. The AT rich –10 sequence facilitates the localized unwinding of DNA, which is an essential prerequisite to the synthesis of a new RNA chain.

2. **Elongation of RNA Chains :** Elongation of RNA chains is catalyzed by the core RNA polymerase enzyme, after the release of the sigma subunit.

   The local unwound segment of DNA forms a transcription bubble as shown in the fig. The extension of RNA chains takes place within the transcription bubble.

   The RNA polymerase molecule contains both DNA unwinding (helicase) and DNA rewinding activities. RNA polymerase continuously unwinds the DNA double helix ahead of the polymerization site and rewinds the complementary DNA strands behind the polymerization site as it moves along the double helix.

   In *E. coli*, the average length of a transcription bubble is 18 nucleotide pairs, and about 40 ribonucleotides are incorporated into the growing RNA chain per second. The nascent RNA chain is displaced from the DNA template strand as RNA polymerase moves along the DNA molecule.

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RNA polymerase moves in 3' to 5' direction along the DNA template. The growth of RNA chain occurs in 5' to 3' direction.

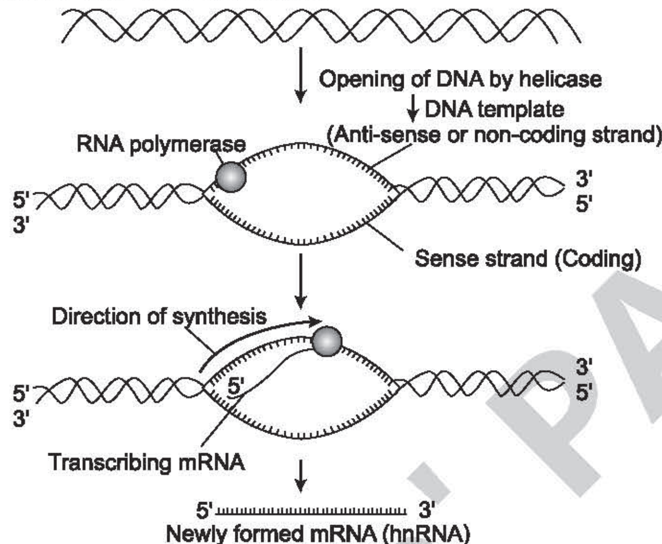

Fig. : Mechanism of transcription

3. **Termination of RNA Chains :** Termination of RNA chains occurs when RNA polymerase encounters a termination signal/terminator.

   At this time, the transcription complex dissociates, releasing the nascent RNA molecule. There are two types of transcription terminators in *E. coli*.

   (i) One type results in termination only in the presence of a protein called *rho*; therefore, such termination sequences are called *rho*-dependent terminators.

   (ii) The other type results in the termination of transcription without the involvement of *rho*; such sequences are called *rho*-independent terminators.

Rho-independent terminators contain a GC rich region followed by six or more AT base pairs, with the A's present in the template strand.

The mechanism by which rho-dependent termination of transcription occurs is similar to that of rho-independent termination in that both involve the formation of a hydrogen-bonded hairpin structure upstream from the site of termination. In both cases, these hairpins impede the movement of RNA polymerase, causing it to stop the process.

Newly Formed *m*RNA : The newly formed mRNA in all organisms consists of three parts :

1. 5' untranslated region (UTR) at 5' end,
2. Protein coding region, and
3. 3' untranslated region (UTR) at 3' end.

Untranslated regions (UTR) do not code for protein, but contain important information regarding the functioning of *m*RNA.

Q.5. Explain the RNA processing with the help of suitable diagram.

Ans.

RNA Processing

The eukaryotic primary transcript or *hn*RNA is highly unstable due to the presence of non-coding regions or introns. Further, the two ends of the *hn*RNA are also not stable and can

be degraded by the attack of nucleases. Hence, the process of RNA processing (conversion of *hn*RNA into active and mature *m*RNA) involves following sub steps :

1. **Stabilisation of 5' end or capping :** The first nucleotide at the 5' end of the *hn*RNA is covered by cap of a molecule-7 methyl guanosine triphosphate (or 7mGTP). It is a modified guanosine triphosphatemol *E. coli*. This event is called **capping**. The cap is linked to the first nucleotide of *hn*RNA by 5' to 5' triphosphate bond. The cap of 7mGTP serves two important purposes :

   (i) It determines the initiation site for translation, and

   (ii) It protects the 5' end of the mRNA against the attack of some nuclease enzymes.

2. **Stabilisation of 3' end or Polyadenylation :** A segment of polyadenylic acid, called Poly A tail or Poly A segment (about 200 to 300 bp of adenylate residues) is added sequentially to the 3' end of the *hn*RNA. This process of addition of Poly A tail to 3' end of the RNA is called **tailing** or **polyadenylation**. Polyadenylation makes the 3' end stable and resistant to any attack by nuclease enzymes.

3. **RNA Splicing :** Removal of introns (non-coding portions) from the *hn*RNA to make it homogeneous is called RNA splicing. Due to the removal of introns, the unusual length of the *m*RNA gets shortened and it becomes more effective and stable.

   In some rare cases (as in a ciliated protozoan Tetrahymena), self splicing takes place due to auto catalytic action of RNA itself. But in majority of the cases, the splicing requires a special structure, called **spliceosomes**. Spliceosome serves to recognise the 'start' and 'end' sequences of introns. It then cuts the *hn*RNA to remove introns and re-ligate (re-join) remaining pieces (exons) of RNA to form mature *m*RNA.

Spliceosomes : The structures, which are responsible for the removal of introns, are called spliceosomes. The spliceosomes are formed by 5 types of small ribonucleo proteins (or *sn*RNPs) are : U1, U2, U4, U5, U6. (note : U3 is not found). In the language of molecular biology, *sn*RNPs are pronounced as "snurps".

Each *sn*RNP consists of specialised small nuclear RNA molecules (known as *sn*RNAs) and special small nuclear proteins.

***sn*RNA and *sc*RNA :** *sn*RNA stands for small nuclear RNA. These are small sized RNA molecules present in nucleus. These are rich in uridine contents. These are different from main classes of RNAs (*i.e.*, *r*RNA, *m*RNA and *t*RNA). *sn*RNA combines with short nuclear proteins, to form *sn*RNP (small ribonucleo proteins), which are also known as 'snurps'. These molecules participate in RNA splicing.

*sc*RNA stands for small cytoplasmic RNA. These are found freely in cytoplasm or may combine with proteins to form signal recognition particles.

Process of Splicing : The *sn*RNP units bind to 5' and 3' ends of the intron and cause an endo-nucleolytic cleavage at the splice junctions. (The binding of RNPs with intronic regions is highly specific). This results in the formation of a loop like spliceosome/intron complex or lariat. Now the adjacent exons (coding regions) get joined with each other and the lariat gets dissolved (*i.e.*, broken into monomers).

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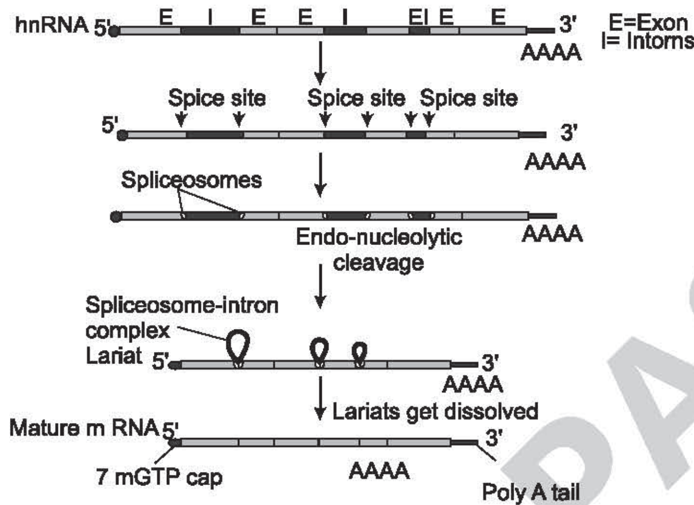

Fig. : Mechanism of splicing

After RNA processing, the *hn*RNA becomes the homogenous, mature, stable and workable *m*RNA. It is now ready to move into the cytoplasm. The highly complicated mechanism of RNA processing is used in eukaryotes to control the gene expression.

RNA processing does not occur in prokaryotes, because Prokaryotic DNA is short, and It does not contain introns or non-coding regions.

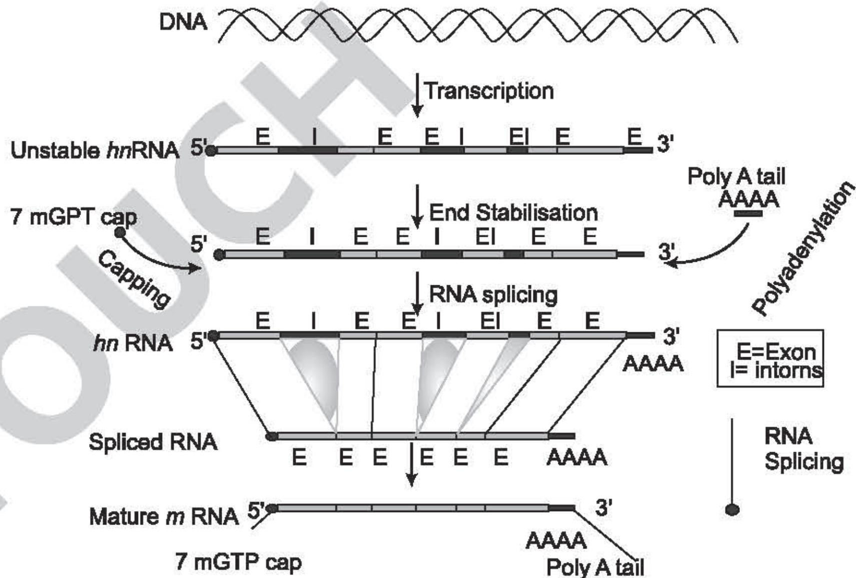

Fig. : Mechanism of splicing

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UNIT-II

Protein Synthesis-II : Process of Transcription

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. What is central dogma?

Ans. The central dogma of molecular biology is a theory stating that genetic information flow only in one direction, from DNA, to RNA to protein or RNA directly to protein.

Q.2. Who gave the central dogma?

Ans. The central dogma of molecular biology was first enunciated by Francis Crick in 1958. Crick proposed explicity that 'once information' has passed into protein it cannot get out again.

Q.3. Why do cells need ribosomes?

Ans. While a structure such as a nucleus is only found in eukaryotes, every cell needs ribosomes to manufacture proteins. Since there are no membrane-bond organelles in prokaryotes, the ribosomes float free in the cytosol.

Q.4. Who discovered ribosome?

Ans. George E. Palade discovered ribosomes in 1955. He identified them a small particles present in the cytoplasm, which often remain associated with the endoplasmic reticulum.

Q.5. Why ribosomes are called protein factory?

Ans. Ribosomes synthesize proteins by gathering and assembling amino acids into protein chain. Since ribosomes are the only cell organelle involved in the synthesis of protein, they are called the protein factory of the cell.

Q.6. Which factor is responsible for the termination of translation?

Ans. There is one-one factor in eukaryotic cells, called eRF_1, whereas in prokaryotic cells there are two factors called RF_1 and RF_2. In termination of translation in mitochondria, process similar to prokaryotes termination, there is only one factor known, called mitochondria release factor 1 (mRF_1).

Q.7. How is translation of termination occur in prokaryotes?

Ans. Termination of translation occurs when a nonsense codon (UAA, UAG or UGA) is encountered. Upon aligning with the A site, these nonsense codons are recognized by protein release factors that resemble *t*RNAs.

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Q.8. What are initiation and termination factors?

Ans. The termination factors or release factors bind with ribosomes instead of a new *t*RNA when a stop codon is reached at the aminoacyl site (A site). Whereas initiation factors, means, proteins that help initiate translation by forming the pre-initiation complex with the initiator *t*RNA and ribosome.

Q.9. What is the role of initiation factors in prokaryotes?

Ans. In prokaryotes translation initiation is mediated by initiation factors (IFs). IF_1, IF_2 and IF_3. Their function is the formation of a 70s ribosome complex that places the *m*RNA and the formyl-Met-*t*RNA (fMet-*t*RNA) in the correct position for starting the elongation process.

Q.10. What are the important steps in the initiation of protein synthesis in prokaryotes?

Ans. Translation is conceptually divided into four phases : initiation, elongation, termination and ribosome recycling. The ribosome is composed of a large and a small subunit, which are assembled on the translation initiation region (TIR) of the *m*RNA during the initiation phase of translation.

Q.11. Where does translation process start?

Ans. Translation begins when an initiator *t*RNA anticodon recognizes a codon on *m*RNA. The large ribosomal subunit joins the small subunits, and a second *t*RNA is recruited. As the *m*RNA moves relative to the ribosome, the polypeptide chain is formed.

Q.12. Which enzymes are involved in translation?

Ans. Peptidyl transferase is the main enzyme used in translation. It is found in the ribosomes with an enzymatic activity that catalyzes the formation of a covalent peptide bond between the adjacent amino acids. The enzyme's activity is to form peptide bonds between adjacent amino acids using *t*RNAs during translation.

Q.13. Where is translation located in eukaryotes?

Ans. Eukaryotes *m*RNA precursors must be processed in the nucleus (*e.g.*, capping, polyadenylation, splicing) in ribosomes before they are exported to the cytoplasm for translation.

Q.14. Where does DNA translation start?

Ans. For translation to begin, the start codon (5'AUG) must be recognised. This codon is specific to the amino acid methiomine, which is nearly always the first amino acid in a polypeptide chain. At the 5' cap of *m*RNA the small 40 s subunit of the ribosome binds.

Q.15. What does translation produce?

Ans. In translation, messenger RNA (*m*RNA) is decoded in a ribosome, outside the nucleus, to produce a specific amino acid chain, or polypeptide. The polypeptide later fold into an active protein and performs its functions in the cell.

Q.16. What type of RNA are involved in translation?

Ans. *t*RNA are an essential component of translation, where their main function is the transfer of amino acids during protein synthesis. Therefore, they are called *t*RNAs. Each of the 20 amino acids has a specific *t*RNA that binds with it and transfers it to the growing polypeptide chain.

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SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. Write about the contribution of H.G. Khorana in the field of molecular biology? What are the different types of codon?

Ans.

Contribution of H.G. Khorana

Dr. H.G. Khorana, was an Indian American biochemist, developed a technique for artificially synthesizing *m*RNA having repeated sequences of known **nucleotides**. Khorana was one of the first scientists to demonstrate the role of nucleotides in protein synthesis and helped crack the genetic code. He was awarded Noble prize in 1968 for his valuable contribution in Physiology or Medicine with **M.W. Nirenberg** and **R.W. Holley**. This they were awarded for their elucidation of the genetic code and its function in protein synthesis.

Khorana's work confirmed Nirenberg's finding that the chemical composition and function of a new cell is determined by how the four nucleotides are arranged on the spiral 'staircase' of a DNA molecule. He also demonstrated that the nucleotide code is always transmitted in groups of three, called **codons**, and that these codons instruct the cell to start and stop the production of proteins.

Khorana and his coworkers developed chains of polyribonucleotide (By using synthetic DNA) with repeated sequence of 2 or 3 nucleotides. One of the developed polyribonucleotide chain poly CUC UCU CUC UCU..., consists of two codons CUC and UCU codes for leucine and serine, hence, this synthesized polynucleotide chain codes for leucine - serine - leucine - serine.

Another polynucleotide chain synthesized was poly CUA, CUA, CUA, CUA, and CUA....., which is an example of homopolymer (chain of nucleotide consisting of repeating units of same codon). Since, CUA codes for leucine this polynucleotide chain codes for leucine leucine-leucine...... leucine.

Types of Codon

The types of codon are as follows :

1. **Sense Codon :** Those codons that code for amino acids are called sense codons. There are 61 sense codons in the genetic code which code for 20 amino acids.
2. **Signal Codons :** Those codons that code for signals during protein synthesis are known as signal codons. There are four codons which code for signal. These are AUG, UAA, UAG and UGA. Signal codons are of two types : Start codons, and Stop codons.

   (i) **Start Codon (Chain Initiation Codons) :** Codon with nucleotide sequence "AUG" is called as start codon. It codes for amino acid methionine in most organisms. The process of translation (protein synthesis) always begins with expression of start codon AUG.

   However, codon AUG can occur later in *m*RNA also, then it will simply code for amino acid methionine. The triplets AUG and GUG, plays double roles in *E. coli*. When they occur in between the two ends of a cistron (intermediate position), they code for the amino acids methionine and valine, respectively in an intermediate position in the protein molecule.

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(ii) **Stop Codon (Chain Termination Codons) :** Out of total 64 genetic codes, the 3 triplets UAA, UAG, UGA do not code for any amino acid. They were originally described as non-sense codons, as against the remaining 61 codons, which are termed as sense codons. During the process of protein Synthesis these codons function to terminate the process.

When any of the three codon is read, the ribosomes pauses and gets separated from *m*RNA and hence the process of protein synthesis is terminated. Due to this reason these codons are also called as termination codons. UAA, UAG and UGA are also known as ochre, Amber and Umber respectively, are believed to be used as signals which end the synthesis of a protein chain.

Q.2. Write a short note on aminoacyl *t*RNA synthetases.

Ans.

Aminoacyl *t*RNA Synthetases

It is the enzyme that helps in combining amino acid to its particular *t*RNA. The enzyme is specific for each amino acid. It is also called **aa-activating enzyme**. Through the process of *t*RNA "charging," each *t*RNA molecule is linked to its correct amino acid by a group of enzymes called aminoacyl *t*RNA synthetases. When an amino acid is covalently linked to a *t*RNA, the resulting complex is known as **aminoacyl-*t*RNA**. At least one type of aminoacyl *t*RNA synthetase exists for each of the 21 amino acids; the exact number of aminoacyl *t*RNA synthetases varies by species.

These enzymes first bind and hydrolyze ATP to catalyze the formation of a covalent bond between an amino acid and **adenosine monophosphate** (AMP); a pyrophosphate molecule is expelled in this reaction. This is called "activating" the amino acid. The same enzyme then catalyzes the attachment of the activated amino acid to the *t*RNA and the simultaneous release of AMP. After the correct amino acid covalently attached to the *t*RNA, it is released by the enzyme. The *t*RNA is said to be charged with its cognate amino acid.

Q.3. Compare between the structure of prokaryotic and eukarotic *m*RNA.

Ans.

Comparison of the Structure of Prokaryotic and Eukaryotic *m*RNA

Eukaryotic *m*RNA is mostly monocistronic. It has a 5' cap, which is recognized by small ribosomal subunit. Protein synthesis, therefore, begins at an initiation codon near the 5' end of the *m*RNA. Upstream of the initiation codon contains a non-translatable sequences called 5' UTR (5'-untranslated region) or leader sequence. Similarly non translatable sequence at 3' end after stop codon is termed as 3' UTR (3'-untranslated region) or trailer sequence, which varies in length and sequence.

In prokaryotes, most of the *m*RNA is polycistronic. In contrast to eukaryotic *m*RNA, the 5' end has no cap-like structure, and there are multiple ribosome-binding sites (called shine Dalgarno sequence) within the polycistronic *m*RNA chain, each resulting in the synthesis of different protein. Just like eukaryotic *m*RNA, prokaryotic mRNA also contains 5' UTR and 3'UTR. All *m*RNA (monocistronic and polycistronic) contain two types of region the coding region (which starts with initiation codon and ends with a stop codon) and untranslated region. (5'- and 3'-UTR). A polycistronic *m*RNA also contain intercistronic regions.

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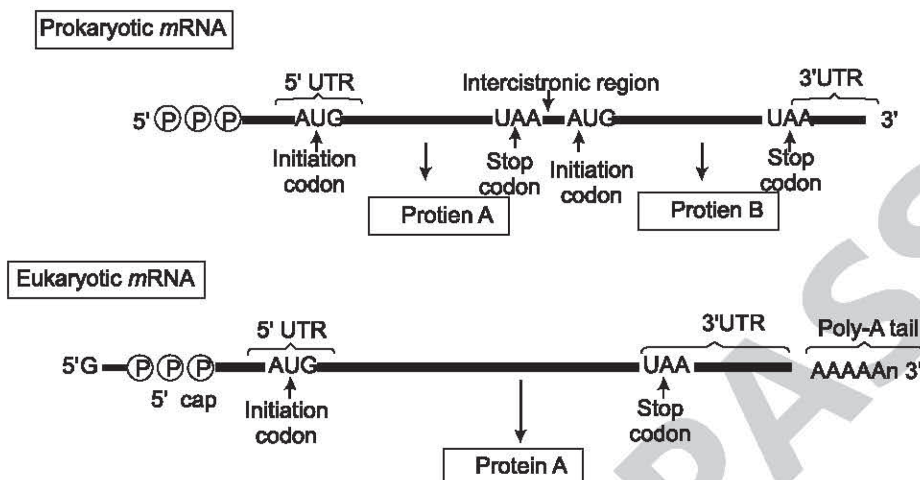

Fig. : Prokaryotic and eukaryotic *m*RNA having coding and untranslated region.

Q.4. What are the differences between prokaryotic and eukaryotic translation?

Ans. Difference between Prokaryotic and Eukaryotic Translation

The basic steps involved in protein synthesis are similar in both prokaryotes and eukaryotes. However, protein synthesis differs in several aspects in these two groups.

S.No.	Particulars	Prokaryotes	Eukaryotes
1.	Polymerase used	One	Several
2.	Ribosomes used	70S with 30S and 50S subunits	80S with 40S and 60S subunits
3.	Transcription and translation	Can overlap	Do not overlap
4.	Starting amino acid	N-formyl methionine	Methionine
5.	Initiation and termination sites	Several	Single
6.	Initiation factors used	IF 1, IF 2 and IF 3.	eIF2, eIF3, eIF 4A, eIF 4E, eIF 4F and eIF 4G.
7.	Chain elongation factors	EF-T4, EF-TS and EF-G	EF1 and EF2.
8.	Release Factors	RF 1, RF2 and RF 3.	RF
9.	Synthesis of *m*RNA.	From DNA or RNA.	From RNA only.

Q.5. Write about the cloverleaf structure of *t*RNA. Also its function.

Ans. Structure of Transfer RNA (*t*RNA)

*t*RNA is the non-coding RNA molecule that carries amino acids to the ribosomes, from the growing peptide chain (*m*RNA) nucleotide sequence. Therefore, the *t*RNA acts as the intermediate between nucleotide and amino acid sequences.

They are ribonucleotides, therefore, they form a hydrogen bond with *m*RNA, and form ester links with amino acids which combine the mRNA and amino acids during translation.

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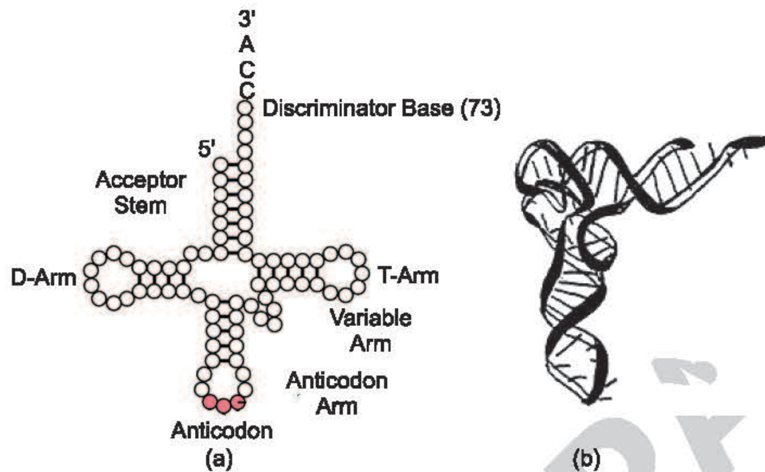

Fig. : *t*RNA : *t*RNA are represented as coverleaf structure

Functions of *t*RNA

Functions of *t*RNA are as follows :

1. It is a small RNA chain of about 80 nucleotides.
2. During translation, *t*RNA transfers specific amino acids corresponding to the *m*RNA sequence to the growing polypeptide chain in the ribosome.
3. *t*RNA pairs with *m*RNA complementarity in a parallel manner with each of its base pairs having three nucleotides paired to *m*RNA.
4. *t*RNAs are coded by short molecules of 70-90 nucleotides (5nm).
5. The set of the three nucleotides on the *m*RNA is known as a **codon**, while the corresponding sequence on *t*RNA is known as an **anticodon**.
6. The base pairing of the codon and the anticodon forms a translation mechanism.
7. At the end of the *t*RNA 3' hydroxyl base, there is an anticodon amino acid sequence that is attached, linking the ribosomes to form a peptide bond, thus elongating the polypeptide chain.
8. Therefore, *t*RNA major parts are the anticodon and the 3' hydroxyl group terminal.
9. Other parts of the *t*RNA structure are the D-arm and the T-arm, which are highly specific and are highly effective.
10. *t*RNAs have a sugar-phosphate backbone which gives it directionality.
11. One end of the *t*RNA has a reactive phosphate group, which is attached to the fifth carbon atom of the ribose (5') and another end which has a free hydroxyl group on the third carbon (3'), giving rise to the 5' to 3' ends of RNA.
12. The 3' terminal end has three bases CCA (Cytosine, cytosine, adenine) which make part of the acceptor arm of the molecule which is covalently attached to the hydroxyl group on the ribose sugar.

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13. The acceptor arm also contains parts of the 5' end of the *t*RNA, made up of 7-9 nucleotides on the opposite ends of the molecule base pairing with each other.
14. The anticodon loop which is recognized by the aminoacyl *t*RNA synthetase (AATS) is paired to *m*RNA and it determines the amino acid that attaches to the acceptor's arm.
15. The AATS reads and recognizes the D-arm from the 5' end of *t*RNA.
16. The D-arm plays a major role in stabilizing the structure of RNA; it affects and influences the kinetics and accuracy of translation at the ribosomes.
17. The T-arm also influences the *t*RNA effect on the translation by interaction with the ribosomes.
18. The D-arm, T-arm, and the anticodon loop combined resembles a cloverleaf. When RNA folds into a tertiary structure, it becomes L-shaped with an extended structure of the acceptor stem, T-arm, anticodon loop, and D-arm.

Q.6. Write a short note on the aminoacylation of *t*RNA.

Ans.

Aminoacylation of *t*RNA

The amino acid's carboxyl group connects to the 3'-hydroxyl of the acceptor arm's terminal adenine. Aminoacyl *t*RNA synthesizes enzymes that catalyze the charging reaction, which requires ATP hydrolysis.

When the correct amino acid is connected to the *t*RNA, it identifies the amino acid's codon in the *m*RNA, allowing it to insert the amino acid in the correct location as determined by the *m*RNA sequence. This ensures that the amino acid sequence encoded by the *m*RNA is correctly translated.

Codon recognition is accomplished through the *t*RNA's anticodon loop, specifically three nucleotides in the anticodon loop that bind to the codon *via* complementary base-pairing.

A 3-pin plug with a socketed base is recognized in the same way as a full codon - anticodon fitting. Both the pin and the socket are highly specific. DNA's four nucleotides can be combined to create 64 codons. Three codons indicate the completion of translation, while the remaining 61 code for the 20 amino acids found in proteins. As a result, most amino acids have multiple codons.

The cytoplasm contains inactive amino acids. When they obtain energy from ATP, they become active. When an amino acid binds to ATR, the reaction is triggered. This step is carried out by aminoacyl RNA synthetase, a specific activating enzyme.

A high-energy acyl bond is formed between the a-phosphate of ATP and the carboxyl group of an amino acid during the production of aminoacyl adenylate. ATP phosphates are converted into inorganic pyrophosphate.

The active amino acid is transported to the corresponding t-RNA. A high-energy ester bond is formed by the carboxyl group of the amino acid and the 3'-hydroxyl group of the terminal adenosine of *t*RNA. When the aminoacyl AMP-enzyme complex combines with the appropriate *t*RNA, the aminoacyl *t*RNA complex is formed.

Aminoacylation is a two-step process that is catalyzed by a group of enzymes known as **aminoacyl *t*RNA synthetases**. There are twenty aminoacyl *t*RNA synthetases in each cell, one for each amino acid in the genetic code. In the first step of aminoacyl *t*RNA production, ATP

and the appropriate amino acid produce an aminoacyl adenylate intermediate. The enzyme inorganic pyrophosphatase catalyzes the breakdown of inorganic pyrophosphate to free phosphate. The aminoacyl adenylate intermediate is "high-energy," and amino acid transfer to the acceptor end of *t*RNA occurs without the need for additional ATP in the second step. The aminoacyl *t*RNA synthetase edits *t*RNA to prevent mis-acylated *t*RNA from being used in protein synthesis. Because the ribosome must treat all aminoacyl *t*RNAs the same in order to form the peptide bond, any *t*RNA containing the wrong amino acid would be used for protein synthesis, potentially resulting in the production of a dangerous protein. A second active site on the aminoacyl *t*RNA synthetase edits aminoacyl *t*RNAs for accuracy.

Enzymes in Acylation : The enzyme's function is to cleave the incorrect aminoacyl *t*RNA and release free amino acids and *t*RNA. This process is similar to the editing that occurs during DNA synthesis due to the 3'-5' exonucleolytic activity of DNA polymerases. Like that procedure, aminoacyl *t*RNA editing results in a "futile cycle", in which the enzyme uses energy to build a link and then breaks it down. Both scenarios preserve information processing fidelity at the expense of energy "wastage". Because mistakes are so harmful to the cell, the cost is reasonable.

SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. Describe the structure, chemical composition and types of ribosome. Also, mention the functions of ribosome.

Ans.

Structure of Ribosome

Ribosomes are the protein synthesising machineries of the cell. These were first observed by Palade. These are found in all living cells, since these are the site of protein synthesis (assembling factory for amino acids).

These are more abundant in cells, which are actively involved in protein synthesis. Each ribosome consists of smaller and larger units. Their composition is different in prokaryotes and eukaryotes.

Ribosomes are designated according to their rates of sedimentation. The 70 S for bacterial ribosome (have two subunits) and 80 S for eukaryotic ribosome (have two subunits).

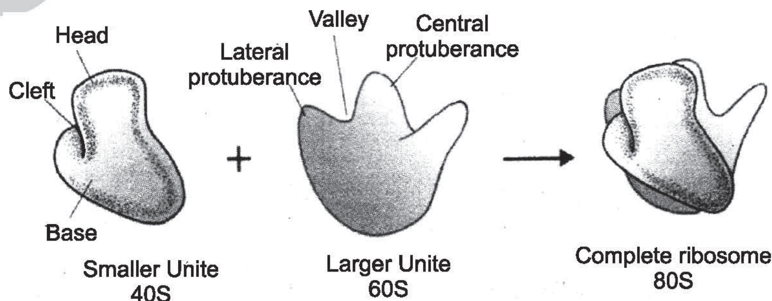

Fig. : Composition of an eukaryotic ribosome

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Each ribosome consists of two units—a larger and a smaller. According to **James A. Lake** (1981), the smaller subunit has a head, a base and a platform. The platform separates the head from the base by a cleft. **Cleft** is the site of codon-anticodon interaction during protein synthesis. The larger unit consists of a valley, a central protuberance and a **stalk**.

Types of Ribosomes

In prokaryotes, the ribosomes are of 70 S type (smaller unit 30 S and larger unit 50 S).

In eukaryotes, the ribosomes are of 80 S type (smaller unit 40 S and larger unit 60 S), where S is 'Sedimentation' coefficient of ribosome in sucrose gradient of **Svedberg Unit**.

This is a sedimentation coefficient which shows how fast cell organelle sediment in an ultra contrifuge.

Chemical Composition

Ribosomes are chemically ribonuclteoproteins (consist of 80% *r*RNA and 20% proteins). The chemical composition in prokaryotes and eukaryotes are as follows :

In RNA :

1. Prokaryotic ribosome contains 3 types of *r*RNA molecules *viz.* 16 S in smaller unit and 23 S and 5 S in larger unit. Eukaryotic ribosome contains 4 types of *r*RNA molecules *viz.* 18 S in smaller unit and 28 S, 5.8 S and 5 S in larger unit.
2. 18 S, 28 S and 5.85 *r*RNAs are synthesised inside the nucleolus while 5S has extra nucleolar origin (but inside the nucleus).

In Ribosomal Proteins : Prokaryotic ribosome contains 21 ribosomal proteins (represented as S1, S2, S3 .....,) in smaller unit and 34 proteins (represented as L1, L2, L3, ...) in larger subunit.

All the proteins in prokaryotes are similar except one, which is same in both units (S20 and 126). So total no. of ribosomal proteins in prokaryotes are (21+ 34) – 1 = 54.

In eukaryotic ribosomes, there are 70 ribosomal proteins, all are different from each other. Smaller unit contains 30, while the larger unit contains 40 ribosomal proteins.

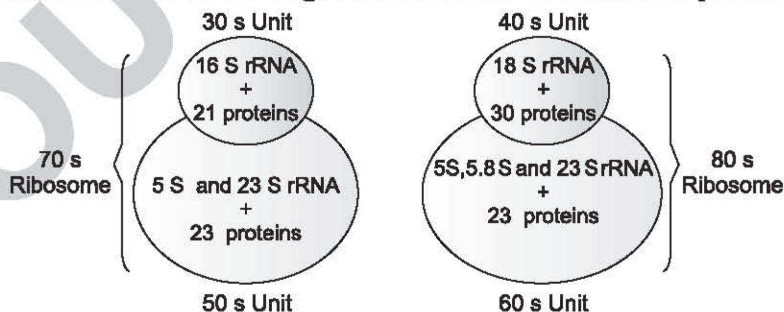

Fig. : Ribosomes in prokaryotes and eukaryotes, and their chemical composition

The 70S ribosome has three *t*RNA-binding sites :

1. **P-site :** P-site, also called the *Peptidyl-tRNA-binding* site, holds the *t*RNA molecule that is linked to the growing end of the polypeptide chain.
2. **A-site :** A-site, also called the *aminoacyl-tRNA-binding* site, holds the incoming *t*RNA molecule charged with an amino acid.

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3. **E-site :** Deacylated *t*RNA (lacking any amino acid) exits via the E site, also called the *exit site.*

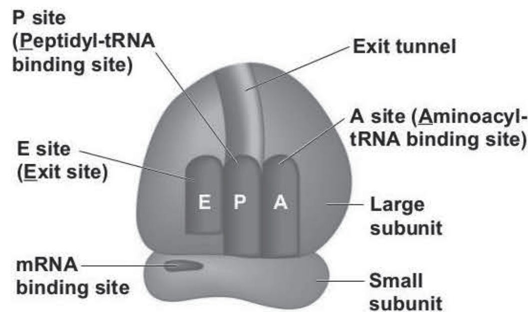

Fig. : Schematic model showing RNA binding site in the ribosome.

Association and Dissociation of Sub-units : The association and dissociation of sub-units depends upon the Mg^{2+} ion concentration.

In low concentration the two units remain separated. But in the higher concentration the units get united.

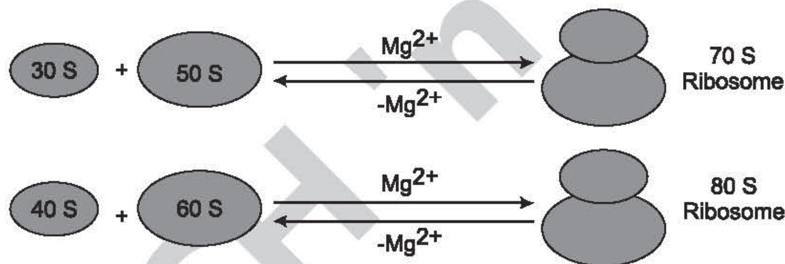

Fig. : Association and dissociation of ribosomal units

Polysome of Ergasome : It is a group or cluster of ribosomes, which are attached to an *m*-RNA molecule. It is also called **polyribosome**. It formation occure in increased Mg^{2+} ion cancentration.

Functions of Ribosome : Ribosomes are minute particles consisting of RNA and associated proteins that function to synthesize proteins. Proteins are needed for many cellular functions such as repairing damage or directing chemical processes. Ribosomes can be found floating within the cytoplasm or attached to the endoplasmic reticulum. Their main function is to convert genetic code into an amino acid sequence and to build protein polymers from amino acid monomers.

Ribosomes act as catalysts in two extremely important biological processes called peptidyl transfer and peptidyl hydrolysis.

Q.2. What is genetic code? Explain about the concept and organization of genetic code.

Ans.

Genetic Code

The genetic code is the set of rules by which information encoded in genetic material (DNA or RNA sequences) is translated into proteins (amino acid sequences) by living cells. The genetic

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code, once thought to be identical in all forms of life, has been found to diverge slightly in certain organisms and in the mitochondria of some eukaryotes. Nevertheless, these differences are rare, and the genetic code is identical in almost all species, with the same codons specifying the same amino acids.

The genetic code consists of 64 triplets of nucleotides. These triplets are called **codons**. With three exceptions, each codon encodes for one of the 20 amino acids used in the synthesis of proteins. This produces some redundancy in the code; most of the amino acids being encoded by more than one codon.

Concept and Organization of Genetic Code

Arrangement of nitrogenous bases in DNA is said to determine by the sequence of amino acid in a protein molecule. Since, there are only 4 nitrogenous bases **Adenine** (A), **Cytosine** (C), **Guanine** (G) and **Thymine** (T). It is obvious that sequence of these four nitrogenous bases on DNA stand directs the synthesis of proteins.

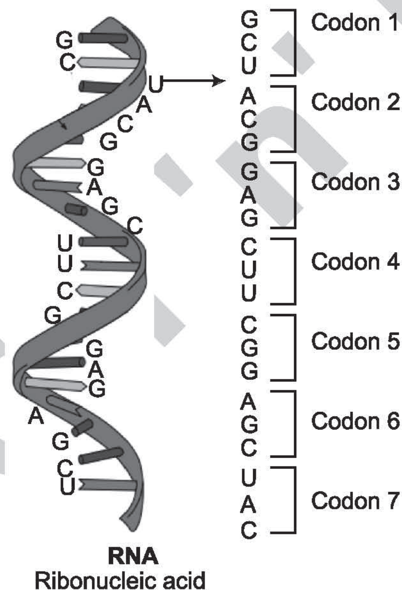

Fig. : Series of codons within a part of an *m*RNA molecule

Different theories have been proposed to predict the mechanism through which sequence of nitrogenous bases present in DNA transcribed into *m*RNA, which eventually determines the position of specific amino acids in protein. Theory proposed by **F.H.C Crick** remains widely accepted. The theory explains existence of genetic code and its smallest unit codon which codes for one amino acid. A codon is defined as nucleotide sequence in *m*RNA which codes for particular amino acid.

When it became clear that codon is a sequence of nucleotide which codes for amino acid, the next question arise was how many nucleotides are present in one codon i.e., it has to be determined the exact number of nucleotides in a codon which codes for an amino acid. Since, there are 20 amino acids and four nitrogenous bases in *m*RNA. Hence, there was requirement

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of sufficient number of codons which could code for 20 amino acids. If you consider that a codon consists of 1 nucleotide *i.e.,* singlet code. Now in this case, since we have 4 nitrogenous bases the total number of codon becomes 4, but how can four codons code for 20 amino acids. Hence, it was clear that each codon consists of more than one nucleotide. Singlet Code = 4 × 1 = 4 Codons (how can 4 codons code for 20 amino acids).

If we consider that a codon consists of 2 nucleotide each *i.e.,* doublet code for each amino acid. Now, in this case the total number of codons becomes 16. Still the problem was not solved because by considering a codon to be a set of two nucleotides only 16 possible codons could be figured out and the amino acids were still 20. Doublet Code = 4 × 4 = 16 codons. Hence, it become clear that codon with one or two nitrogenous bases could not provide sufficient number of codon combinations to code for 20 known amino acids.

Gamow (1954) proposed possibility of a three letter code *i.e.,* each codon consisting of three nitrogenous bases (Triplet code). Now, in this case the total possible combination of codon becomes 4 × 4 × 4 = 64.

Since there are only 20 amino acids, hence, 64 codons are more than enough to code for amino acids. Here you should also note that now total no. of codons becomes 64 and amino acids for which they will code is 20. Hence, several different codons will code for same amino acids. Hence, genetic code is a nucleotide base sequence consisting of codons, each codon made up of three nitrogenous bases. Genetic code is translated into a sequence of amino acids which combine to form proteins.

First Letter	Second Letter: U	C	A	G	Third Letter
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Composition of different codons comprising genetic code

Q.3. Write about the characteristics of genetic code.

Ans.

Characteristics of Genetic Code

The characteristics of genetic code are as follows :

1. **Triplet nature :** Each codon (genetic code) is made up of three nitrogenous bases hence the genetic code is a triplet codon. A triplet code could make a genetic code for 64 different combinations (4 × 4 × 4) genetic code and provide plenty of information in the DNA molecule to specify the placement of all 20 amino acids. When experiments were performed to crack the genetic code it was found to be a code that was triplet. These three letter codes of nucleotides (AUG, AAA, etc.) are called codons.
2. **Non-overlapping :** Non-overlapping codons means that each nitrogenous base in *m*RNA which be used for one codon only. One nitrogenous base cannot be a part of two codons or the same letter is not used for two different codons. In other words, no single base can take part in the formation of more than one codon. Hence genetic code is non-overlapping, *i.e.,* the adjacent codons do not overlap.
3. **Commaless code :** A commaless code means that no nucleotide or comma (or punctuation) is present in between two codons. There is no signal to indicate the end of one codon and the beginning of the next. Therefore, code is continuous and commaless and no letter is wasted between two words or codons. Thus, the genetic code is commaless (or comma-free).
4. **Non-ambiguity :** A particular codon will always code for the same amino acid, i.e. genetic code is specific for e.g. UUU codes for amino acid Phenylalanine, it cannot code for any other amino acid. While the same amino acid can be coded by more than one codon (the code is degenerate), the same codon shall not code for two or more different amino acids. This property of genetic code makes them non ambiguous. However, there are some exceptions.
   (i) AUG and GUG both may code for methionine although GUG codes for valine.
   (ii) GGA is another codon which codes for two amino acid glycine and glutamic acid.
5. **Universality :** Although the code is based on work conducted on the bacterium *Escherichia coli* but it is valid for other organisms. This important characteristic of the genetic code is called its universality. It means that the same sequences of 3 bases encode the same amino acids in all life forms from simple microorganisms to complex, multi-celled organisms such as human beings. All types of living organisms use same type of genetic code.

   This means that codons specifying 20 amino acids are same in bacteria, fungi, plant and animal.

   Occurrence of similar genetic codes in diverse organisms indicates common origin of life on earth. However, there are very few exceptions where same codon codes for different amino acid. Most prominent exception to universality of genetic code is seen in animal mitochondrial DNA where UGA codes for tryptophan however normally UGA is a stop codon.

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Similarly in animal mitochondrial DNA, codon AGA and AGG are used as stop codons which are different from normal stop codons. As compared to animal mitochondrial DNA plant mitochondrial DNA utilizes universal (normal) gene code pattern. In yeast (Saccharomyces cerevisiae), all codons begins with CU code for amino acid threonine, whereas, in other organisms it codes for leucine.

Enlists Exceptions to universal genetic code

DNA (Organism)	Genetic Code	Universally codes for	Altered expression
Human Mitochondrial DNA	UGA	Stop	Tryptophan
	AUA	Isoleucine	Methionine
All vertebrate Mitochondrial DNA	AGA	Arginine	Stop
	AGG		
Yeast (*Saccharomyces cerevisiae*)	UGA	Stop	Tryptophan
	CUA	Leucine	Threonine
	CUG		
	CUU		
	CUA		
Plants	CGG	Arginine	Tryptophan
Nuclear DNA (*Paramecium*)	UAG	Stop	Glycine
Bacterial DNA (*Mycoplasma*)	UGA	Stop	Tryptophan

6. **Degeneracy :** The code is degenerate which means that the same amino acid is coded by more than one base triplet. Genetic code is degenerate *i.e.,* more than one codon can code for same amino acid. For example, the three amino acids arginine, alanine and leucine each have six synonymous codons. There are a total of 64 codons and 20 known amino acids.

   Hence, if you are asked to assign one amino acid to each codon then it is obvious that same amino acids will be assigned too many amino acids. Hence a single amino acid can be coded by many codons except tryptophan and methionine which are coded by one codon each. Degeneracy of genetic code is also known as redundancy. Degeneracy of genetic code can be two types :

   (i) **Partial degeneracy :** When the first two nitrogenous bases are same but the third base is different, the degeneracy is called partial degeneracy, *e.g.,* CUU and CUC (codes for amino acid leucine).

   (ii) **Complete degeneracy :** Complete degeneracy occurs when the third position in the genetic code can be taken by any of the four bases and the codon in each case codes for same amino acid. *i.e.,* UCU, UCA, UCC, UCG (codons codes for serine).

Q.4. Describe the mechanism of translation process in eukaryotic cell.

Ans.

Translation Process in Eukaryotes

The process of protein synthesis from amino acid sequences specified by the sequence of codons in messenger RNA is called **translation**. Translation is the first stage of protein biosynthesis. The main points about translation in eukaryotes are given below :

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1. **Site :** Translation occurs in the cytoplasm where the ribosomes are located. Ribosomes are made of a small and large subunit which surrounds the *m*RNA. In eukaryotic translation 80S ribosomes with 40 S and 60 S subunits are used. The *m*RNA is synthesized from DNA only. In eukaryotes, there is single initiation and termination site.
2. **Template :** This uses an *m*RNA sequence as a template to guide the synthesis of a chain of amino acids that form a protein. Many types of transcribed RNA, such as transfer RNA, ribosomal RNA, and small nuclear RNA are not necessarily translated into an amino acid sequence.
3. **Requirements :** The translation process requires *m*RNA, *r*RNA, ribosomes, 20 kinds of amino acids and their specific *t*RNAs.
4. **Factors involved :** In eukaryotes, several factors are used in chain initiation such as eIF2, eIF3, eIF 4A, eIF 4E, eIF 4F and eIF 4G. Two factors (EF-1 and EF-2) are used in chain elongation. There is a single release factor RF for recognition of three termination codons (UAA, UAG and UGA).
5. **Enzymes involved :** In eukaryotes, two types of enzymes are used in translation. Aminoacyl *t*RNA synthetase (an enzyme) catalyzes the bonding between specific *t*RNAs and the amino acids. The enzyme peptidyl transferase connect A site and P site by forming a peptide bond (the nitrogen carbon bond) during elongation phase.
6. **Codons involved :** In the process of translation two types of codons, *viz.,* start codons and stop codons are involved. The codon, AUG, initiates the process of translation and one of three stop codons i.e. UAA, UAG, or UGA is used for chain termination. In eukaryotes and archaea, the amino acid encoded by the start codon is methionine.
7. **Starting amino acid :** In eukaryotes, starting amino acid is methionine. Moreover, there is no overlapping of transcription and translation.

Mechanism of Translation in Eukaryotes

The mechanism of translation in eukaryotes is similar to that of prokaryotes in several aspects. Translation process consists of three phases or stages, *viz* :

(1) Initiation, (2) Elongation and (3) Termination.

1. **Initiation :** The process of initiation of translation in eukaryotes is of two types, *viz :*

   (i) **Cap-Dependent Initiation :** Initiation of translation usually involves the interaction of certain key proteins with a special tag bound to the 5'-end of an *m*RNA molecule, the 5' cap. The protein factors bind the small ribosomal subunit (also referred to as the 40S subunit), and these initiation factors hold the *m*RNA in place.

   The eukaryotic Initiation Factor 3 (eIF_3) is associated with the small ribosomal subunit, and plays a role in keeping the large ribosomal subunit from prematurely

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binding. The factor eIF_3 also interacts with the eIF_4F complex which consists of three other initiation factors (eIF_4A, eIF_4E and eIF_4G). The factor eIF_4G is a protein which directly associates with both eIF3 and the other two components.

The eIF_4E is the cap-binding protein. It is the rate-limiting step of cap dependent initiation, and is often cleaved from the complex by some viral proteases to limit the cell's ability to translate its own transcripts. The eIF_4A is an ATP-dependent RNA helicase, which aids the ribosome in resolving certain secondary structures, formed by the *m*RNA transcript. There is another protein associated with the e1F $_4F$ complex called the Poly-A Binding Protein (PABP), which binds the poly-A tail of most eukaryotic *m*RNA molecules. This protein is considered to play a role in circularization of the *m*RNA during translation.

This pre-initiation complex ($_{43}S$ subunit, or the $_{40}S$ and *m*RNA) along with protein factors move along the *m*RNA chain towards its 3'-end. It scans for the 'start' codon (typically AUG) on the *m*RNA. The start codon indicates the site where the *m*RNA will begin coding for the protein. In eukaryotes and archaea, the amino acid encoded by the start codon is **methionine**. The initiator *t*RNA charged with Met forms pan of the ribosomal complex and thus all proteins start with this amino acid. The Met-charged initiator *t*RNA is brought to the P-site of the small ribosomal subunit by eukaryotic Initiation Factor 2 (eIF_2). It hydrolyzes GTP, and signals for the dissociation of several factors from the small ribosomal subunit which results in the association of the large subunit (or the 60S subunit).

The complete ribosome (80S) then commences translation elongation, during which the sequence between the 'start' and 'stop' codons is translated from *m*RNA into an amino acid sequence. In this way a protein is synthesized.

(ii) **The Cap-Independent Initiation :** This is lesser known method of translation in eukaryotes. This method of translation has been recently discovered. It has been found to be important in conditions that require the translation of specific *m*RNAs. It works despite cellular stress or the inability to translate most *m*RNAs. Examples of such type of translation are factors responding to apoptosis and stress-induced responses.

The best studied example of the cap-independent mode of translation initiation in eukaryotes is the Internal Ribosome Entry Site (IRES) approach. The main difference between cap independent translation and cap-dependent translation is that the former does not require the ribosome to start scanning from the 5' end of the *m*RNA cap until the start codon.

The ribosome can be trafficked to the start site by ITAFs (IRES trans-acting factors) bypassing the need to scan from the 5' end of the un-translated region of the *m*RNA.

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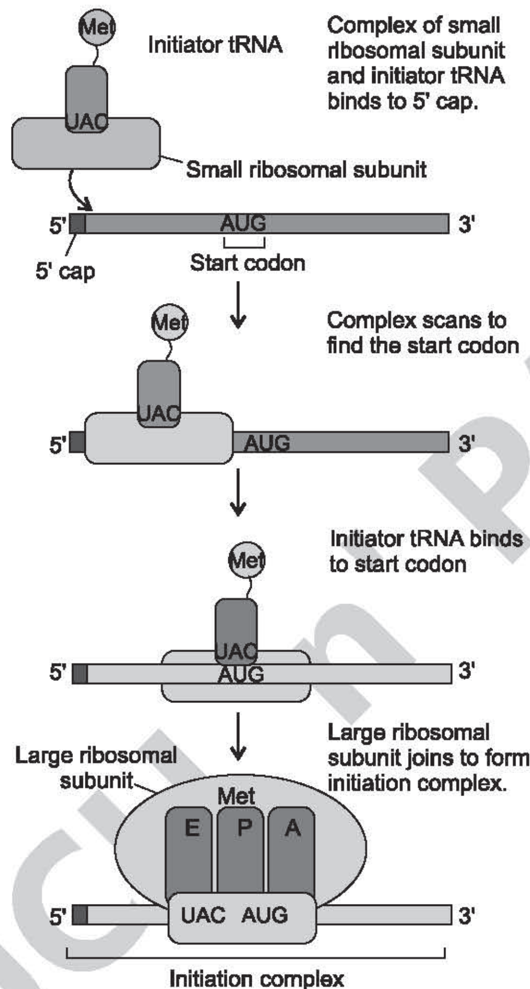

Fig. : Eukaryotic translation initiation

2. **Elongation :** Elongation is dependent on eukaryotic elongation factors At the end of the initiation step, the *m*RNA is positioned so that the next codon can be translated during the elongation stage of protein synthesis.

   The initiator *t*RNA occupies the P site in the ribosome; and the A site is ready to receive an aminoacyl-*t*RNA. During chain elongation, each additional amino acid is added to the nascent polypeptide chain in a three-step micro cycle. The steps in this micro-cycle are :

   (i) Positioning the correct aminoacyl-*t*RNA in the A site of the ribosome;

   (ii) Forming the peptide bond and

   (iii) Shifting the *m*RNA by one codon relative to the ribosome.

   The translation machinery works relatively slowly compared to the enzyme systems that catalyze DNA replication. Proteins are synthesised at a rate of only 18 amino acid

residues per second, whereas bacterial replisomes synthesize DNA at a rate of 1,000 nucleotides per second. This difference in rate reflects, in part, the difference between polymerizing four types of nucleotides to make nucleic acids and polymerizing 20 types of amino acids to make proteins. Testing and rejecting incorrect aminoacyl-*t*RNA molecules takes time and slows protein synthesis.

The rate of transcription in prokaryotes is approximately 55 nucleotides per second, which corresponds to about 18 codons per second, or the same rate at which the *m*RNA is translated. In bacteria, translation initiation occurs as soon as the 5' end of an *m*RNA is synthesized, and translation and transcription are coupled. This tight coupling is not possible in eukaryotes because transcription and translation are carried out in separate compartments of the cell (the nucleus and cytoplasm).

Eukaryotic *m*RNA precursors must be processed in the nucleus (e.g., capping, polyadenylation, splicing) before they are exported to the cytoplasm for translation.

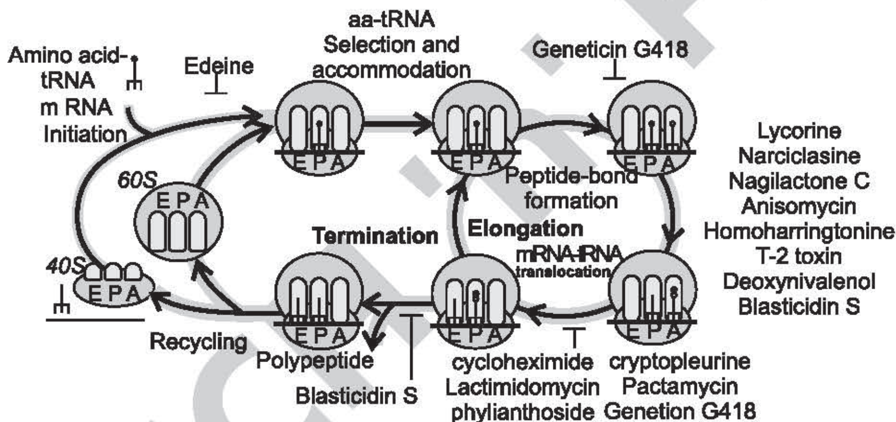

Fig. : Translation process in eukaryotes

3. **Termination :** This is the last phase of translation. Termination occurs when one of the three termination codons moves into the A site. These codons are not recognized by any tRNAs.

   Termination of elongation is dependent on eukaryotic release factors In eukaryotes, there is only one release factor that is eRF, which recognizes all three stop codons [in place of RF_1, RF_2, or RF_3 factors in prokaryotes]. However, the overall process of termination is similar to that of prokaryotes.

Q.5. Explain the mechanism of translation process in prokaryotes with suitable diagram.

Ans.

Translation Process in Prokaryotes

The process by which proteins are produced with amino acid sequences specified by the sequence of codons in messenger RNA is called translation. Translation is the first stage of protein biosynthesis. The main points about translation in prokaryotes are given below :

1. **Site :** Translation occurs in the **cytoplasm** where the ribosomes are located. Ribosomes are made of a small and large subunit which surrounds the *m*RNA. In

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prokaryotic translation 70S ribosomes with 30S and 50S subunits are used. The *m*RNA is synthesized from DNA only. In prokaryotes, there are several initiation and termination sites.

2. **Template :** In translation, messenger RNA (*m*RNA) is decoded to produce a specific polypeptide according to the rules specified by the genetic code. This uses an *m*RNA sequence as a template to guide the synthesis of a chain of amino acids that form a protein. Many types of transcribed RNA, such as transfer RNA, ribosomal RNA, and small nuclear RNA are not necessarily translated into an amino acid sequence.
3. **Requirements :** The translation process requires *m*RNA, *r*RNA, ribosomes, 20 kinds of amino acids and their specific *t*RNAs.
4. **Factors involved :** In prokaryotes, three factors are involved in the initiation of translation (IF_1, IF_2 and IF_3), one factor in the elongation of polypeptide chain and three factors in chain termination (RF_1, RF_2 and RF_3).
5. **Enzymes involved :** Two types of enzymes are used in translation. Aminoacyl *t*RNA synthetase (an enzyme) catalyzes the bonding between specific *t*RNAs and the amino acids. The enzyme peptidyl transferase connects A site and P site by forming a peptide bond (the nitrogen carbon bond) during elongation phase.
6. **Codons involved :** In the process of translation two types of codons, *viz.,* start codon and stop codons are involved. The codon, AUG, initiates the process of translation and one of three stop codons *i.e.,* UAA, UAG or UGA is used for chain termination.
7. **Starting amino acid :** In prokaryotes, starting amino acid is N-formyl methionine. Moreover, there is overlapping of transcription and translation.

Mechanism of Translation in Prokaryotes

Translation process consists of three major phases or stages, *viz.* : (1) Initiation, (2) Elongation and (3) Termination.

1. **Initiation :** This is the first phase of translation. Start or initiation codon (AUG) is responsible for initiation of translation process. Initiation of translation in prokaryotes involves the assembly of the components of the translation system which are : the two ribosomal subunits (small and large), the *m*RNA to be translated, the first (formyl) aminoacyl *t*RNA (the *t*RNA charged with the first amino acid), GTP (as a source of energy), and three initiation factors (IF_1, IF_2 and IF_3) which help the assembly of the initiation complex.

   The ribosome consists of three sites, the A site, the P site, and the E site. The A site is the point of entry for the aminoacyl *t*RNA (except for the first aminoacyl *t*RNA, fMet-*t*RNA f^{met}, which enters at the P site). The P site is where the peptidyl *t*RNA is formed in the ribosome. And the E site which is the exit site of the now uncharged *t*RNA after it gives its amino acid to the growing peptide chain.

   Translation Translation begins with the binding of the small ribosomal subunit to a specific sequence on the *m*RNA chain. Initiation of translation begins with the 50S and 30S ribosomal subunits. IF1 (initiation factor 1) blocks the A site to ensure that the

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IMet-*t*RNA can bind only to the P site and that no other aminoacyl-*t*RNA can bind in the A site during initiation, while IF_3 blocks the E site and prevents the two subunits from associating.

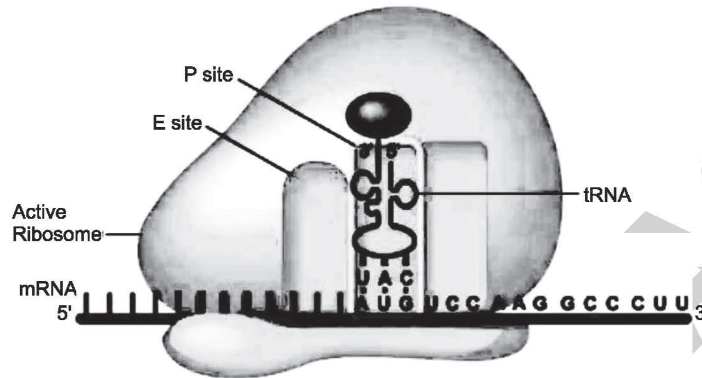

Fig. : Initiation in translation

IF_2 is a small GTPase which binds fmet-*t*RNA f^{met} and helps its binding with the small ribosomal subunit. The 3' end of the 16S *r*RNA of the small 30S ribosomal subunit recognizes the ribosomal binding site on the *m*RNA (Shine-Dalgarno sequence or SD), through its anti-SD sequence, 5-10 base pairs upstream of the start codon. The Shine-Dalgarno sequence is found only in prokaryotes.

This helps to correctly position the ribosome onto the *m*RNA so that the P site is directly on the AUG initiation codon. IF3 helps to position fMet-*t*RNA f^{met} into the P site, such that fMet *t*RNA f^{met} interacts via base pairing with the *m*RNA initiation codon (AUG). Initiation ends as the large ribosomal subunit joins the complex causing the dissociation of initiation factors. The small subunit binds *via* complementary base pairing between one of its internal subunits and the ribosome binding site. This site a sequence of about ten nucleotides on the *m*RNA. It is located anywhere from 5 and 11 nucleotides from the initiating codon (AUG). After binding of the small subunit, a special *t*RNA molecule, called *N*-formyl methionine, or fMet, recognizes and binds to the initiator codon. Then the large subunit binds resulting in the formation of the initiation complex. As soon as the initiation complex is formed, the fMet-*t*RNA occupies the P site of the ribosome and the A site is left empty. This entire initiation process is facilitated by extra proteins, called initiation factors that help with the binding of ribosomal subunits and *t*RNA to the *m*RNA chain.

2. **Elongation :** This is the second phase or middle phase of translation. Elongation begins after the formation of the initiation complex. Elongation of the polypeptide chain involves addition of amino acids to the carboxyl end of the growing chain. The growing protein exits the ribosome through the polypeptide exit tunnel in the large subunit.

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Elongation starts when the fmet-*t*RNA enters the P site, causing a conformational change which opens the A site for the new aminoacyl-*t*RNA to bind. This binding is facilitated by elongation factor-T4 (EF-T4), a small GTPase. Now the P site contains the beginning of the peptide chain of the protein to be encoded and the A site has the next amino acid to be added to the peptide chain.

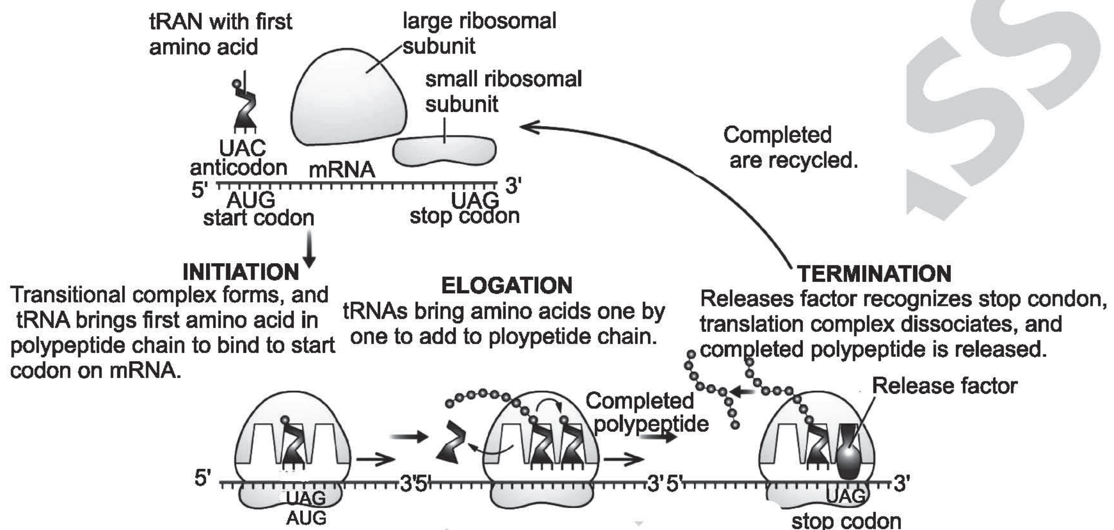

Fig. : Translation process in Prokaryotes.

The growing polypeptide connected to the *t*RNA in the P site is detached from the *t*RNA in the P site and a peptide bond is formed between the last amino acids of the polypeptide and the amino acid still attached to the *t*RNA in the A site.

This process, known as **peptide bond formation**, is catalyzed by a ribozyme, peptidyltransferase, an activity intrinsic to the 23S ribosomal RNA in the 50S ribosomal subunit. Now, the A site has newly formed peptide, while the P site has an unloaded *t*RNA (*t*RNA with no amino acids). In the final stage of elongation, translocation, the ribosome moves 3 nucleotides towards the 3' end of *m*RNA. Since *t*RNAs are linked to *m*RNA by codon-anticodon base-pairing, *t*RNAs move relative to the ribosome taking the nascent polypeptide from the A site to the P site and moving the uncharged *t*RNA to the E exit site.

This process is catalyzed by elongation factor G (EF-G). The ribosome continues to translate the remaining codons on the *m*RNA as more aminoacyl-*t*RNA binds to the A site, until the ribosome reaches a stop codon on *m*RNA(UAA, UGA, or UAG). When the A site opens again, the next appropriate aminoacyl *t*RNA can bind there and the same reaction takes place, yielding a three amino acid peptide chain. This process repeats, creating a polypeptide chain in the P site of the ribosome. A single ribosome can translate 60 nucleotides per second. This speed can be vastly augmented when ribosomes unite together to form polyribosomes.

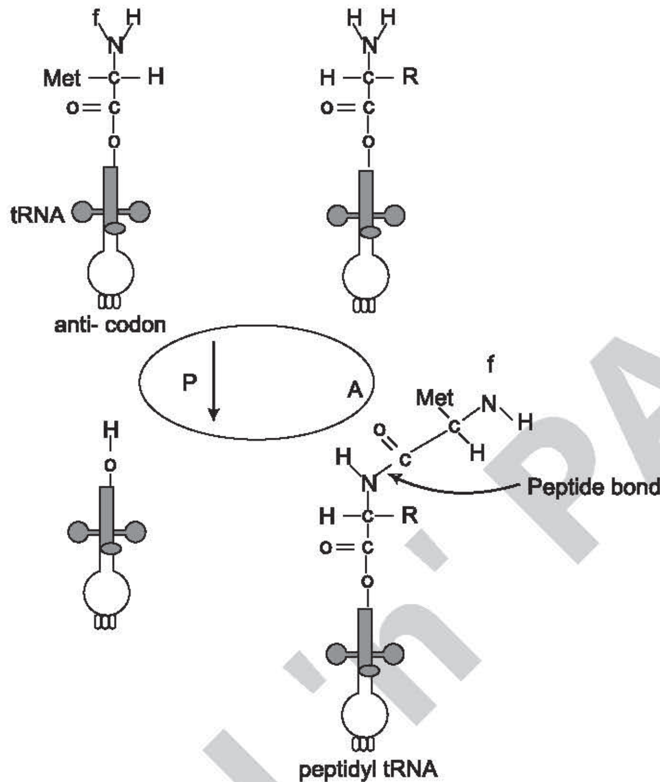

Fig. : Peptide Formation

3. **Termination :** This is the last phase of translation. Termination occurs when one of the three termination codons moves into the A site. These codons are not recognized by any *t*RNAs. Instead, they are recognized by proteins called **release factors**, namely RF1 (recognizing the UAA and UAG stop codons) or RF2 (recognizing the UAA and UGA stop codons). These factors trigger the hydrolysis of the ester bond in peptidyl-*t*RNA and the release of the newly synthesized protein from **the ribosome**. A third release factor RF-3 catalyzes the release of RF-1 and RF-2 at the end of the termination process.

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UNIT-III

Regulation of Gene Expression-I

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. What do you mean by gene expression?

Ans. Gene expression is the process by which the information encoded in a gene is used to either make RNA molecules that code for protein or to make non-coding RNA molecules that serve other functions.

Q.2. What controls gene expression?

Ans. Specifically, gene expression is controlled on two levels. First, transcription is controlled by limiting the amount of *m*RNA that is produced from a particular gene. The second level of control is through post-transcriptional events that regulate the translation of *m*RNA into proteins.

Q.3. What is gene expression in eukaryotes?

Ans. Gene expression in eukaryotes in influenced by a wide variety of mechanisms including the loss, amplification and rearrangement of genes. Genes are differentially transcribed and the RNA transcripts are variably utilized. Multigene families regulate the amount, the diversity and the timing of gene expression.

Q.4. What is the difference between gene expression in prokaryotes and eukaryotes?

Ans. Prokaryotes gene expression (both transcription and translation) occurs within the cytoplasm of a cell due to the lack of a defined nucleus; thus the DNA is freely located within the cytoplasm. Eukaryotes gene expression occurs in both the nucleus (transcription) and cytoplasm (translation).

Q.5. What controls gene expression in eukaryotes?

Ans. Gene expression in eukaryotic cells is regulated by repressors as well as by transcriptional activators. Like their prokaryotic outerparts, eukaryotic repressors bind to specific DNA sequence and inhibit transcription.

Q.6. What is *lac* operon?

Ans. The *lac* operon is one of the best known that in the obsence of inducer lactose, the regulator gene *R* produce a repressor protein which binds to the operator site and presents transcription of structural genes. When inducer lactose is introduced in the medium, it binds to the represor and presents it from binding to the operator.

Q.7. What is the structure of operon?

Ans. An operon is made up of several structural genes arranged under a common promotor and regulated by a common operator. It is defined as a set of adjacent structural genes plus the adjacent regulatory signals that affect transcription of the genes.

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Q.8. What is called as the *trp* operon?

Ans. Tryptophan (trp) is one of the 20 amino acid building blocks that cells need for making proteins. The genes for the bio synthesis of trptophan are clustered together under the control of a single promotor. This cluster of genes and their regulatory sequences is called *trp* operon.

Q.9. What is the function of *trp* operon?

Ans. TRP (Transient receptor potential) ion channels functions as signal integrators through their ion conductance properties and is some cases kinases activity. They mediate processes such as vision, taste, oyaction, hearing, touch and thermo and osmosensation.

Q.10. Is trp operon positive or negative?

Ans. The trp operon is negatively controlled by the trp repressor, a product of the trp R gene. The trp repressor binds to the operator and blocks transcription of the operon.

Q.11. What is RNA editing?

Ans. RNA splicing is a biological process where a newly synthesized pre-*m*RNA transcript is processed and transformed into *m*RNA. It involves the removing of non-coding region of RNA (introns) and the joing of the coding regions (exons).

Q.12. Why is *m*RNA splicing important?

Ans. Precursor *m*RNA (pre-*m*RNA) splicing is a critical step in gene expression that results in the removal of intronic sequences from immature *m*RNA, leading to the production of mature *m*RNA that can be translated into protein.

Q.13. What is chromatin and how does it affect gene expression?

Ans. Chromatin serves as a plat form for numerous cellular signals to influence gene expression. Post-translational modifications (PTMs) of histone proteins or covalent modifications of nucleotides influence a cell's transcriptional program, which ultimately impacts cellular behaviour and cell fate.

Q.14. How does chromatin modify regulate gene expression?

Ans. Eukaryotic DNA is packaged and wrapped around proteins known as histones which protect and regulate gene expression. The structure of DNA wrapped histone octamers is known as chromatin. Chromatin at the first level of its organization appears a a linear array of uniform structural units, nucleosomes.

Q.15. How does chromatin increase transcription?

Ans. As chromatin condensed into the primary nucleosome structure, DNA becomes less accessible for transcription factors. With the loosening of this chromatin structure, however, transcription machinery is better able to access the genetic DNA, and transcription is thus promoted.

Q.16. What is the process of capping?

Ans. Capping is a three-step process that utlizes the enzymes RNA triphosphatase, quanylyl transferase and methyltransferase. Through a series of three steps, the cap is added to the first nucleotide's 5' hydroxyl group of the growing *m*RNA strand while transcription is still occurring.

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Q.17. What is capping and tailing of *m*RNA?

Ans. There is an addition of 7-methylyuanylate at the 5' end of the *m*RNA. This process is known as the capping of the *m*RNA. At the 3' end of the *m*RNA, there is an addition of a chain of adenine nucleotides. This is known as the poly-A tail or the tailing mechanism.

Q.18. What is the function of polyadenylation?

Ans. Then, an enzyme called poly-A polymerase adds a chain of adenine nucleotides to the RNA. This process, called polyadenylation, adds a poly a tail that is between 100 and 250 residues long. The poly-A tail makes the RNA molecule more stable and prevents its degradation.

SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. What do you mean by regulation of gene expression?

Ans.

Regulation of Gene Expression

A cell contains a complete set of genes, which code for all the proteins required by the organism, but only a few proteins are needed at a time. All the genes are not expressed simultaneously, *i.e.,* a cell is not always flooded with all the necessary and unnecessary proteins. Proteins are formed only when they are required by the body.

In multi-cellular organisms all cells have same genetic make up. Despite this fact, they do not function in asimilar manner. For instance, in human body, the cells of salivary glands synthesise ptyalin, but not insulin.

Similarly the beta cells of **islets of Langerhans** synthesise insulin, but not ptyalin. Why, when they have same genetic material?

In these organisms, there occurs a process called **differentiation**, in the early embryonic period. During differentiation, the cells of the body get specialised for a particular function. Since the functions of the cell are associated with enzymatic activities and proteins, so the differentiation process occurs at the level of genes. This process is not very well understood, however, in this process certain mechanisms are involved due to which only selected genes are expressed.

So by phrase 'regulation of gene expression' we mean the induction of a few genes and the suppression of the others gene. This is also called as **differential gene expression**.

The mechanism of protein synthesis or gene expression is highly organised and well controlled.

Q.2. What are the differences between prokaryotic and eukaryotics gene regulation?

Ans.

Difference between Prokaryotic and Eukaryotic Gene Regulation

S.No.	Prokaryotic	Eukaryotic
1.	Lack nucleus	Contain nucleus
2.	DNA is found in the cytoplasm.	DNA is in the nucleus.
3.	Transcription and translation occur almost simultaneously.	Transcription occurs in the nucleus prior to translation, which occurs in the cytoplasm.

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4.	Gene expression is regulated primarily at the transcriptional level.	Gene expression is regulated at many levels : epigenetic, transcriptional, nuclear shuttling, post-transcriptional, translational and post-translational.

Q.3. Differentiate between Lac Operon and Tryptophan operon.

Ans.

Difference between *Lac* Operon and Tryptophan Operon

S.No.	*Lac* Operon	Tryptophan Operon
1.	It follows catabolic pathway.	It follows anabolic pathway.
2.	It shows inducible control of gene activity.	It shows repressible control of gene activity.
3.	Enzymes for the catabolism of substrate are synthesised.	Enzymes for the production of tryptophan are to synthesised.
4.	Repressor molecules alone is able to bind to the operator gene.	Repressor molecules alone is unable to bind to the operator gene.
5.	Inducer can deform the repressor making it unable to get attached to the operator gene. Thus, the lactose acts as inducer. It induces the production of enzymes.	Tryptophan combines with the repressor and both together bind to the operator. Here, tryptophan does not act as an inducer, instead it causes the repression (inhibition) of the enzyme synthesis.

Q.4. Write about the inducible and repressible regulation.

Ans.

Inducible and Repressible Regulation

In prokaryotes and eukaryotes, all genes are not expressed simultaneously. Genes are expressed according to the cellular requirements In *E. coli*, there are about 3,000 genes. All these genes do not express at the same time. This shows that, the expression of gene will be **'switched on'** when the protein (which, the gene forms), is required by the cell. This 'switching on' is called **'induction'**. The substance that causes the induction of gene, leading to the formation of a protein (necessary for the cell) is called **inducer.** The mechanism involved is called inducible control or **positive control**.

When the requirement of the cell is over or fulfilled, the same gene will be turned off. This is called **repression 'switched off'**. Some substances (usually the end products of a metabolic pathway) when added to the medium, can inhibit the expression of all the genes, which are concerned with this metabolic pathway. Such substances are called **repressors**. The mechanism involved is called repression or repressible control or **negative control**.

Q.5. What is negative and positive gene regulation?

Ans.

Negative and Positive Gene Regulation

The regulation of gene activity can be negative or positive :

1. **Negative control :** Both the control system inducible as well as repressible the structural genes are expressed only when the operator is free. But when operator is blocked by repressor protein, synthesis of proteins is inhibited. Such control mechanism for protein synthesis is called as **negative type control system.** *Lac* operon model of *E. coli* is an example of negative control.
2. **Positive control :** Regulator protein produces repressor protein which binds to operator and blocks transcription and translation. However, in some cases regulator

protein may act as an activator and enhances protein synthesis. The site at which activator attached are called **initiator site.**

Operator can also act as initiator site; however, it is not necessary that initiator site will always be the operator. Since, in the case where regulator protein enhances the synthesis of proteins, it is called **positive control system**. Arabinose operon of *E. coli* is an example of positive control of gene regulation.

SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. Describe the composition and working of tryptophan in prokaryotes.

Ans.

Tryptophan Operon (Repressible Control)

Basically, it is similar to lac operon. However, instead of catabolism, it operates on anabolic pathway, because it is associated with the production of enzymes, which are involved in the formation (*i.e.,* anabolism) of amino acid tryptophan. The enzymes are synthesised only when the medium does not contain tryptophan.

When tryptophan is present in the medium, the production becomes inhibited due to repression of genes. It is therefore clear that the substance itself acts as a repressor and inhibits the genes associated with its own production. This kind of regulation is called **repressible regulation** or **control**.

This operon (trp Operon) also consists of regulator, promoter, operator and structural genes. In this case, there are five genes, which are involved in the production of enzymes (related to the formation of tryptophan). These five structural genes are referred to as trp E, trp D, trp C, trp B and trp A.

In this case, the repressor molecule (produced by *R*-gene) by itself is unable to bind to operator gene. This repressor is better known as **'apo-repressor'** Tryptophan in this case, itself acts as repressor (not inducer). It is better known as co-repressor.

Working Of Tryptophan Operon

Working process of tryptophen operon are as follows :

Switching off : When tryptophan is added to the medium, it (tryptophan) binds with apo-repressor to form a complex. This complex binds with the trp O gene. Due to this binding the gene trp O gets arrested and RNA polymerase is unable to cross it.

Consequently, the transcription of structural genes is inhibited.

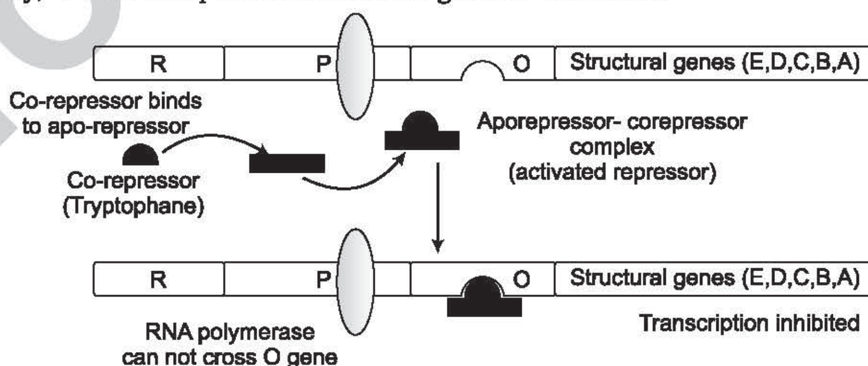

Fig. : Working of Tryptophan Operon (Switching off control)

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Switching on : When tryptophan is absent in the medium, the apo-repressor alone is unable to bind to the operator gene. Due to this the trp O gene remains free.

The RNA polymerase, under this situation, can easily cross the O gene and can carry out the transcription of the structural genes. This results in the synthesis of enzymes, concerned with the production of tryptophan.

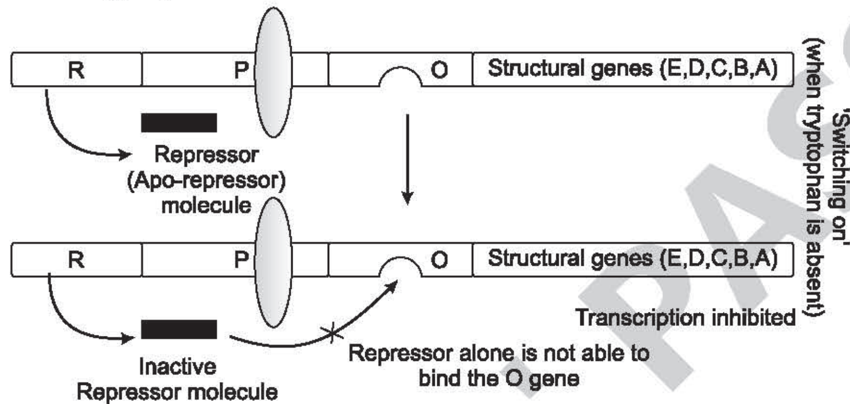

Fig. : Working of Tryptophan Operon (Switching on control)

Q.2. Define the *lac* operon concept. Also describe its various gene, components and working process.

Ans.

Operon Concept

Francois Jacob (geneticist) and **Jacques Monad** (biochemist) in 1961, proposed 'Operon concept' to explain the mechanism of inducible and repressible regulation of gene expression in prokaryotes. For this achievement, they were awarded Nobel prize of Medicine in 1965 (along with Lowff).

Jacob and **Monad** (1961), at the Pasteur Institute, Paris, described their Operon model, by analysing the metabolism of lactose in bacterium *E. coli*. It is the best-understood mechanism in any organism.

According to the hypothesis of Jacob and Monad, any metabolic reaction, in prokaryotes, is regulated by a setof genes, rather than a single gene. All the components (genes) that together control a particular metabolic reaction, together form an operon. Thus, operon is a DNA segment, containing a few genes, that together (in a co-ordinated manner), serve to regulate a metabolic reaction or operon is a group of genes,which are present adjacently and are expressed together as a unit.

An open consists of genes are as follows :

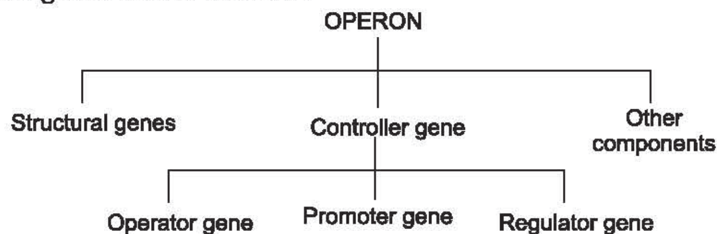

Fig. : Components of an operon.

Example of Lac Operon : A set of genes that together control the catabolism of lactose sugar in *E. coli* constitutes the *lac* operon. This operon is an example of **inducible type** of catabolic pathway operon.

1. **Structural genes :** These are the genes, which actually contain the genetic information, for the synthesis of protein (enzyme). These genes determine the sequence of amino acids in the mature protein. These genes are of three types—*z*, *y* and *a*. *z*-gene codes for an enzyme beta galactosidase (β-gal), which is mainly responsible for the hydrolysis of lactose into glucose and galactose units. *y*-gene codes for permease enzyme, which increases permeability of cell membrane for β-gal. These respectively code for the synthesis of enzymes, β-galactosidase (*lac z*), permease (*lac y*) and transacetylase (*lac A*). These enzymes together control the metabolism of lactose in *E. coli*.

   When lactose is absent from the medium in which *E. coli* grows, few molecules of each enzyme are produced. If lactose is added to the medium and glucose is absent, the rate of synthesis of all three enzymes simultaneously increases about a thousand fold within 2 to 3 minutes. This boost in enzyme synthesis results from the transcription of *lac Z*, *lac Y*, and *lac A*.

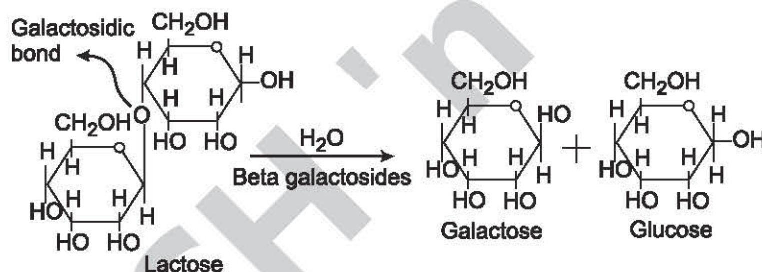

Fig. : Catabolism of lactose sugar in *E. coli*

2. **Controller genes :** These genes serve to control the expression of the structural genes. These are of three main types : (i) Regulator, (ii) Promoter and (iii) Operator.

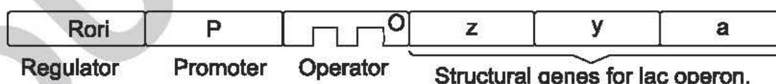

Fig. : A part of DNA in *E. coli*, showing *lac* operon and its components

(i) **Regulator gene :** It is called *R* gene. In *E. coli*, it is referred to as *lac* i, because its expression is maintained by an inducer molecule (lactose in this case). Regulator gene leads to the formation of a protein molecule, called 'repressor molecule'. This repressor molecule has a great affinity for operator gene. Therefore it binds specifically to the operator gene.

(ii) **Promoter gene :** It is also called *P* gene. It marks the promoter site or transcription initiation site. It provides a platform to RNA polymerase to commence transcription.

(iii) **Operator gene :** It is called *O* gene. It is a short DNA segment (about 27 bp long in *E. coli* for *lac* operon). It is present between the promoter site and first structural

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gene (*i.e.,* it is just adjacent to the first structural gene). This gene contains the site for the specific binding of the repressor molecule (which is synthesised by the regulator gene). When repressor molecule binds to this site, RNA polymerase can not cross itand thus transcription cannot occur.

3. **Other components :** These include following components :
   (i) **Repressor molecule :** It is a protein molecule (consisting of 4 subunits, each with a MW 40,000). Its formation is coded by the *R* gene. It has a high affinity for operator gene. It thus binds the operator genes and thereby prevents the movement of RNA polymerase through the operator gene. This prevents the transcription of structural genes.
   (ii) **Inducer :** In many cases the substrate molecule, (for which the cell is to prepare enzyme or protein), itself acts as an inducer (*allo*-lactose in case of *lac* Operon). The small inducer molecules bind specifically the repressor molecule. This binding brings out a conformational change in the structure of the repressor molecule. Due to this change the repressor molecule becomes unfit to get attached with the operator gene. Consequently, the operator gene remains free. In such condition, the RNA polymerase can easily pass through the operator gene and thus transcription can occur.

It is therefore clear that, transcription occurs only when the inducer is present in the medium. When the inducer (substrate) is missing, the transcription does not occur, leading to the turning off of the associated genes.

Working of *Lac* Operon

The working process of *lac* operon are as follows :

1. **Switching on :** When lactose is added in the medium, it acts as an inducer for the operons. It binds specifically to the repressor molecule. Due to this binding, it prevents the binding of repressor with the operator gene.

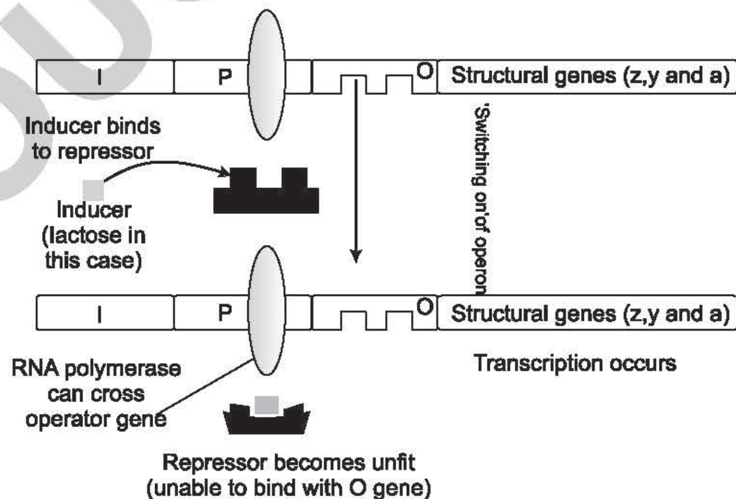

Fig. : 'Switching on' of lac operon

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As a result of this the operator gene remains free. Under such a condition, the enzyme RNA polymerase (associated with P gene), can pass through the operator gene without any difficulty and can carry out transcription of structural genes (*z*, *y* and *a*). So on adding the lactose (inducer) in the culture medium the synthesis of enzymes (associated with lactose metabolism) is switched on.

2. **Switching off :** On the other hand, when lactose (or inducer) is absent in the medium, the repressor molecule is free to bind the operator gene.

   Due to this binding, the operator gene gets arrested. Under this condition, the enzyme RNA polymerase cannot cross such an arrested operator gene and consequently transcription is inhibited. *Lac* Operon is an example of catabolic pathway, as it involves the control of genes, which produce a proteinfor the catabolism of the substrate (*i.e.*, lactose to glucose and galactose).

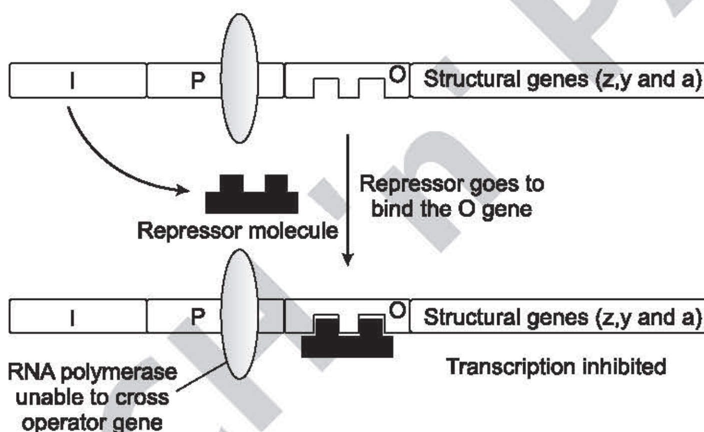

Fig. : 'Switching off' of *lac* operon

Q.3. Describe in detail the role of chromatin in gene regulation in eukaryotes.

Ans.

Role of Chromatin in Gene Expression or Regulation

One type of gene control in eukaryotic cells is accomplished through the modification of chromatin (gene) structure. The chromatin is the loose form of chromosome in interphase. It consists of DNA and histone proteins. Histones form octamers (8 units of histones), around which helical DNA tightly coils to create chromatin. The histone octamer, along with DNA is called **nucleosome.** Histones are of 5 types - H1, H2A, H2B, H3 and H4. Except H1, two units of remain 4 types of histones form a histone octamer. Histone H1 helps in sealing off the entire structure. A single DNA molecule is associated with several thousand histone octamers to form same number of nucleosome. This chromatin structure represses gene expression. For a gene to be transcribed, transcription factors, activators, and RNA polymerase must bind to the DNA.

Before transcription, chromatin structure changes and the DNA becomes more accessible to the transcriptional enzymes and proteins. So, if histones get modified, the DNA may not accessible to the transcriptional machinery.

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Histone modification : Histones are basic proteins and they have positively charged tails that interact with the negatively charged phosphate groups on the backbone of DNA.

The tails of histone proteins are often modified by the addition or removal of phosphate groups, methyl groups, or acetyl groups. These modifications are called histone coding, because codes on histones, encode information that affects how genes are expressed.

One type of histone modification is the addition of methyl groups to the tails of histone proteins. These modifications can bring about either the activation or the repression of transcription, depending on which particular amino acids in the histone tail are methylated.

A common modification is the addition of three methyl groups to lysine-4 in the tail of the H3 histone protein. This is abbreviated as H3K4me3 (where, K is the abbreviation for lysine).

The H3K4me3 modification is frequently found near the transcription start site of many genes in eukaryotes. Proteins such as **NURF** (nucleosome-remodeling factor) can recognize H3K4me3 and bind to the H3 histone tail and then alter the chromatin packing. This allows transcription to take place.

Another type of histone modification that affects chromatin structure is acetylation, the addition of acetyl groups(CH_3CO^-) to histone proteins. Acetyl groups destabilize the chromatin structure, allowing transcription to take place. An important example is the addition of a single acetyl group to lysine-16 in the tail of the H4 histone prevents the formation of the 30-nm chromatin fibre, causing the chromatin to be in an open configuration and available for transcription. Acetyl groups are added to histone proteins by acetyl transferase enzymes.

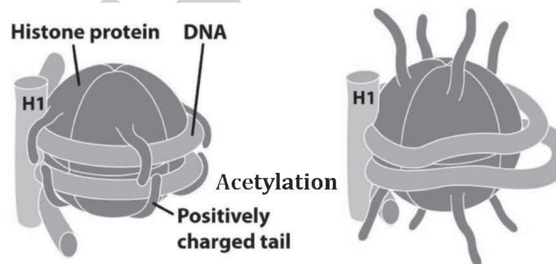

Fig. : The acetylation of histone proteins alters chromatin structure and permits some transcription factors to bind to DNA.

Some enzymes called **deacetylases**, remove the acetyl groups from histones and restore chromatin structure. This represses transcription. Certain proteins that regulate transcription either have acetyl transferase activity or attract acetyl transferases to the DNA. In this way gene expression may be regulated.

The importance of histone acetylation in gene regulation is demonstrated by the control of flowering in Arabidopsis thaliana. The expression of the gene responsible for flowering in this plant is controlled by acetylation of associated histones.

Chromatin re-modelling : Besides altering the structure of histones, the cells can also reposition the location of nucleosomes. This exposes a particular area of the DNA and thereby regulates the gene expression. Some proteins, which are called **chromatin re-modelling complexes** can alter chromatin structure without altering the chemical structure of the

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histones. They bind directly to particular sites on DNA and reposition the nucleosomes. This allows transcription factors to bind to transcription promoters and initiate transcription.

The best example of this type of regulation is SWI-SNF Complex (Switch/sucrose non-fermentable complex), which is a chromatin remodelling complex in yeast, humans, *Drosophila* and other organisms. This complex utilizes ATP energy to reposition nucleosomes. This exposes promoters in the DNA and initiates transcription.

DNA methylations : One more way in which gene expression is regulated is the methylation of DNA bases. The methylation of cytosine bases (forming 5-methyl cytosine) helps in repression of transcription invertebrates and plants. It has been observed that the DNA which is transcriptionally active, is usually un-methylated.

Q.4. Explain the regulation at the level of transcription in eukaryotes.

Ans. Regulation at the Level of Transcription in Eukaryotes

Transcription is the process by which *m*RNA is prepared from DNA template. This is a highly regulated enzymatic process. Most of the gene regulation in eukaryotes occurs by altering the transcription machinery. Transcription machinery includes DNA template, RNA polymerase and several transcription factors and proteins. Any change in the transcription machinery can change the transcription rate. Gene regulation at level of transcription is achieved by following steps :

1. **Enhancers and silencers :** These are certain DNA sequences which are present some distance away from the structural gene. For example, an enhancer that regulates the gene encoding the alpha chain of the T-cell (T-lymphocyte) receptor is located 69,000 bp downstream of the gene's promoter. These sequences provide attachment to specific protein molecules which can either promote transcription or repress transcription. DNA sequences that provide attachment to transcription promoting proteins are called enhancers.

   On the other hand, the sequences which provide attachment to transcription prepressing proteins, are called silencers or insulators.

   Most eukaryotic silencer proteins do not directly block RNA polymerase, instead they compete with activators for DNA binding sites.

2. **Gene regulation through RNA splicing :** In eukaryotes, the newly formed *m*RNA is not homogenous and functional because it contains both coding (exons) and non-coding (introns) regions. Such eukaryotic primary transcript *m*RNA is called *hn*RNA. It is and needs stabilization and homogenisation. The RNA processing mechanism converts this *hn*RNA into active and mature *m*RNA.

   The introns which are non-coding regions are removed off during this process and all exons (coding regions) are joined together to form mature *m*RNA. This process is referred to as splicing. Alternative splicing allows a pre-*m*RNA to be spliced in multiple ways, generating different proteins in different tissues or at different times in development from the same *hn*RNA.

   In such cases, the normal exons are sometimes treated as introns and are removed off. This can be done is several different ways to produce different kind of *m*RNAs that result in different proteins.

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Best example of such splicing comes from calcitonin and CGRP (calcitonin gene-related peptide) gene in mammals.

Calcitonin is mainly produced by thyroid C cells whereas CGRP is secreted and stored in the nervous system.

The mammalian CGRP pre-*m*RNA is alternatively spliced in a tissue-specific manner, leading to the production of :

(i) Calcitonin *m*RNA in thyroid C cells, which contains exons 1, 2, 3 and 4.

(ii) CGRP *m*RNA in neurons, which contains exons 1, 2, 3, 5 and 6.

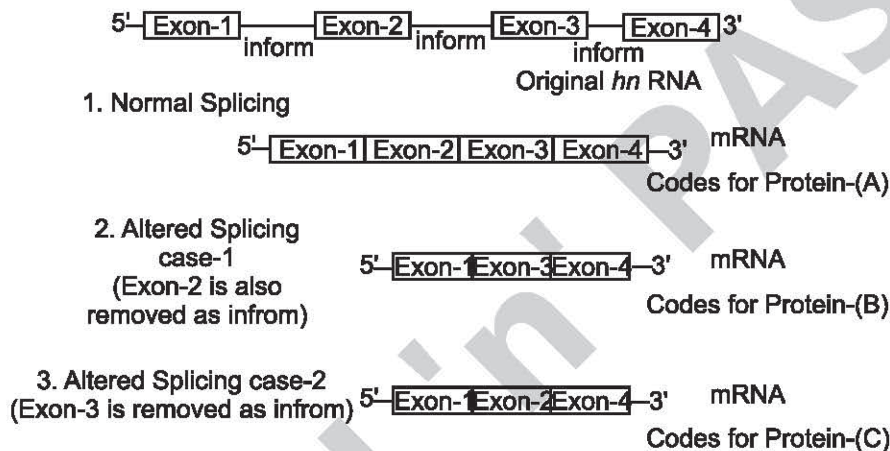

Fig. : Normal splicing and altered splicing compared to see different *m*RNA product

Many eukaryotic genes undergo alternative splicing, and the regulation of splicing is probably an important means of controlling gene expression in eukaryotic cells.

3. **Capping and polyadenylation :**

(i) The first nucleotide at the 5' end of the *hn*RNA is covered by cap of a molecule-7 methyl guanosine triphosphate (7mGTP). It is a modified guanosine triphosphate molecule.This event is called **capping.** The cap is linked to the first nucleotide of *hn*RNA by 5' to 5' triphosphate bond. The cap of 7mGTP determines the initiation site for translation, and it also protects the 5' end of the *m*RNA against the attack of some nuclease enzymes.

(ii) A segment of polyadenylic acid, called PolyA-tail or PolyA segment (about 200 to 300 bp of adenylate residues) is added sequentially to the 3' end of the *hn*RNA. This process of addition of PolyA-tail to 3' end of the RNA is called tailing or polyadenylation.

Polyadenylation makes the 3' end stable and resistant to any attack by nuclease enzymes. The place where PolyA-is added to the *m*RNA also provides a tool to regulate protein synthesis. Poly(A)-binding proteins (PABPs) normally bind to the poly(A) tail and contribute to its stability-enhancing effect. The presence of these proteins at the 3' end of the *m*RNA protects the 5' cap.When the poly(A) tail has been shortened below a critical limit, the 5' cap is removed, and the *m*RNA is degraded by removal of nucleotides from the 5' end.

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Q.5. Describe in detail the regulation of gene expression in prokaryotes with suitable diagram.

Ans. Regulation of Gene Expression in Prokaryotes (Bacteria)

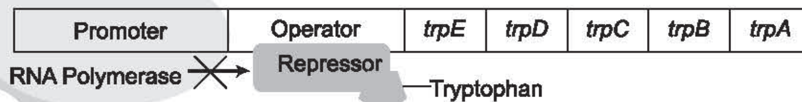
The DNA of prokaryotes is organized into a circular chromosome that resides in the cell's cytoplasm. Proteins that are needed for a specific function, or that are involved in the same biochemical pathway, are often encoded together in blocks called **operons.** For example, all five of the genes needed to make the amino acid tryptophan in the bacterium E. coli are located next to each other in the trp operon. The genes in an operon are transcribed into a single *m*RNA molecule. This allows the genes to be controlled as a unit: either all are expressed, or none is expressed. Each operon needs only one regulatory region, including a **promoter**, where RNA polymerase binds, and an **operator**, where other regulatory proteins bind.

In prokaryotic cells, there are three types of regulatory molecules that can affect the expression of operons. **Activators** are proteins that increase the transcription of a gene. **Repressors** are proteins that suppress transcription of a gene. Finally, inducers are molecules that bind to repressors and inactivate them. Below are two examples of how these molecules regulate different operons.

1. **The *trp* Operon : A Repressor Operon :** Like all cells, bacteria need amino acids to survive. Tryptophan is one amino acid that the bacterium E. coli can either ingest from the environment or synthesize. When E. coli needs to synthesize tryptophan, it must express a set of five proteins that are encoded by five genes. These five genes are located next to each other in the tryptophan (trp) operon (Fig.)

   When tryptophan is present in the environment, E. coli does not need to synthesize it, and the trp operon is switched off. However, when tryptophan availability is low, the trp operon is turned on so that the genes are transcribed, the proteins are made, and tryptophan can be synthesized.

When trytophan is present the trp repressor binds the operator, and RNA synthesis is blocked.



In the absence of tryptophan, the repressor dissociates form the operator, and RNA synthesis proceeds.

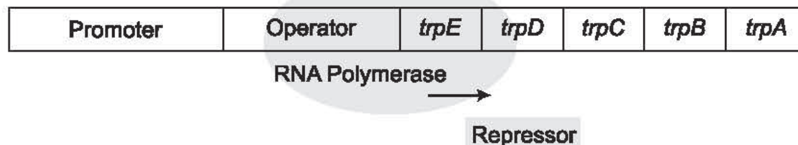


Fig. : The five genes that are needed to synthesize tryptophan in *E. coli* are located next to each other in the trp operon.

DNA sequence called the **operator** is located between the promoter and the first trp gene. The operator contains the DNA code to which the repressor protein can bind. The repressor protein is regulated by levels of tryptophan in the cell.

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When tryptophan is present in the cell, two tryptophan molecules bind to the trp repressor. This causes the repressor to change shape and bind to the trp operator. Binding of the tryptophan-repressor complex at the operator physically blocks the RNA polymerase from binding, and transcribing the downstream genes. Thus, when the cell has enough tryptophan, it is preventing from making more.

When tryptophan is not present in the cell, the repressor has no tryptophan to bind to it. The repressor is not activated and it does not bind to the operator. Therefore, RNA polymerase can transcribe the operon and make the enzymes to synthesize tryptophan. Thus, when the cell does not have enough tryptophan, it synthesizes it.

2. **The *lac* Operon : An Inducer Operon :** The *lac* operon in *E. coli* has more complex regulation, involving both a repressor and an activator. *E. coli* uses glucose for food, but is able to use other sugars, such as lactose, when glucose concentrations are low. Three proteins are needed to break down lactose; they are encoded by the three genes of the lac operon.

   When lactose is not present, the proteins to digest lactose are not needed. Therefore, a repressor binds to the operator and prevents RNA polymerase from transcribing the operon.

   When lactose is present, lactose binds to the repressor and removes it from the operator. RNA polymerase is now free to transcribe the genes necessary to digest lactose (Fig.)

   In the absence of lactose, the lac repressor binds the operator, and transcription is blocked.

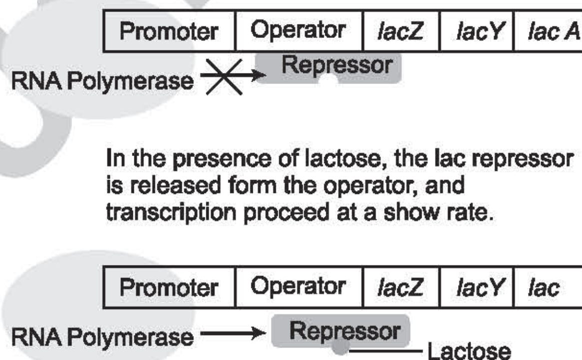

Fig. : Transcription of the lac operon only occurs when lactose is present. Lactose binds to the repressor and removes it from the operator.

However, the story is more complex than this. Since *E. coli* prefers to use glucose for food, the lac operon is only expressed at low levels even when the repressor is removed. Now the bacterium needs to ramp up production of the lactose-digesting proteins. It does so by using an activator protein called catabolite activator protein (CAP).

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When glucose levels drop, cyclic AMP (CAMP) begins to accumulate in the cell. CAMP binds to CAP and the complex binds to the lac operon promoter (Fig.). This increases the binding ability of RNA polymerase to the promoter and ramps up transcription of the genes.

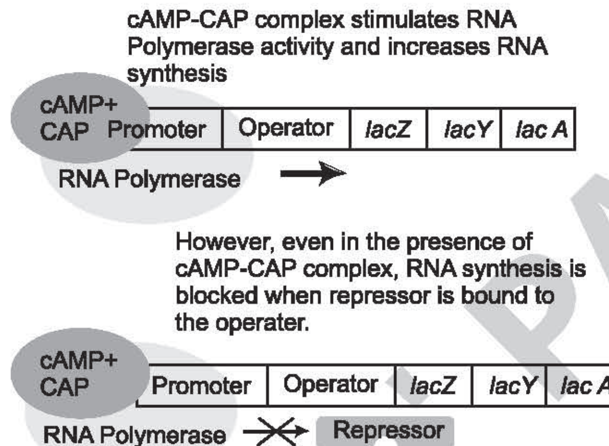

Fig. : When there is no glucose, the CAP activator increases transcription of the lac operon. However, if no lactose is present, the operon is not activated

In summary, for the lac operon to be fully activated, two conditions must be met. First, the level of glucose must be very low or non-existent. Second, lactose must be present. Only when glucose is absent and lactose is present will the lac operon be transcribed maximally. This makes sense for the cell, because it would be energetically wasteful to create the proteins to process lactose if glucose was plentiful or lactose was not available.

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UNIT-IV

Regulation of Gene Expression-II

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. What is gene expression in eukaryotes?

Ans. Gene expression in eukaryotes is influenced by a wide variety of mechanisms including the loss, amplification and rearrangement of genes. Genes are differentially transcribed and the RNA transcripts are variably utilized. Multigene families regulate the amount, the diversity and the timing of gene expression.

Q.2. What is the first step in eukaryotic gene expression?

Ans. Eukaryotic gene expression begins with control of access to the DNA. This form of regulation, called epigenetic regulation, occur even before transcription is initiated.

Q.3. What is the main difference between aukaryotic and prokaryotic gene regulation?

Ans. Prokaryotic gene expression is primarily controlled at the level of transcription. Eukaryotic gene expression is controlled at the level of epigenetics, transcription, post transcription, translation and post-translation.

Q.4. Where does post-translational modification occur in eukaryotes?

Ans. Post translational modification (PTM) is the covelent and generally enzymatic modification of proteins following protein biosynthesis. This process occurs in the endoplasmic reticulum and golgi apparatus.

Q.5. What contributes to protein folding?

Ans. Protein folding is a very sensitive process that is influenced by several external factor including electric and magnetic fields, temperature, ph, chemicals, space limitation and molecular crowding. These factors influence the ability of proteins to fold into their correct functional forms.

Q.6. Where does gene silencing occur?

Ans. Gene silencing is a negative feedback mechanism that regulates gene expression to define cell fate and also regulates metabolism and gene expression throughout the life of an organism. In plants, gene silencing occurs *via* transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS).

Q.7. How do chaperones fold proteins?

Ans. Chaperones are a functionally related group of proteins assisting protein folding in the cell under physiological and stress conditions. They share the ability to recognize and bind non-native protein thus preventing unspecific aggregation.

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Q.8. What is ubiquitin and what is its function?

Ans. Ubiquitin is a small protein that is found in almost all cellular tissues in humans and other eukaryotic organisms, which helps to regulate the processes of other proteins in the body.

Q.9. What are transposons used for gene silencing?

Ans. Transposon silencing is a form of transcriptional gene silencing targeting transposons. Transcriptional modifications that prevent the transcription of a particular area of DNA. Transcriptional silencing of transposons is crucial to the maintenance of a genome.

Q.10. Who gene RNA interference?

Ans. In 1998, Fire and Coll coined the term RNA interference (RNAi) refferring the phenomenon of post-translational silencing of gene expression that occur in response to the introduction of *ds*RNA into a cell. This phenomenon can result in highly specific suppression of gene expression.

Q.11. Which RNA type is involved in RNA interference?

Ans. Two types of small ribonuclecic acid (RNA) molecules Micro RNA (miRNA) and small interfering RNA (*si*RNA) are central to components to the RNAi pathway.

Q.12. How are intracellular proteins degraded?

Ans. The major pathway of protein degradation in eukaryotic cells involves the uptake of proteins by lysosomes. Lysosomes are membrane-enclosed organelles that contain an array of digestive enzyme, including several proteases.

Q.13. What are the steps of protein degradation?

Ans. Degradation of a protein via the ubiquitin pathway proceeds in two discrete and successive steps : (i) covalent attachment of multiple ubiquitin molecules to the protein substrate and (ii) degradation of the targeted protein by the 26*s* proteasonse complex with the release of free and reusable ubiquitin.

Q.14. What happens to proteins after translation?

Ans. Polypeptides often need some 'edits'. During and after translation amino acids may be chemically altered or removed. The new polypeptide will also fold into a distincts 3D structure and may join with other polypeptides to make a multi-part protein.

Q.15. Why is regulation of gene expression important in eukaryotes?

Ans. Gene regulation is essential for viruses, prokaryotes and eukaryotes as it increases the versatility and adaptability of an organism by allowing the cell to express protein when needed.

Q.16. What are types of post-translational modifications?

Ans. Types of post-translational modification are as follows :

Phosphorylation, acetylation, hydroxylation, methylation.

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SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. What do you mean by gene silencing?

Ans.

Gene Silencing

Gene silencing is the regulation of gene expression in a cell to prevent the expression of a certain gene. Gene silencing can occur during either transcription or translation and is often used in research. Gene silencing is often considered the same as gene knock down.

When genes are silenced, their expression is reduced or inhibited. The common methods used to silence genes, such as RNAi, CRISPR, or *si*RNA, generally reduce the expression of a gene by at least 70% but do not eliminate it.

Gene silencing simply means not allowing expression of a particular gene *i.e.,* no synthesis of protein from that gene. This can be done at various stages of gene expression *i.e.,* at the level of gene (DNA) itself, at transcriptional level or at translational level.

Q.2. Explain the two important pathways of intracellular protein degration in eukaryotes.

Ans.

Intracellular Protein Degradation

The levels of proteins within cells are determined not only by rates of synthesis, but also by rates of degradation. The protein life within the cells vary widely, from minutes to several days. The rate protein synthesis as well as rate of protein degradation are important aspects of cell regulation. Many rapidly degraded proteins function as regulatory molecules, such as transcription factors. The rapid turn over of these proteins is necessary to allow their levels to change quickly in response to external stimuli. Other proteins are rapidly degraded in response to specific signals, providing another mechanism for the regulation of intracellular enzyme activity.

In addition, faulty or damaged proteins are recognized and rapidly degraded within cells, thereby eliminating the consequences of mistakes made during protein synthesis. In eukaryotes, there are two important pathways of protein degradation.

1. **The Ubiquitin-Proteasome Pathway :** Ubiquitin is a small, 76-amino acid, regulatory protein, which is present in all eukaryotic cells. It can be attached with any protein as a flag and it then directing the movement of protein.

   The major pathway of selective protein degradation in eukaryotic cells uses ubiquitin as a marker that targets cytosolic and nuclear proteins for rapid proteolysis.

   Proteins are marked for degradation by the attachment of ubiquitin to the amino group of the side chain of a lysine residue. Additional ubiquitins are then added to form a multi ubiquitin chain. Such poly-ubiquinated proteins are recognized and degraded by a large, multi sub-unit protease complex, called the **proteasome.** Ubiquitin is released in the process, so it can be reused in another cycle.

   The attachment of ubiquitin and the degradation of marked protein, both processes require energy in the form of ATP. Since the attachment of ubiquitin marks proteins for rapid degradation, the stability of many proteins is determined by whether they become ubiquitinated.

   Ubiquitin is activated by the ubiquitin-activating enzyme (E_1). The ubiquitin is then transferred to a second enzyme, called ubiquitin-conjugating enzyme (E2).

The final transfer of ubiquitin to the target protein is then mediated by a third enzyme, called **ubiquitin ligase** or E3, which is responsible for the selective recognition of appropriate substrate proteins.

2. **Lysosomal Proteolysis :** The other major pathway of protein degradation in eukaryotic cells involves the uptake of proteins by lysosomes. Lysosomes are membrane-enclosed organelles that contain an array of digestive enzymes, including several proteases.

   The containment of proteases and other digestive enzymes within lysosomes prevents uncontrolled degradation of the contents of the cell. Therefore, in order to be degraded by lysosomal proteolysis, cellular proteins must first be taken up by lysosomes. One pathway for this uptake of cellular proteins, autophagy, involves the formation of vesicles (autophagosomes) in which small areas of cytoplasm or cytoplasmic organelles are enclosed in membranes derived from the endoplasmic reticulum.

These vesicles then fuse with lysosomes, and the degradative lysosomal enzymes digest their contents. The uptake of proteins into auto-phagosomes appears to be non-selective, so it results in the eventual slow degradation of long-lived cytoplasmic proteins.

Q.3. Discuss in briefly the enzymes involved in protein folding.

Ans.

Enzymes Involved in Protein Folding

In addition to chaperones, which facilitate protein folding by binding to and stabilizing partially folded intermediates, cells contain at least two types of enzymes that catalyse protein folding by breaking and re-forming covalent bonds.

The first enzyme is 'Protein di-sulphide isomerase' (PDI), which works on disulphide bonds between cysteine residues.

Protein di-sulphide isomerase catalyzes the breakage and re-formation of these bonds. For proteins that contain multiple cysteine residues, protein di-sulphide isomerase (PDI) plays an important role by promoting rapid exchanges between paired di-sulphides, thereby allowing the protein to attain the pattern of di-sulphide bonds that is compatible with its stably folded conformation.

The second enzyme is 'peptidyl prolyl isomerase' (PPI) that plays a role in protein folding catalyzes the isomerization of peptide bonds that involve proline residues. Proline is an unusual amino acid in that the equilibrium between the cis and trans conformations of peptide bonds that precede proline residues is only slightly in favour of the trans form. In contrast, peptide bonds between other amino acids are almost always in the trans form. Isomerization between the cis and trans configurations of prolyl peptide bonds can change the protein folding pattern.

Q.4. Describe the regulation at translational level in eukaryotes.

Ans.

Regulation at Translational Level

Translation is the process in which *m*RNA binds to the ribosomes and amino acids are joined together by peptide bonds to form a polypeptide chain. This process can be checked and controlled by several mechanisms in eukaryotes to regulate gene expression *i.e.,* amount of the protein to be formed.

1. **Control by Changing *m*RNA Longevity :** When *m*RNA is formed, it is transported to the cytoplasm for translation. Inside the cytoplasm, *m*RNA longevity and sustainability plays an important role in regulation of protein synthesis. Some *m*RNAs are degraded

very rapidly and thus produce only a limited amount of protein on ribosomes, while others live longer and may get associated with several ribosomes simultaneously (polysomes) to yield a large amount of protein.

*m*RNA longevity can be influenced by several factors. Poly (A) tails seem to stabilize *m*RNAs. The sequence of the 3 untranslated region (3 UTR) preceding a poly(A) tail also seems to affect *m*RNA stability. The presence of multiple copies of sequence 5-AUUUA-3, in the poly A tail (3-noncoding/untranslated region) makes the *m*RNA unstable and short lived. Several short-lived *m*RNAs have the sequence 5-AUUUA-3 repeated several times in their 3 untranslated regions.

2. **Control by Chemical Factors :** Chemical factors, such as hormones, may also affect *m*RNA stability. In the toad Xenopuslaevis, the vitellogeningene is transcriptionally activated by the steroid hormone estrogen. However, in addition to inducing transcription of this gene, estrogen also increases the longevity of its *m*RNA.

   Another form of translational control involves proteins binding directly to the *m*RNA and preventing translation. This RNA is called 'masked *m*RNA'. In appropriate circumstances, the *m*RNA can be translated when the protein dissociates.

3. **Control by Regulating the Amount of Iron in the Cells :** Iron is essential for the activity of some proteins, but it is harmful in excess. Iron is transported into cells by the transferrin receptor protein and is stored within cells bound to the storage protein ferritin. The *m*RNA for each of these proteins contains noncoding sequences that can form stem-loop structures, called the **iron response element** (IRE). These provide attachment (binding) to **'iron sensing protein'** (ISP) can bind. However, the position of the IRE and the action of the bound ISP is very different.

   In the transferrin receptor *m*RNA, the IRE is in the 3'noncoding region. When iron is scarce, the ISP binds to IRE of *m*RNA and stabilizes the *m*RNA and allows more translation. But when iron levels are high the ISP dissociates from the IRE to make *m*RNA unstable which can then be attacked by nucleases, thus reducing translation.

Control by micro RNAs : Other examples of translational control include microRNAs in eukaryotes that bind to *m*RNAs to which they are complementary, and either cause degradation or translational repression of the *m*RNA.

eIF4E inhibitory proteins prevent cap-dependent initiation of translation of some *m*RNAs in eukaryotes. Some molecules, for example thiamine, can bind to the **Shine-Dalgarno** sequence of prokaryotic *m*RNAs and prevent ribosome binding.

Q.5. What are the differences between chaperones and chaperonins?

Ans. **Difference between Chaperones and Chaperonins**

S.No.	Chaperones	Chaperonins
1.	The proteins, which assist the covalent folding or unfolding and assembly and disassembly of other macromolecular structures.	The proteins, which provide favourable conditions for the correct folding of denatured proteins, preventing aggregation.
2.	Monomers with a molecular weight of 70-100 kDa.	Oligomers with a molecular weight of 800 kDa.

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3.	Most of them are heat shock proteins (HSPs).	Have a shape of two donuts stacked on top of one another to create a barrel.
4.	Responsible for the folding, unfolding, assembly and disassembly of proteins. **Examples :** DnaK, DnaJ, GrpE, HtpG, and Hsp33.	Responsible for the correct folding of denatured proteins, which prevent aggregation. **Examples :** GroEL/GroES and TRiC.

Q.6. How do you analyze post-translational modification?

Ans.

Post-translational Modification Analysis

Post-translational modification (PTM) refers to the covalent, usually enzymatic modification of proteins, and protein process during or after protein biosynthesis. Protein post-translational modification (PTM) increases the functional diversity of the proteome by the modifying proteins with functional groups, such as phosphate, acetate, amide groups, or methyl groups, and influences almost all the aspects of normal cell biology and pathogenesis. It plays a key role in many cellular processes such as cellular differentiation, protein degradation, signaling and regulatory processes, regulation of gene expression, and protein-protein interactions. The modifications genererally include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis and influence almost all aspects of normal cell biology and pathogenesis. Therefore, characterization of PTM, including the modification categories and modified sites, is critical in the study of cell biology and disease diagnostics and prevention.

Identification of Post-Translational Modifications (PTM) is a tedious process. It can be affected by many factors. For example, most of the post-translational modifications are present in very low level. Therefore, enrichment steps are necessary before identification process. Additionally, stability of modification, and detection efficiency of mass spectrometry are also critical factors in PTM identification process. Creative Proteomics offers an advanced analytical platform for the characterization of various post-translational modifications (PTM).

SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. Describe the eukaryotic translational and post translational gene regulation with suitable diagram.

Ans.

Eukaryotic Translational and Post-translational Gene Regulation

After RNA has been transported to the cytoplasm, it is translated into protein. Control of this process is largely dependent on the RNA molecule. As previously discussed, the stability of the RNA will have a large impact on its translation into a protein. As the stability changes, the amount of time that it is available for translation also changes.

The Initiation Complex and Translation Rate

Like transcription, translation is controlled by proteins that bind and initiate the process. In translation, the complex that assembles to start the process is referred to as the translation initiation complex. In eukaryotes, translation is initiated by binding the initiating met-*t*RNAi to the 40S ribosome. This *t*RNA is brought to the 40S ribosome by a protein initiation factor, eukaryotic initiation factor-2 (eIF-2). The eIF-2 protein binds to the high-energy molecule

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guanosine triphosphate (GTP). The *t*RNA-eIF2-GTP complex then binds to the 40S ribosome. A second complex forms on the *m*RNA. Several different initiation factors recognize the 5' cap of the *m*RNA and proteins bound to the poly-A tail of the same *m*RNA, forming the *m*RNA into a loop. The cap-binding protein eIF4F brings the *m*RNA complex together with the 40S ribosome complex. The ribosome then scans along the *m*RNA until it finds a start codon AUG. When the anticodon of the initiator *t*RNA and the start codon are aligned, the GTP is hydrolyzed, the initiation factors are released, and the large 60S ribosomal subunit binds to form the translation complex. The binding of eIF-2 to the RNA is controlled by phosphorylation. If eIF-2 is phosphorylated, it undergoes a conformational change and cannot bind to GTP. Therefore, the initiation complex cannot form properly and translation is impeded (Fig). When eIF-2 remains unphosphorylated, the initiation complex can form normally and translation can proceed.

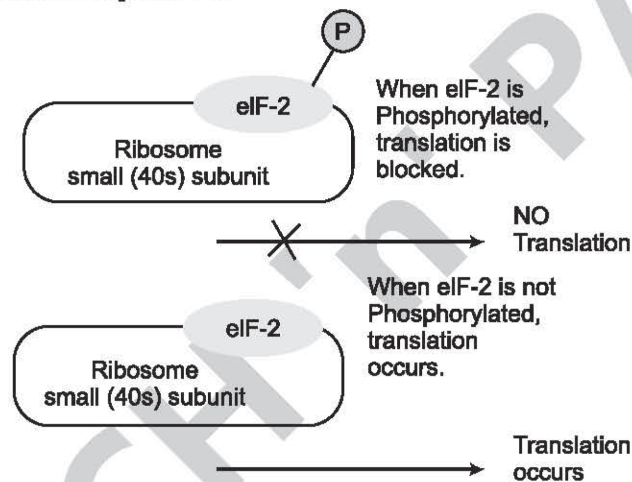

Fig. : Gene expression can be controlled by factors that bind the translation initiation complex.

An increase in phosphorylation levels of eIF-2 has been observed in patients with neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's.

Chemical Modifications, Protein Activity, and Longevity

Proteins can be chemically modified with the addition of groups including methyl, phosphate, acetyl, and ubiquitin groups. The addition or removal of these groups from proteins regulates their activity or the length of time they exist in the cell. Sometimes these modifications can regulate where a protein is found in the cell-for example, in the nucleus, in the cytoplasm, or attached to the plasma membrane.

Chemical modifications occur in response to external stimuli such as stress, the lack of nutrients, heat, or ultraviolet light exposure. These changes can alter epigenetic accessibility, transcription, *m*RNA stability, or transiation-all resulting in changes in expression of various genes. This is an efficient way for the cell to rapidly change the levels of specific proteins in response to the environment. Because proteins are involved in every stage of gene regulation, the phosphorylation of a protein (depending on the protein that is modified) can alter accessibility to the chromosome, can alter translation (by altering transcription factor binding or function), can change nuclear shuttling (by influencing modifications to the nuclear pore complex), can alter RNA stability (by binding or not binding to the RNA to regulate its

stability), can modify translation (increase or decrease), or can change post-translational modifications (add or remove phosphates or other chemical modifications).

The addition of an ubiquitin group to a protein marks that protein for degradation. Ubiquitin acts like a flag indicating that the protein lifespan is complete. These proteins are moved to the proteasome, an organelle that functions to remove proteins, to be degraded (Fig.). One way to control gene expression, therefore, is to alter the longevity of the protein.

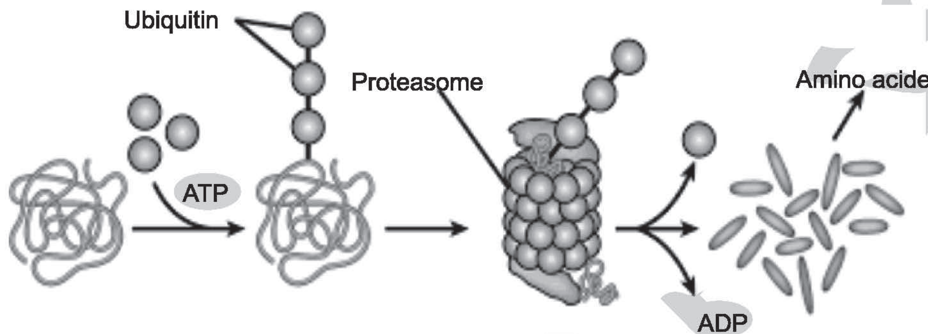

Fig. : Proteins with ubiquitin tags are marked for degradation within the proteasome.

Q.2. Write a note on the role of double stranded RNA in RNAi (RNA interference) mechanism with example.

Ans.

RNA Interference (RNAi)

It has been found that some long double-stranded RNAs (*ds*RNAs, longer than 200 bp) can be used to silence the expression of target genes in a variety of organisms and cell types (*e.g.,* worms, fruit flies, and plants). These *ds*RNAs are produced in the cell itself or can be introduced into a target cell using artificial methods. This mechanism of silencing the activity of a gene (DNA) by using an RNA molecule is called **RNA interference** or **RNAi.** It is a popular mechanism of controlling the expression of certain genes. **Andrew Fire** and **Craig Mello**, first reported the process of RNA interference (RNAi) in 1998.

The expression of a number of eukaryotic genes is controlled through RNA interference, also known as **RNA silencing**. It is also called post-transcriptional gene silencing. RNA interference appears to be widespread, existing in fungi, plants, and animals. As much as 30% of human genes are regulated by RNA interference.

This technique is also widely used as a powerful tool for artificially regulating gene expression in genetically engineered organisms.

Mechanism

RNAi takes place in all eukaryotic cells as a method of cellular defence. This method involves silencing of a specific *m*RNA due to a complementary *ds*RNA molecule, the *ds*RNA undergoes denaturation (separation of strands) and the complementary strand binds to the *m*RNA and prevents its translation. In artificial silencing, the complementary *ds*RNA can be introduced in the cell with the help of a virus or mobile genetic elements (transposons). RNA interference is triggered by small RNA molecules know as micro RNAs (*mi*RNAs) and small interfering RNAs (*si*RNAs), depending on their origin and mode of action.

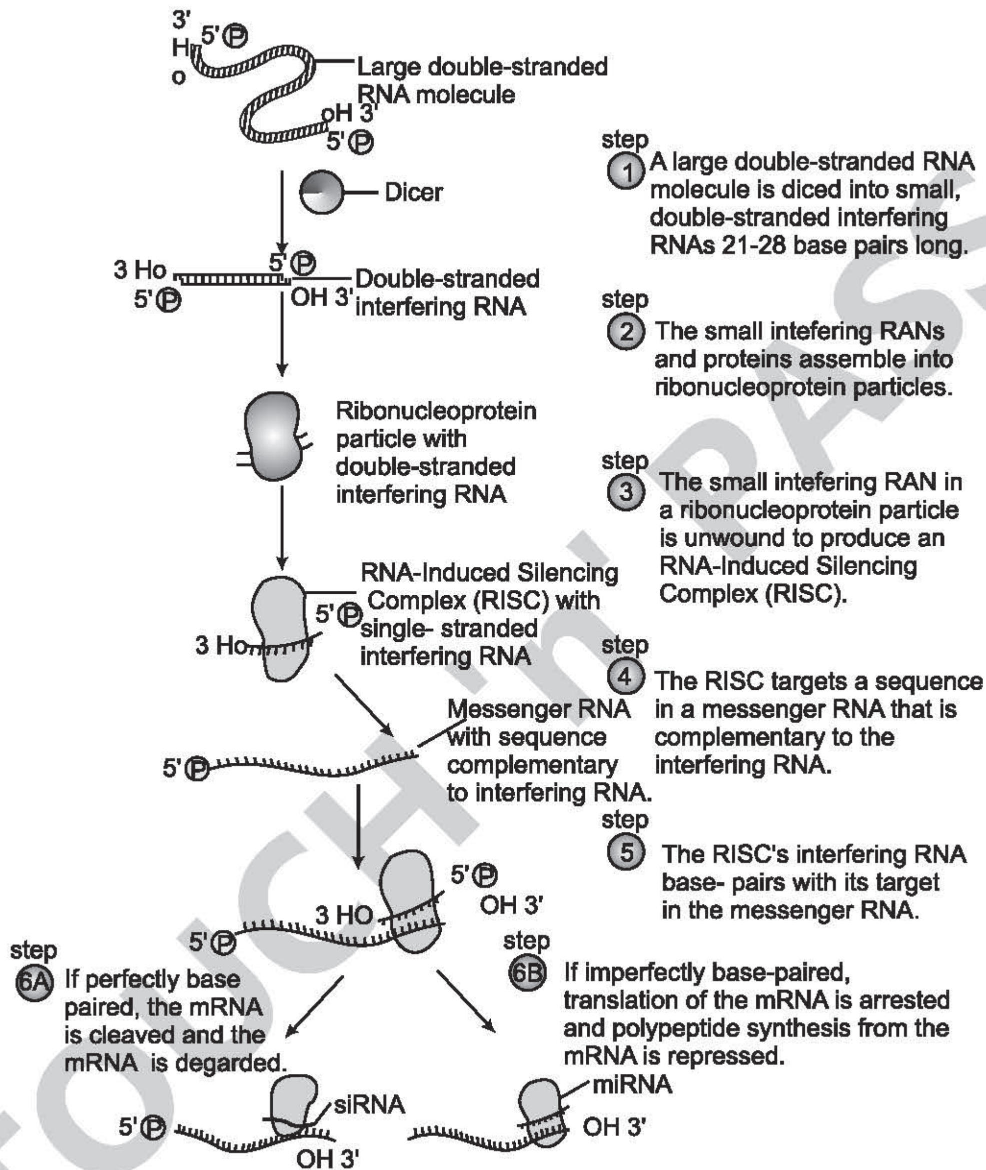

Fig. : Structure of events involved in RNA interference pathways

An enzyme called **Dicer** (an endonuclease enzyme) cleaves and processes *ds* RNA to produce *si*RNAs or *mi*RNAs that are mostly 21 nucleotides in length. These *si*RNAs or *mi*RNAs (which are now small *ds*RNA molecules) combine with certain proteins (called Argonaut proteins or slicers) to form an RNA-induced silencing complex (RISC).

The *si*RNA and micro RNA in the RISC, guide it to bind with specific *m*RNA molecule. After binding, the Argonaut proteins catalyse the cleavage of the specific *m*RNA molecule. In this way, *m*RNA is degraded and translation is blocked.

An example of Artificial RNAi : There are several nematodes parasites which adversely affect the growth of various plants and animals including human beings. One such parasite is

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Meloidegyne incognitia, which infects the roots of tobacco plants and causes a great reduction in the yield.

For this, certain parasite (nematode *Meloidegyne incognita*) specific genes were introduced in tobacco plant cells (using Agrobacterium vector). These genes produced both sense and anti-sense RNA in the tobacco cells. These two RNA's being complementary to each other formed a *ds*RNA.

When parasite infection takes place in tobacco cells, the *ds*RNA becomes active that initiates RNAi of the nematode friendly genes. As a result, the nematode cannot survive in the tobacco plant cells.

Q.3. Throw light on the post translational modifications of proteins with suitable diagram.

Ans.

Post-Translational Modification of Proteins

Translation is the "last step" of the "central dogma" of biology, whereby DNA is converted to RNA and then to protein. However, there are additional steps involved after protein synthesis that are necessary for a cell, tissue and organism to achieve its functional biology and diversity. Post-translational modifications are changes that are made to proteins after synthesis, typically mediated by enzymes.

The genome (DNA) is essentially constant across different cell types in the human body but the human proteome (the collection of proteins that are or can be expressed in a human) is extremely diverse. Different cells generally produce different proteins in order to achieve diverse functions. For example, beta (β) cells of islets of Langerhans produce insulin, while alpha (α) cells produce glucagon.

Individual cells have to perform their individual functions, and respond to environmental stimuli. So, a variety of different proteins must be expressed at different time points across cells, tissues and organs. It is estimated that there are approximately 25,000 to 30,000 genes but number of proteins that are expressed in human body is over 1,000,000.

There are severs different mechanisms to achieve this diversity. One such important method is the post-translational modifications of proteins.

Post-translational Modification Methods

After there formation on ribosomes, the newly form proteins are transported to Golgi bodies through endoplasmic reticulum (ER). Golgi is the major centre where raw proteins are modified and given their final shape. Different chemical changes in protein structure mediated by different enzymes are used for this purpose. Some important modification methods are phosphorylation, acetylation, methylation, etc.

1. **Protein Phosphorylation :** Protein phosphorylation is the one of the most commonly occurring and most-studied post-translational modifications. It entails the phosphorylation of a specific amino acid residue through the addition of a phosphate group to a polar group *R* with the help of a kinase enzyme and ATP. This modification most commonly occurs at serine, tyrosine or threonine residues.

   The addition of the phosphate group results in a modification of the protein, whereby it changes from hydrophobic a polar to hydrophilic polar. This change enable the modified protein to interact with other molecules.

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A reversible post-translational modification, protein phosphorylation is important for cell regulation and the activation and deactivation of enzymes and receptors, which can be implicated in disease processes such as **cancer**.

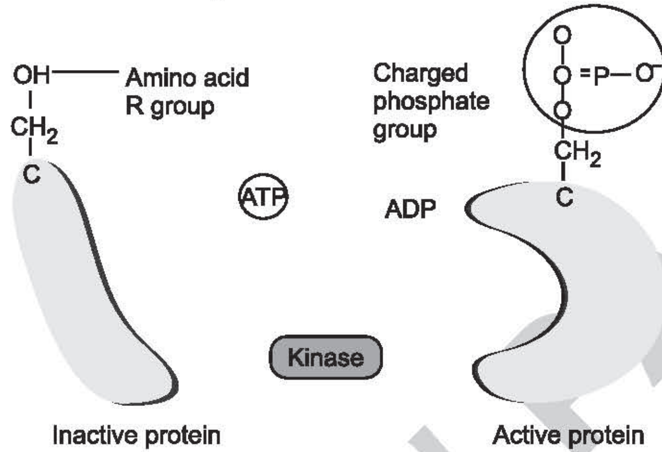

Fig. : Phosphorylation of Proteins

2. **Protein Glycosylation :** Protein glycosylation is recognized as one of the most complicated" yet most commonly occurring post-translational modifications. It involves the covalent addition of a carbohydrate residue to an amino acid, forming a glycoprotein.

   Glycosylation reactions are diverse and catalyzed by various different enzymes, which attach specific glycans to specific amino acids. Glycoproteins are estimated to make up 50% of the proteome.

   Glycosylation of eukaryotic proteins is usually categorized into two major types; N-linked, whereby a sugar molecule is attached to the amide nitrogen of asparagine, and CO-linked, where a sugar molecule is attached to the oxygen atom oi serine or threonine.

3. **Protein Ubiqultination :** Ubiquitin is a small protein - approximately 8 kDa in size - that can bind to a substrate protein in a process known as **ubiquitination**, a type of post-translation modification.

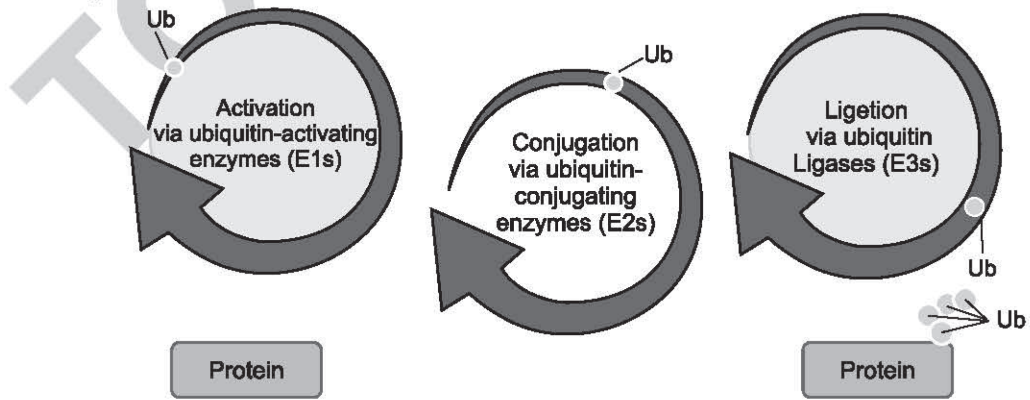

Fig. : Ubiquitination of Proteins

Ubiquitination serves to regulate a protein's function or mark it for degradation. Ubiquitination occurs in three sequential steps that are catalysed by three groups of enzymes.

This process generally culminates with an isopeptide bond forming between ubiquitin and the lysine residue of the protein substrate. Monoubiquitination refers to the addition of one ubiquitin molecule, where as the addition of several ubiquitin proteins is known_as polyubiquitination.

Ubiquitination serves several functions, the most common being to flag proteins for degradation by the proteasome, but there are others including: immune and inflammatory response, organelle biogenesis and signalling roles in DNA repair.

4. **Protein Methylation :** Protein methylation has a regulatory role in many essential cellular processes, including gene transcription and signal transduction. In protein methylation, enzymes known as methyl-transferases add a methyl group (most commonly donated by S-adenosyl-l-methionine or SAM) to specific amino acids on a protein molecule, such as the lysine and arginine residues. Protein methylation helps in :
   (i) Protein stability
   (ii) Protein subcellular localization
   (iii) Protomer binding affinity and
   (iv) Protein-protein interactions.
5. **Protein Acetylation :** Protein acetylation is a common post-translational modification in eukaryotes and involves the addition of an acetyl group to nitrogen *via* reversible and irreversible processes. Acetylation has been studied largely in histones, the proteins that pack DNA into chromosomes, where the acetylation of the lysine side chain (-NH2) on the N-terminus of histones is associated with the regulation of gene expression. If lysine is acetylated, it is no longer positively charged. In turn, the binding of DNA to the histone is relaxed, which facilitates the transcription of genes.

Q.4. Discuss the role of chaperones in protein folding and protection of nascent proteins.

Ans.

Protein Folding

The synthesis of a polypeptide is not equivalent to the production of a functional protein. To be useful, polypeptides must fold into distinct three-dimensional conformations, and in many cases multiple polypeptide chains must assemble into a functional complex. In addition, many proteins undergo further modifications, including cleavage and the covalent attachment of carbohydrates and lipids that are critical for the function and correct localization of proteins within the cell.

Chaperones and Protein Folding

The three-dimensional conformations of proteins is the result of interactions between the side chains of their constituent amino acids.

The classic principle of protein folding is that all the information required for a protein to adopt the correct three-dimensional conformation is provided by its amino acid sequence. Protein folding is therefore a self-assembling process. However, recent studies suggests that actual protein folding is mediated by various other proteins and factors.

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Proteins that facilitate the folding of other proteins are called **molecular chaperones**. The term "chaperone" was first used by **Ron Laskey** and his **colleagues** to describe a protein that is required for the assembly of nucleosomes from histones and DNA.

Chaperones act as catalysts that facilitate assembly without being part of the assembled complex. Subsequent studies have extended the concept to include proteins that mediate a variety of other assembly processes, particularly protein folding.

It is important to note that chaperones do not convey additional information required for the folding of polypeptides into their correct three-dimensional conformations; the folded conformation of a protein is determined solely by its amino acid sequence. Rather, chaperones catalyze protein folding by assisting the self-assembly process.

They appear to function by binding to and stabilizing unfolded or partially folded polypeptides that are intermediates along the pathway leading to the final correctly folded state. In the absence of chaperones, unfolded or partially folded polypeptide chains would be unstable within the cell, frequently folding incorrectly or aggregating into insoluble complexes.

The binding of chaperones stabilizes these unfolded polypeptides, thereby preventing incorrect folding or aggregation and allowing the polypeptide chain to fold into its correct conformation. A good example is provided by chaperones that bind to nascent polypeptide chains that are still being translated on ribosomes, thereby preventing incorrect folding or aggregation of the amino-terminal portion of the polypeptide before synthesis of the chain is finished.

Chaperones also stabilize unfolded polypeptide chains during their transport into subcellular organelles-for example, during the transfer of proteins into mitochondria from the cytosol.

Many of the proteins now known to function as molecular chaperones were initially identified as heat-shock proteins (a group of proteins which are active when the cell is subjected to high temperature or other forms of environmental stress).

The heat-shock proteins (abbreviated Hsp) are highly conserved in both prokaryotic and eukaryotic cells. These stabilize and facilitate the refolding of proteins that have been partially denatured as a result of exposure to elevated temperature.

The Hsp70 and Hap60 families of heat-shock proteins appear to be particularly important in the general pathways of protein folding in both pockanotie and eukaryotic cells.

Members of the Hsp70 family stabilize unfolded polypeptide chains during translation, as well as during the transport of polypeptides into a variety of subcellular compartments, such as mitochondria and the endoplasmic reticulum.

Members of the Hsp60 family (also called **chaperonins**) facilitate the folding of proteins into their native conformations. Unfolded polypeptide chains are shielded from the cytosol by being bound within the central cavity of the chaperonin cylinder. In this isolated environment protein folding can proceed while aggregation of unfolded segments of the polypeptide chain is prevented by their binding to the chaperonin.

The binding of unfolded polypeptides to the chaperonin is a reversible reaction that is coupled to the hydrolysis of ATP as a source of energy.

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UNIT-V

Principle and Types of Microscopes

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. Who invented a microscope?

Ans. A Dutch father-son team named **Hans** and **Zacharias Janssen** invented the first so-called compound microscope in the late 16th century when they discovered that, if they put a lens at the top and bottom of a tube and looked through it, objects on the other end became magnified.

Q.2. What is the formula for magnification?

Ans. Magnification = image size/actual size
Actual size = image size/magnification
Image size = magnification × actual size

Q.3. Who is known as the father of microscope?

Ans. Antoni van Leeuwenhoek (1632-1723) is known as the father of microscope.

Q.4. What is resolution of a microscope?

Ans. In microscope, the term 'resolution' is used to describe the ability of a microscope to distinguish detail. In other words, this is the minimum distance at which two distinct points of a specimen can still be seen either by the observer or the microscope camera - as separate entities.

Q.5. What is the microscope used for?

Ans. A microscope is an instrument that is used to magnify small objects. Some microscopes can even be used to observe an object at the cellular level, allowing scientists to see the shape of a cell, its nucleus, mitochondria, and other organelles.

Q.6. What is microscopy? What are the types of microscopy?

Ans. Microscopy is the act of using a microscope to view tiny things that cannot be seen with the unaided eye. There are three main types : optical microscopy, scanning probe microscopy and electron microscopy. Optical microscopes bounce light up objects and use lenses or mirrors to magnify the image.

Q.7. What is the advantage and disadvantage of microscope?

Ans. **Advantage :** Light microscopes have high resolution. Electron microscopes are helpful in viewing surface details of a specimen.

Disadvantage : Light microscopes can be used only in the presence of light and are costly. Electron microscopes uses short wavelength of electrons and hence have lower magnification.

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Q.8. What are the limitations of microscope?

Ans. The resolution of the light microscope cannot be small than the half of the wavelength of the visible light, which is 0.4-0.7 μm. When we can see green light (0.5 μm), the objects which are, at most, about 0.2 μm. Below this point, light microscope is not useful, as wavelength smaller than 400 nm is needed.

Q.9. How many parts are there in microscope?

Ans. There are three structural parts of the microscope *i.e.,* head, base, and arm.

Q.10. Why TEM is better than SEM?

Ans. TEM allows you to observe details as small as individual atoms, giving unprecedented levels of structural information at the highest possible resolution. As it goes through objects it can also give you information about internal structures, which SEM cannot provide.

Q.11. Does dark field microscopy use staining?

Ans. Resolution by dark-field microscope is somewhat better than bright-field microscope. It improves image contrast without the use of stain and thus do not kill cells.

Q.12. What type of specimen are best suited for the dark field miscroscope?

Ans. The best specimens for dark field are those that have refractive objects scattered about with empty space between them. No dark field occurs if objects are too crowded or if a thick solid specimen turns light into the microscope.

Q.13. What is the principle of fluorescence?

Ans. Fluorescence is based on the property of some molecules that when they are hit by a photon, they can absorb the energy of that photon to get into an excited state. Upon relaxation from that excited state, the same molecule releases a photon fluorescence emission.

Q.14. What is the application of fluorescence?

Ans. Fluorescence has many practical application including mineralogy, gemology, medicine, chemical sensors, fluorescent labelling dyes, biological detectors, cosmic-ray detection, vacuum fluorescent displays and cathode-ray tubes.

Q.15. What are the limitations of electron microscope?

Ans. An electron microscope is operated only in high vaccum. This prohibits the use of the microscope to study living organisms which would evaporate and disintegrate under such conditions.

Q.16. What is the principle on which electron microscope works?

Ans. Electron microscope is based on the principle of De-Broglie hypothesis of wave-nature of electrons.

SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. Write about the history of microscope.

Ans.

History of Microscope

The credit for the invention of the first microscope goes to **Dutch** eyeglass makers **Zacharias Janssen** and his father **Hans Janssen** (1590) who lived in Middleburg, Holland. However, some science historians regard another Dutch watchmaker **Hans Lippershey** as a concurrent but independent inventor of microscope. All these three workers had used a crude form of

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microscope which comprised of an eye-piece and an objective lens fitted at two ends of a tube. **Robert Hooke** made some improvement to the initial design of the instrument and was able to make one of the earliest break throughs using microscope when he observed dead cells for the first time in a section of Cork (*Quercus spp*).

After Hooke, it was Anton Van Leeuwenhoek who made significant progress in the field of microscopy by observing a number of living organisms such as bacteria, protozoa, and unicellular plants or animals. **Leeuwenhoek** is often wrongly regarded as the inventor of microscope and **Father of microscopy**. He was not the inventor of microscope but his instrument was more sophisticated than other microscopes of that period. He used a single lens in his microscope and was able to achieve 270X magnification. Since the invention of the first microscope, there have been several improvements in the basic design and capability of microscopes and the technique of microscopy has gone through significant refinements to have found some novel applications in biology.

Q.2. Write about the parts of a compound microscope.

Ans.

Parts of a Compound Microscope

The compound microscope has the following important components :

1. **Light Source :** If sunlight is a available then a mirror is used to project the light onto the sample. However, if solar light is not available then illuminator (light bulb) may be used.
2. **Base :** A metallic base supports the body of the microscope. It has a mirror attached to it and also possesses a pair of elevated pillars which connect the base with the body.
3. **Inclination joint :** The body of the microscope can be inclined as required because of the presence of a movable inclination joint which connects the body with the pillar.
4. **Curved tube :** This is a very important component of the microscope because the body tube, the stage and the adjustments are all assembled in the curved tube.
5. **Stage :** It is the platform attached to the C-tube and is meant for keeping slides to be observed. The mechanical stage contains rock and pinion along with two knobs which are used to move the slide in vertical as well as horizontal directions.
6. **Body Tube :** It is a vertical tube having eye-piece at the upper end and objective at the lower end. The body tube is about 14-18 cm long. Its upper part which holds the eye-piece is slightly narrow and is called **'draw tube'**.
7. **Revolving Nosepiece :** It is a revolving disc attached to the base of the body tube. It can hold three or more objectives of different magnifications. The objective lens of desired magnification can be set by rotating the revolving nosepiece.
8. **Coarse Adjustment :** This is a large knob on the side arm which moves the body tube up and down by making use of rack and pinion mechanism so as to bring the specimen in focus. Slight rotation of coarse adjustment moves the body tube by a large distance.
9. **Fine Adjustment :** This is the smaller knob which also moves the body tube but its rotation causes slight movement of the tube.
10. **Diaphragm and Condenser :** A condenser lens is fixed above the diaphragm and lies below the stage. The function of diaphragm is to regulate the amount of light entering the condenser which then focuses it onto the specimen.

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Q.3. Write a short note on the fluorescence microscope.

Ans.

Fluorescence Microscope

Fluorescence microscope is a type of optical microscope which uses fluorescent materials (dyes) to stain objects and forms image by focusing the light emitted from such materials. The phenomenon of fluorescence which was discovered by **Fredrik Herschel** (1845) involves absorption of a particular wavelength of light by a fluorophore (fluorochrome) followed by emission of a longer wavelength. **George Stokes** (1852) used the word 'fluorescence' for the first time to describe the difference between the absorbed and emitted wavelengths of light. The application of fluorescence in microscopy started around 1900 and the first fluorescence microscope was developed by **Oskar Heimstadt** in 1911. Today, the technique of fluorescence microscopy has emerged as one of the indispensable tools for a biologist.

Principle

In fluorescent microscope, the tissue to be viewed has to possess fluorescent components. Some biological materials are naturally fluorescent (autofluorescent) whereas others have to be stained with appropriate dyes. In a recent development, fluorescent genes can also be expressed in the desired cells. The sample is excited with the exactly the same wavelength of light which is absorbed by the fluorophore. This is achieved by passing the light entering the sample through a filter which blocks all other wavelengths and allows only the desired wavelength of light to reach the specimen. The fluorophore absorbs a characteristic wavelength and emits a different (longer) wavelength which is again filtered by letting it pass through an emission filter to prevent all wavelengths except the one emitted by the fluorophore. Thus the image is formed only by the light emitted from the fluorophore.

The most common form of fluorescent microscopes is called the **epifluorescence microscope**. In this design the light from excitation filter that passes through the objective lens falls straight downwards to reach the sample returns after striking the sample through the same objective and goes upwards to reach the eye-piece.

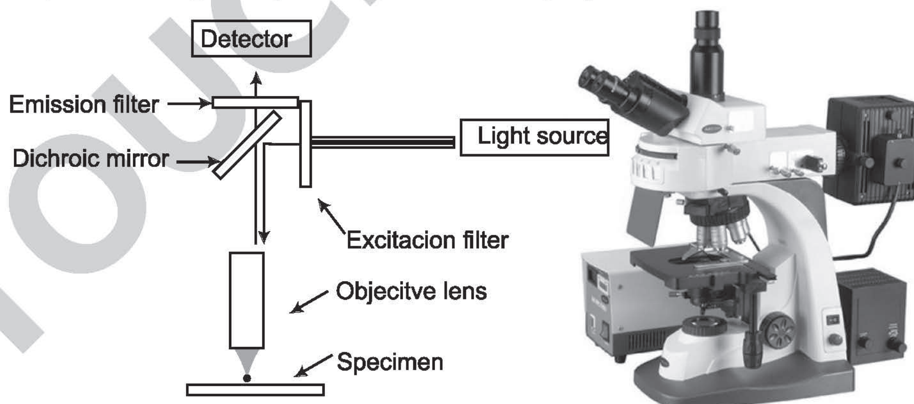

Fig. : Fluorescence Microscope

Q.4. Write about the components, advantages and disadvantages of fluorescence microscope.

Ans.

Components of Fluorescence Microscope

The fluorescence microscope has following essential components in its design :

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1. **Light source :** Monochromatic light of high intensity is a perquisite for fluorescence microscope. Some of the common light sources comprise of lamps (*e.g.,* Xenon arc lamp or Hg-vapour lamp), strong LEDs or lasers.
2. **Excitation filter :** This is a high quality optical glass filter which is used to select the wavelength that is to be absorbed by the fluorophore present in the sample. It blocks all the remaining wavelengths coming from the light source.
3. **Dichroic mirror :** It acts as a beam splitter and bends the light beam received from the excitation filter at right angle and directs it to pass through the objective lens. The light rays that return from the sample pass again through the objective and dichroic mirror.
4. **Objective lens :** Light rays of specific wavelength pass through the objective lens before reaching the specimen. Besides, the light emitted by the fluorochromes of the specimen also goes past this lens.
5. **Emission filter :** It is just like the excitation filter but is placed at right angle to the excitation filter. It receives light beam emitted by the sample which passes through the objective lens and the dichroic mirror which blocks all the background radiation and allows only the wavelength of light emitted from the fluorophore.
6. **Ocular lens :** This is the last component of fluorescence microscope which focuses the beam of light onto the image plane.

Advantages

There are following advantages of fluorescence microscope :

1. Fluorescence microscope is specially useful in observing very fine details of subcellular structures such as microtubules and micro filaments.
2. The images formed by fluorescence microscope are brightly coloured and have excellent contrast.
3. Fluorescent probes can be designed against specific target molecules and can detect their presence.
4. Reporter genes can be designed to monitor the expression of certain target proteins.

Disadvantages

There are following disadvantages of fluorescence microscope

1. Fluorescence microscope can view only those tissues or molecules which can be bound to fluorophores.
2. There is loss of activity of fluorophores due to strong light. This is called photon leaching.

Q.5. Briefly explain about electron microscope.

Ans.

Electron Microscope

The first electron microscope (EM) was constructed by **Knoll** and **Ruska** of Germany in 1932. **Ruska was awarded Nobel Prize in physics for this work in 1986**. The need of an improved microscope was felt because resolving power of the light microscopes was insufficient. This limitation was due to source of illumination. In compound microscopes the light source is the ordinary light which consists of longer wavelengths. In electron microscopes a beam of electrons is used as light source. This is of much shorter wavelengths.

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Electrons are emitted by placing a metal filament in vacuum tube and heated. High voltage electric current of 50,000 volts having a wavelength of 0.05 A is provided. The actual numerical aperture of electron microscope is small.

The limit of resolution, therefore, theortically comes to 0.25 Å (0.2 nm). In practice, however, the limit reaches to about 2-10 Å. The magnification obtained by electron microscope is also very high. This is due to introduction of one or more intermediate lenses. Direct magnification as high as 1,000,000 X may thus be obtained. The microphotographs from electron photographs can be further enlarged to 10,000,000 or more times.

Electron microscopes exist in two common variants, both working on different principles :

1. Transmission Electron Microscope (TEM)
2. Scanning Electron Microscope (SEM)

1. **Principle and Construction of Electron Microscopes :** Electron microscopes are designed to provide a beam of electrons instead of a beam of light from glass lens. The beam of electrons has properties of electromagnetic waves of very short wavelengths.

   In electron microscopes a beam of electrons, projected from an electron gun, is focussed on the specimen by means of a condenser lens and the image obtained is further enlarged by a series of magnifying lenses. The final image appears on a fluorescent screen. The entire path through the instrument is under high vacuum in order to minimise both the scattering of electrons through collision with air particles and the subsequent heating that would occur.

2. **Construction of Electron Microscopes :** Electron microscope consists of following parts.

   (i) **Electron gun or cathode :** It emits beam of electrons.

   (ii) **Ray tube :** Through this tube electrons travel and reach the condenser lens.

   (iii) **Condenser lens :** It is a magnetic coil which generates electromagnetic field and focusses the electron beam in the plane of the object.

   (iv) **Objective lens :** Like condenser lens, it is also an electromagnetic coil which, with the help of an intermediate lens produces the first magnified image of the object.

   (v) **Projector lens :** It is also an electromagnetic coil which further enlarges the image formed by the objective lens.

   (vi) **Fluorescent screen :** Since the electrons can not be observed with the naked human eye, therefore, they are focussed on a fluorescent screen which produces a visible image. To obtain a photograph, the beam of electrons can be focussed on a photographic film instead of fluorescent screen. The photograph thus obtained is called electron micrograph.

Q.6. Write a short note on the scanning electron microscope and also its limitations.

Ans.

Scanning Electron Microscope (SEM)

Scanning electron microscope was invented by **Knoll** (1953). Both light microscopes and transmission electron microscope produce images that are essentially two dimensional. A modification of the electron microscope, called scanning electron microscope, makes it possible to obtain three dimensional images of the microscopic objects. Here a finely focussed

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beam of electrons scans to and fro across the specimen and the electrons reflected from the surface are collected and transferred to a television tube which scans and forms the image on the screen.

Though the resolving power of scanning electron microscope is poorer than that of TEM (5-20 nm), it is very useful in the study of surface topography of the specimen. The clarity and depth of field obtained by SEM is not possible by any other method. The surface of the object to be examined is coated with a thin layer of heavy metal to obtain a good contrast.

Limitations of Electron Microscopy

Despite the fact that electron microscopes provide highly magnified images of the object, there are following limitations.

1. The object being examined must be dry because it is placed under complete vacuum. Thus electron microscope is not useful for observing metabolically active cells.
2. The drying process may alter morphological characteristics of the object.
3. The material for study under electron microscope needs special preparation otherwise it is quickly heated up and would be destroyed by the electron beam.

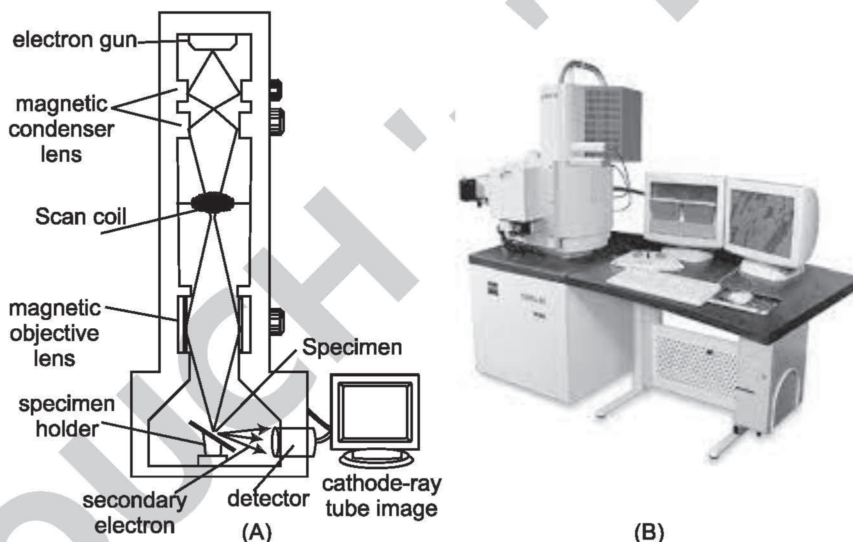

Fig. A-B : Scanning electron microscope, A. Photograph of SEM, B. Sectional view

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SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. Explain the basic principles and applications of microscopes.

Ans.

Basic Principles of Microscopes

The basic purpose of a microscope is to form enlarged images of various objects (e.g., cells and tissues) such that the finer details of the specimen under observation are revealed to the observer. There are two basic principles involved in the working of a microscope :

1. **Magnification :** It indicates the extent of enlargement of images compared to the size of objects. Thus, it is expressed as a unit less figure which is given by the following formula :

$$\text{Magnification} = \frac{I}{O}$$

Where, I = Size of Image; O = Size of Object.

If the magnification of a lens is 20X, it implies that the size of image is 'twenty times the image of the object. If there are two lenses with magnifications m_1 and m_2 then the magnification of their combination will be the product $m_1 m_2$. Besides, magnification of a lens is inversely proportional to its focal length (f) :

$$\text{Magnifying power} = \frac{1}{f}$$

2. **Resolution :** Resolution of a microscope is a measure of how clearly the details are resolved in the specimen. The minimum distance between two points which can be seen as distinct point is called the limit of resolution (d). It is given by the following formula which is called Abbe's limit of Resolution :

$$d = \frac{0.61\lambda}{n.\sin\alpha} = \frac{0.61\lambda}{NA}$$

Where, λ = wavelength of the light (400-750 nm for visible spectrum of light)

n = refractive index of medium between objective lens and objective;

a = semiangle of refraction (the angle that marginal light rays entering the eyepiece make with the axis *i.e.,* it is the half of the angle of the cone of light that enters the microscope). This angle cannot be more than 90°.

$$\text{NA} = \text{Numerical Aperture} = n\sin\alpha$$

The inverse of the limit of resolution (d) is called the Resolving Power (R). From the above equation it can be inferred that :

(i) The limit of resolution (d) is directly proportional to the wavelength (λ).

$$d \propto \frac{1}{n\sin\alpha}$$

It implies that the details will be better resolved in the presence of lower wavelengths (*i.e.,* in violet light).

(ii) The limit of resolution (d) is inversely proportional to the Numerical Aperture. Thus, the limit of resolution is inversely proportional to the refractive index (n) of medium between the object and the objective as well as to the semiangle of refraction (α)

$$d \propto \frac{1}{n\sin\alpha}$$

It implies that : $d \propto \frac{1}{n}$ $\qquad$ $d \propto \frac{1}{\sin\alpha}$

Applications of Microscopes

Microscopes were initially used to observe cells and tissues from plants and animals. This led to the discovery of first cells in a slice of cork by **Robert Hooke** and pioneered the field of cytology. However, with the passage of time microscopes have found a number of diverse applications in various fields of study some of which are being summarized here :

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1. Observation of tissues and their thin sections in order to understand their micro-architecture.
2. Study of cells and subcellular structures as a cytological exercise.
3. Identification of microbes for taxonomic and pathological purpose.
4. Detection of disease-causing germs and parasites in samples of blood, body fluids and tissues from patients.
5. Diagnosis of genetic diseases through karyotype and study of chromosomes.
6. Study of crystalline structures and molecular processes.
7. Detection of evidence of a crime in forensic samples such as traces of blood, semen or hair.
8. Creation of electronic devices and circuits.
9. Stereomicroscope is used for soldering, watch-making and many other purposes.

Q.2. Describe the principle of compound light microscope. What are the steps involved in sample preparation for compound microscope?

Ans. **Compound Light Microscope (Bright Field Microscope)**

This is perhaps the most commonly used microscope in biology laboratories of educational institutions (Fig.). The compound microscope possesses following two distinct lenses having different focal lengths :

1. Eye-piece or ocular lens which remains close to eyes and has large focal length (fe).
2. Objective lens which is kept close to the object and has small focal length (fo).

The magnification of compound microscope is given by the following relationship :

$$M = DL/fo \cdot fe$$

Where, D = Least distance of distinct vision

L = Length of the microscope tube,

fo = Focal length of objective lens,

fe = Focal length of eye-piece

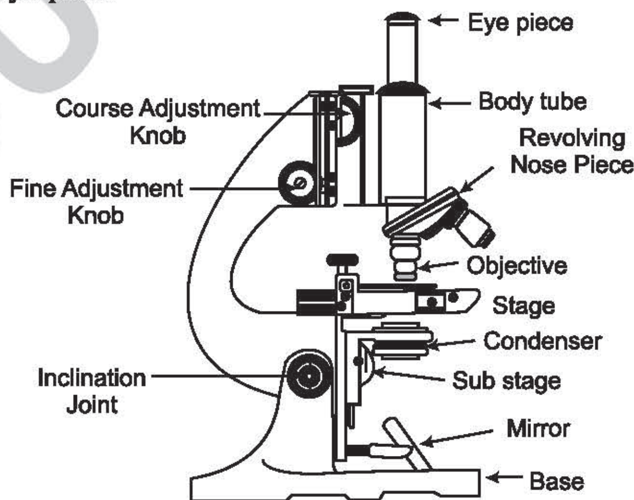

Fig. : Compound Microscope

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Principle

In bright field light microscopy the light beam that passes through the sample illuminates it in such a way that it appears dark against a bright background and there is not much contrast in the sample. Hence, staining of the object to be viewed is required to produce desired contrast and a number of stains are used for this purpose.

The magnification is achieved by both the lenses. The object has to be placed between the focus and the centre of curvature of the objective lens so that an enlarged, inverted and real image is obtained on the other side of the objective lens. If the image so formed by the objective lens happens to lie within the focus of the eye-piece the eye-piece will form a further enlarged and virtual image of the object on the same side as the object. Since the image formed by the objective is inverted and the eye-piece does not form an inverted image, therefore the final image is highly enlarged, inverted, and lies on the same side as the object. The ray diagram of image formation by the compound microscope is shown in Fig.

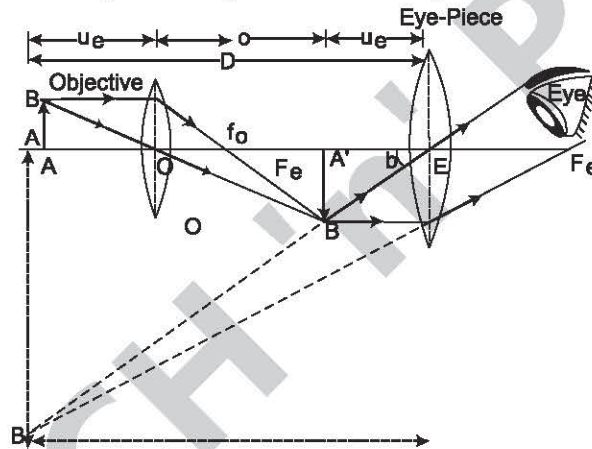

Fig. : The ray diagram of image formation by the compound microscope

Sample Preparation

The process of preparing samples for observation under microscope involves a series of steps which are as follows :

1. **Fixation :** The tissues to be observed by the microscope need to possess stable cellular organization. A fixative hardens the tissues and preserves normal cell architecture. It also kills microorganisms. The most common fixative used in light microscopy is formalin which is 40% formaldehyde. 10% neutral buffered formalin is obtained by dissolving 4% formalin in phosphate buffered saline (PBS). Corrosive sublimate and glutaraldehyde are other examples of fixative.
2. **Washing :** When the tissue has been properly fixed, it is washed with water so as to remove any trace of the fixative from the sample.
3. **Embedding and Sectioning :** After fixation the tissue is embedded in some rigid medium that holds it strongly so that sections of appropriate thickness may be cut using a microtome. Of all the substances, paraffin wax is used for embedding the specimen. Epoxyplastic resin is another embedding material which is sometimes used. Sectioning is done after the paraffin wax hardens.

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4. **Dehydration :** It is the process of removal of excess water from the tissues which is performed to prevent them from decomposition. Different grades of alcohol are used to replace water in a gradual step-by-step manner so as to control the rate of diffusion of alcohol into cells in exchange of an equal amount of water that leaves cells. If the tissues are directly kept in higher concentration of alcohol, it may rupture the cell membranes due to rapid influx of alcohol and outflux of water.
5. **Staining :** Stains are used to produce colour contrast in tissue samples. On the basis of solvent, the stains may be aqueous or alcoholic. Besides, cytoplasmic stains are distinct from nuclear stains. Borax carmine, haematoxylin, Janus green and safranin, etc. are some commonly used stains in processing biological specimens.
6. **Destaining :** This is the process of removal of excess stains from tissue samples. Acid water and acid alcohol are used to remove aqueous and alcoholic stains, respectively.
7. **Clearing :** It is performed after dehydration to remove the ethanol left in the tissue. Clove oil or xylene is commonly used for this purpose. The process of clearing makes the tissue transparent.
8. **Mounting :** After clearing, the tissue is mounted on a slide in a mountant and is covered by a cover glass. For temporary preparations, glycerin is used as a mounting medium whereas for permanent slides, Canada balsam or DPX mountain is commonly used. An ideal mountant must be sticky, transparent and its refractive index should be close to that of glass.

Q.3. Describe the working principle and components of transmission electron microscope.

Ans.

Transmission Electron Microscope [TEM]

This was the very first type of electron microscope which was developed by **Max Knoll** and **Ernst Ruska** in 1932, an invention which earned them the 1986 Nobel Prize in Physics. The Transmission electron microscope had a much higher resolution compared to optical microscopes. It uses a beam of electrons to project on to the sample. The electron beam is focused by electromagnets and an image is formed by transmitted electrons on a phosphorescent screen.

Challenges : Using electrons as a source of illumination comes with a number of challenges which are addressed in designing the electron microscope. Some of the constraints encountered in building an electron microscope are as follows :

1. Requirement of high energy electrons and of high electric potential in the electron gun.
2. Necessity of maintaining vacuum to prevent the loss of energy of electrons.
3. Low penetration power of electrons which can be compensated by using ultrathin sections of the tissue with the help of ultramicrotome.
4. Focusing of electrons onto the sample. Electromagnets can be used for this purpose.
5. Formation of image by electrons which are not visible to eyes. This can be achieved by projecting the electron beam on a phosphorescent screen.

Components of TEM

The Transmission electron microscope has a number of components and some of these are comparable to those found in light microscope. Following are the main components of TEM :

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1. **Electron Gun :** This is the part that produces a beam of high energy electrons. A very high (50,000-100,000 V) voltage is applied in the electron gun in order to generate electrons of high velocity and low wavelength (λ) which is a desired condition for obtaining high resolution. The four important parts of the electron gun comprise of a filament, a biasing circuit, a Wehnelt cap and an extraction anode.
2. **Vacuum chamber :** This part is meant to maintain vacuum which is needed to prevent the scattering of electrons by particles of the air. A very low pressure of 10.4 Pa (Pascal) is maintained by the vacuum pump. Pressure may vary in the range of 10.4-10.7 Pa in different parts if the microscope. In high voltage TEMs, ultra low vacuum in the range of 10.7-10.9 Pa is required to prevent the formation of an electric arc at TEM cathode.
3. **Specimen stage :** This is the place to hold a sample grid made up of copper, Molybdenum, gold or platinum. The grid has a standard diameter of 3.05 mm with a thickness and mesh size ranging from a few to 100 μm. The mesh area where samples are kept has a diameter of about 2.5 mm. The specimen stage design includes air locks so as to minimize the loss of vacuum.

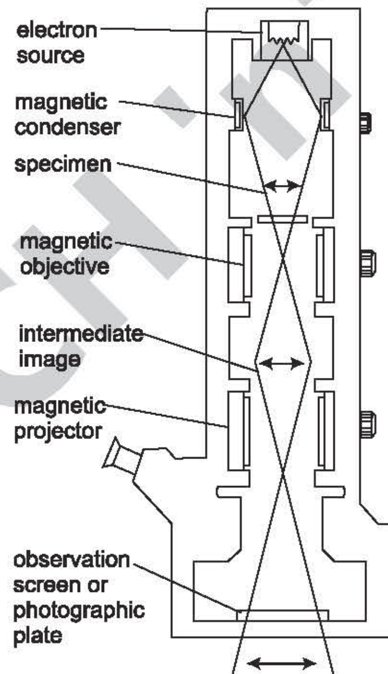

Fig. : Transmission electron microscope

4. **Electron Lens :** Electron lenses are electromagnets made from Fe-Co or Ni-Co alloys. They are designed to act as convex lenses by converging the beam of parallel rays of electrons at some focal distance. Their components include a yolk, a magnetic coil, a magnetic pole piece and a regulatory circuit. The condenser lens collects light from the electron gun and projects it on the sample. The electrons emerging from the sample have to pass through an objective lens, two intermediate lenses and a projector lens before forming an image.

TOUCH 'n' PASS

5. **Apertures :** These are annular metallic plates with small metallic discs which allow axial electrons to pass through but prevent those that are distant from optical axis. This reduces the intensity of electron beam and prevents the entry of electrons which have scattered too much.
6. **Projection Screen :** Electrons are not detectable by human eyes making it impossible for humans to directly view the image formed by electron microscope. However, when electrons are projected onto a Zinc sulphide screen they produce phosphorescence which can be directly viewed. All images can also be captured by Charge-Coupled Device (CCD) camera which is placed under the stage.

Q.4. Discuss the design and principle of dark field microscope. How does it differ from bright field microscope? Also write its advantages and disadvantages.

Ans.

Dark Field Microscope

Although the normal bright field microscope described above is the most common optical microscope yet it can be made more useful for observing certain specimens by applying a different illumination scheme. The normal bright field microscope suffers from the defect of a bright background which interferes with an equally bright fore ground and necessitates the use of stains to produce colour contrast in the specimen so as to differentiate its details. However it is not always possible to stain the sample specimen and dark field microscope can eliminate this requirement by applying some modification in the optical components. The dark field microscope is used to observe unstained objects such as living cells of bacteria, spirochetes, Protozoa and other small organisms.

Principle

In bright field microscope, the light beam is used to form a dark image against a bright background. This happens because the lenses used in this set up focus the entire beam of light onto the specimen and the image is formed by the transmitted light whereas the scattered light is not available to form an image. However, in a dark field microscope, the illumination scheme undergoes following modifications :

1. The light emitted from a source is made to pass through a 'patch stop' which blocks the central part of the light beam and allows the light to pass through an outer ring (annulus). This cuts a major portion of background light which would have passed through the specimen.
2. The circular beam of light that passes through the annulus ring is focused by the condenser lens onto the specimen.
3. The coming from the specimen is made up of two components direct light and scattered light.
4. Direct light is prevented from reaching the objective lens by an objective aperture (direct illumination block). Therefore, the only light that reaches the objective is that which is scattered (diffracted) by the specimen.

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The light path and various optical components used in a dark field microscope are shown in Fig. below :

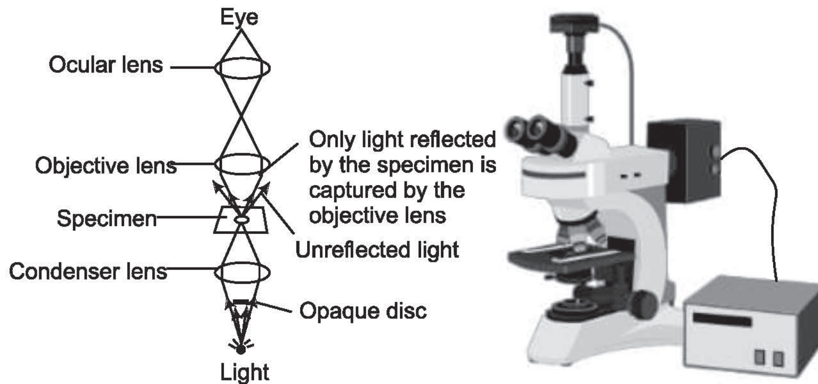

Fig. : The light path and various components used in a dark field microscope

Advantages and Disadvantages

Dark field microscope has the advantage of being able to produce excellent contrast even without the use of any stain. It offers a chance to observe live cells and organisms which is not possible with bright field microscope.

However, a big drawback of dark field microscope is that the intensity of incident light decreases considerably due to blocking of central light beam and the use of annulus. Hence, very strong light is needed to illuminate the sample and form image by dark field microscope. This may obviously damage the sample.

Q.5. Describe the working principle and design of phase contrast microscope. Also write its application.

Ans.

Phase Contrast Microscope

This is a very useful type of microscope that was invented by Franz Zernike Frits Zernike in 1930 who won Nobel Prize in Physics (1953) for this remarkable leap in microscopy. Phase contrast microscope enables viewing of unstained objects and is helpful in observing living cells. The microscope produces contrast in the specimen by introducing phase shifts and converting them into differences of amplitude which are perceptible to human eyes.

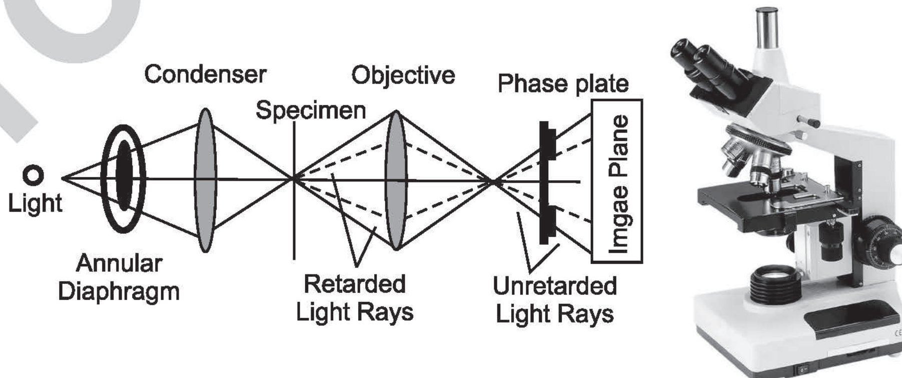

Fig. : Phase Contrast Microscope

Principle

Light is an electromagnetic wave and possesses wave properties like frequency wavelength, amplitude and phase. Phase describes the relationship between the position of amplitude crests and troughs of two waveforms. Two waves interact to produce characteristic interference pattern depending on their phase difference. If the waves are in same phase their combined amplitude adds up producing constructive interference but when waves have opposite phase, they produce destructive interference because the two waves cancel out. Thus, phase difference can be converted into amplitude difference by altering the phase difference between two waves. This phenomenon is exploited in phase contrast microscope by making use of phase changes induced by the thickness and refractive index of different parts of an object and by creating a phase shift between background and transmitted radiation. The phase shift causes amplitude differences in the radiation. Though the human eye can't detect the phase of waves yet it can easily discern amplitude differences which produce contrast.

Phase contrast microscope separates the illuminating light background) from the specimen-scattered (foreground) light and induces phase shift in two different ways to increase contrast in images :

1. **Negative Phase Contrast :** The beam of light from the light source passes through a condenser annulus to form a ring of light which is projected onto the specimen. The light from the specimen is either diffracted (scattered) by it or is unaffected. Normally the specimen-scattered light loses intensity and also becomes phase shifted due to thickness of tissue thereby making the foreground appear nearly as bright as the background which gives poor contrast. Thus, the background light is phase shifted by $-90°$ with the help of a phase ring so as to eliminate the phase angle difference between the light diffracted by the specimen and the background light. This causes positive interference and increased brightness of points lying in the sample field. Besides, the background light is reduced up to 90% by grey filter ring. This results in an image formed mainly by specimen-scattered light against a dark background.
2. **Positive Phase Contrast :** In this variant of the technique, the background light is phase shifted by $+90°$ so that its difference of phase from the scattered light is $+180°$ enabling destructive interference between the two. Since the scattered light is subtracted from the background light, the image formed appears dark against a lighter background.

Design

The phase contrast microscope has following components in its design :

1. **Light source :** It is a lamp of appropriate luminosity as used in compound microscope.
2. **Annular diaphragm :** An annular diaphragm (phase condenser) is placed in the focal plane of the substage to control the illumination. It blocks the central zone of light beam and creates a hollow cone of light.
3. **Phase plate :** It is located in the back focal plane and is used to retard the phase of light. For this purpose the phase plate has light retarding material such as Magnesium Fluoride (MgF_2) in areas where phase has to be retarded and normal glass in the remaining part.

TOUCH n PASS

4. **Specimen stage :** This is similar to that used in compound microscope except that live specimens can be kept for observation on the stage.

Applications

Phase contrast microscope is used for following purposes :

1. It is used to observe unstained objects which makes it ideal for viewing live cells.
2. It is helpful in resolving the details of transparent objects. Thus, it can be effectively used to differentiate the cell organelles and cytoplasm in living cells.
3. Movements of cell organelles such as flagella, centrosome or chromosomes can be tracked using phase contrast microscope.

Q.6. Describe the optical path in a confocal microscope. Also explain the component parts and applications of confocal microscope.

Ans.

Confocal Microscope

This is a very sophisticated variation of fluorescence microscope which uses optical sectioning to resolve very fine details of the objects. The first confocal microscope was developed by **Marvin Minskyin** 1955 who filed a patent for the same in 1957. Alternatively called **Laser Scanning Confocal Microscopy** (LSCM), this technique has become an extensively used method in life sciences.

Principle

The technique of confocal microscopy is an advancement of fluorescence microscopy and overcomes some of its major shortcomings. In the usual wide-field fluorescence microscope, the whole sample is illuminated at the same time and the fluorescence captured simultaneously from the whole sample is used to form images. Hence, a large fraction of out-of-focus light is also involved in image formation leading to poor contrast. Hence, unlike conventional fluorescence microscope, the confocal microscope uses laser beams to illuminate the object for precise control. The resolution and contrast of the image is increased by a spatial pinhole which blocks but-of-focus light and allows point illumination. The name 'confocal' is used because of the specific configuration of pinholes in which a pinhole in a conjugate plane is placed in front of the detector to prevent out-of-focus light rays.

Another advantage of confocal microscope is that it permits formation of 3D images of an object. Fluorescence collected from all the points in a plane can be assembled to provide details of a section of the object. Similarly details from all the sections of an object can be put together to form a 3D image through this process called **'optical sectioning'**.

Components

The laser scanning confocal microscope has following important components in its design :

1. **Light source :** Lasers of high intensity are among the essential requirements of confocal microscope. One advantage of using a laser is that it can penetrate deep and form image of various points in the depth of the sample.
2. **Pinholes :** The laser beam from the source of illumination is allowed to pass through a pinhole situated close to it. It lets light to fall on a dichroic mirror. Another pinhole is placed in a conjugate plane close to detector and is used to focus the light coming from a point at desired depth in the sample and blocks out-of-focus light rays.

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3. **Detector :** A highly sensitive detector is used to compensate for the loss of signal intensity at pinholes. Photo-multiplier tubes or Avalanche photo-diodes are used as detectors of choice in Laser Scanning Confocal Microscope (LSCM).

Applications

Confocal microscope is of special significance in modern biology because of its high resolution and the ability to perform optical sectioning of the object. It is specially used for the following purposes :

1. It is helpful in observing molecular processes taking place inside the cell. Interactions of proteins, lipids and nucleic acids can be observed in real time.
2. Movements of ions in the cell can be tracked with the help of confocal microscope. For instance, it can monitor the movement of Ca^{2+} ions in the oocyte.
3. Optical sectioning enables viewing of 3D structure of cells and subcellular structures.
4. In medicine it can be used to detect the endothelial cells of cornea and for monitoring endoscopic procedures (endomicroscopy).
5. Confocal microscope is also used to study biofilms which are complex porous structures inhabiting microbes.

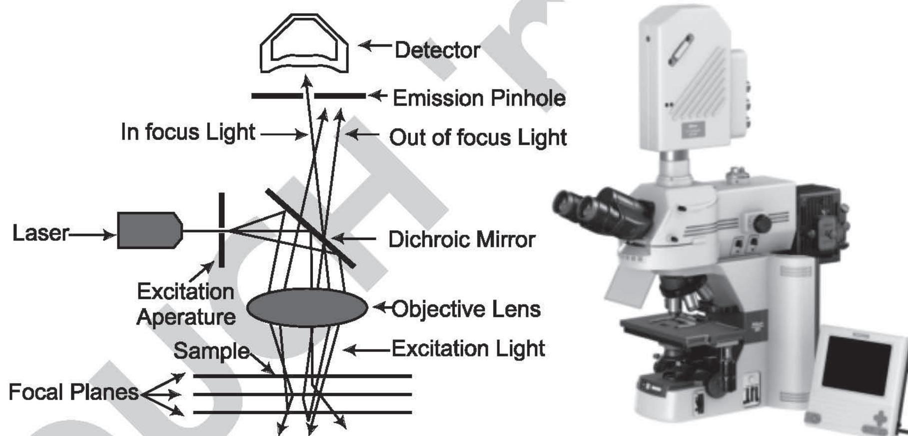

Fig. : Confocal Microscope

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UNIT-VI

Centrifugation and Chromatography

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. Who created the centrifuge?

Ans. The first continous centrifuge, designed in 1878 by the Swedish inventor De Laval to separate cream from milk, opened the door to a broad range of industrial application.

Q.2. What is *G* force in centrifuge?

Ans. The relative centrifugal force (RCF) or the radial force generated by the spinning rotor as expressed relative to the earth's gravitational force. The *g* force acting on particles is exponential to the speed of rotation defined as revolution per minute (RPM).

Q.3. What is RPM in centrifuge?

Ans. RPM stands for 'Revolutions per minute'. This is how centrifuge manufactures generally describe how fast the centrifuge is going. The rotor, regardless of its size, is revolving at that rate. The force applied to the centents varies by the size, is revolving at that rate. The force applied to the contents varies by the size of the centrifuge rotor.

Q.4. What is the principle of centrifugation?

Ans. Centrifuge works using the sedimentation principle, due to the centrifugal acceleration denser substances move outward in the radial direction and that are less dense are displaced and move to the center.

Q.5. What is meant by ultracentrifuge.

Ans. A high-speed centrifuge able to separate out colloidal and other small particles and used especially in determining the sizes of such particles or the molecular weights of large molecules.

Q.6. Who invented ultracentrifuge?

Ans. The analytical ultracentrifuge was invented in 1925 by Theodor Svedberg who was awarded the nobel prize in chemistry for his research on colloids and proteins using the ultracentrifuge.

Q.7. What is the role of rotor in centrifugation?

Ans. The centrifuge is composed of a rotor, which is used to house the tubes where separation occurs. There are two main types of centrifuge rotors : fixed angle or swinging bucket. Fixed angle rotor hold tubes at a stable angle (typically 45°) relative to the axis of rotation.

TOUCH 'n' PASS

Q.8. What is chromatography with examples?

Ans. Chromatography is a group of laboratory technique used to separate the components of a mixture by passing the mixture throught a stationary phase. Typically, the sample is suspended in the liquid or gas phase and is separated or identified based on how it flows through or around a liquid or solid phase.

Q.9. Who discovered chromatography?

Ans. Chromatography was invented about ninety years ago by M.S. Tsmett, a Russian scientist studying plant pigments.

Q.10. What is the principle of HPLC?

Ans. The specific intermolecular interactions between the molecules of a sample and the packing material define their time 'on-column'. Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved.

Q.11. What is polar and non-polar in HPLC?

Ans. The stationary phase is non-polar, like C18 bonded silica. The mobile phase is polar, usually being wates and polar organic solvent. Compounds with the most hydrophobicity elute later in the chromatogram and those with the least hydrophobicity elute earlier.

Q.12. What is difference between GC and HPLC?

Ans. GC is typically used to measure oils, organic compounds, air samples, toxins and drugs (both pharmaceutical and recreational). HPLC is more commonly used for inorganic ions and food substances like sugars, protein and vitamins as well as other compounds like, polymers, nucleotides and tetracyclines.

Q.13. What do you understand by paper chromatography?

Ans. Paper chromotography is a technique which is used to separate low-molecular-mass compounds based on their distribution between stationary phase and mobile phase. Due to its low cost and availability of various protocols for the separation of compounds, paper chromatography is considered a powerful analytical technique.

Q.14. What are the advantages of paper chromatography?

Ans. The main advantages that paper chromatography offers are simplicity, low cost and unattended hassle-free-operation. It can be run in various modes and quantitation may be achieved without the use of expensive instrumentation.

TOUCH in PASS

SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. Write a short note on the types of centrifuges.

Ans.

Types of Centrifuges

The types of centrifuges are mainly depending on the particular application, generally centrifuges differ in their overall size and design. In all types of centrifuges there is a common feature, a central motor that spins a rotor holding the samples to be separated. Generally, they are divided into four groups based on maximum attainable speed of sedimentation, temperature control refrigeration, capacity of centrifugation tubes, the presence or absence of vacuum and volume of sample, etc.

1. Small benchtop centrifuge.
2. Low-speed centrifuge.
3. High-speed centrifuge.
4. Ultracentrifuges.
5. Microcentrifuge.
6. Continuous flow centrifuge.
7. Refrigerated centrifuges
8. Vacuum centrifuge/Concentrators.
9. Hematocrit centrifuge.
10. Gas centrifuge.

Q.2. Write about the ultracentrifuge.

Ans.

Ultracentrifuge

The ultracentrifuge works on the same principle as all other centrifuges. The working of an ultracentrifuge is based on the sedimentation principle, which states that the denser particles settle down faster when compared to less dense particles under gravity.

Ultracentrifuges are the most sophisticated type of centrifuge that allows the separation of particles that cannot be separated with other separators. These centrifuges operate at enormously high speeds resulting in the separation of much smaller particles like viruses, ribosomes and proteins. Besides separation process, ultracentrifuges can also be used for the determination of macromolecules properties like shape, size and densities. The speed of ultracentrifuge can reach up to 150,000 rpm. Due to this intense spinning, heat is produced so refrigeration systems are existing in such centrifuges that support to balance the excessive heat.

Generally, ultracentrifuges are classified into two main classes, **preparative** and **analytical**. In polymer science, molecular biology and biochemistry, both types of devices are useful. Bioparticles, organelles, viruses, membranes, and biomolecules such as lipoproteins, RNA and DNA, are isolated or pelleted by means of preparative ultracentrifuges.

On the other hand, analytical ultracentrifuges use real-time detection devices to determine equilibrium and sedimentation velocity, which are used to determine the mass and shape of macromolecules.

Q.3. Write note on high speed centrifuge and also write the factors affecting centrifugation.

Ans.

High Speed Centrifuge

A high speed centrifuge is a device that use centrifugal force to separate particales of different mass or densities suspended in a liquid. Rotating the solution in the tube at high speeds gives the angular momentum of each particle a centrifugal force proportional to its mass.

High-speed centrifuge operates at the speed range of 15,000 to 30,000 rpm. They are generally used in more sophisticated laboratories that involves a high speed of processes. These centrifuges are equipped with a system for regulatory the speed and temperature of the whole process, which is mandatory for the examination of sensitive biotic particles. These high-speed centrifuges with refrigeration are undeniably vital for the sedimentation of

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cellular debris derived from micro organisms and homogenisation, large unbroken organelles and protein precipitates. High-speed centrifuges come with different types of adapters that to accommodate the sample tubes of several volumes and sizes. In these centrifuges can used all three types of rotors. However, the majority of tasks are carried out in fixed angle rotors.

Uses : These centrifuges are classically used to separate viruses, lysosomes, micro organisms, mitochondria, intact tubular Golgi membranes and peroxisomes.

Factors affecting centrifugation

The factors affecting centrifugation are as follows :

1. Density of both samples and solution.
2. Temperature and nescosity.
3. Distance of particales displacement.
4. And rotation speed.

Q.4. Explain the term chromatography also discuss the basis principle of chromatography.

Ans.

Chromatography

Chromatography is a vital biophysical technique for separating, identifying, and purifying the constituents of a mixture for qualitative and quantitative investigation. **Mikhail Tswett**, a Russian-Italian botanist known as the **"Father of Chromatography"**, is credited with the invention of the technique and coined the term chromatography in 1906. By running solutions of different plants pigments through a glass column packed with finely divided calcium carbonate, he was able to separate distinct plant pigments such as chlorophylls and xanthophylls. The separated species displayed as coloured bands on the column, which explains the method's name (chroma means "colour" and graphein means "writing" in Greek). Differences in binding affinities, size, charge, and other features are used in a variety of chromatographic methods to separate materials. It is a powerful separation technique utilised in all fields of science, and it is frequently the only way to separate components from complex mixtures.

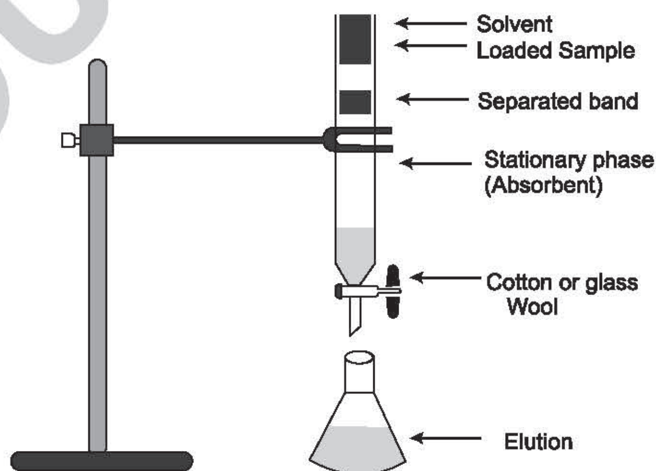

Fig. : Column Chromatography

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Principle of Chromatography

Chromatography works on the idea of allowing the sample to be studied (also known as the **solute** or **analyte**) to interact with two immiscible phases: the **mobile phase** and **the stationary phase**. A solid and a liquid, a gas and a liquid, or a liquid and another liquid could be the two immiscible phases. The stationary phase also known as the **sorbent**, which can be a solid or a liquid supported on a solid, does not move. The sample is moved through the stationary (immobile) phase by the mobile phase (Liquid in liquid chromatography and gas in gas chromatography).The two phases are selected in such a way that the sample's components are distributed differently across the mobile and stationary phases. Molecular characteristics associated to affinity, adsorption (liquid-solid), partition (liquid-solid), or variations among their molecular weights are effective on this separation process. Some components of the mixture linger longer in the stationary phase and move slowly through the chromatographic system as a result of these differences, while others flow quickly into the mobile phase and leave the system faster.

Q.5. Write a note on the modes of paper chromatography.

Ans.

Modes of Paper Chromatography

Modes of paper chromatography are as follows :

1. **Ascending : Consden, Gordan** and **Martin**, were the first to invent ascending chromatography, which was then improved by **Williams** and **Kirby**. Because the mobile phase moves upward, the chromatogram of this technique slowly ascends. The solvent reservoir is held at the bottom of the beaker in this approach, and a piece of paper with the loaded sample is dipped in the solvent. It is always advisable to ensure that the spot remains above the solvent system. Further more, the size of the paper must be considered to avoid crumpling and bending.
2. **Descending :** The descending technique refers to the process of developing paper chromatogram by allowing the solvent to go down the paper. The mobile phase is held at the top of the chromatogram in this kind, and the mixture components separate downward due to gravity and the filter paper's capillary action. This approach is favoured over ordinary ascending chromatography because the solvent flow rate is constant, easier to separate solutes with low *Rf* values and takes less time. The sole disadvantage of this technique over ascending chromatography is the need exclusive apparatus.
3. **Ascending-Descending :** This is a modified variant of paper chromatography that involves the flow of solvent ascending and descending on the same sheet of paper. Only the distance between them has grown longer; climbing comes first, followed by descending so longer flow distance gives better resolution. The main advantages of this technique over other are: (i) the run time is reduced, *i.e.,* only a short period of time is required; (ii) components with *RF* values greater than 0.50 can be detected individually because they will have their own channel.
4. **Circular/Radial :** Rutter coined the phrase radial chromatography, which involves the use of circular filter paper to separate components into concentric rings rather than a

TOUCH 'n PASS

single spot. This technique allows the radiating mixed component to expand out until all of the components have separated. A petri dish is used to cover the entire system as a precaution. A list of benefits was also provided, including better resolution, separation sharpness, simplicity, rapid separation, control over solvent flow rate, compactness of the used apparatus reproducibility and effortlessness of removal of test samples before and after development.

5. **Two-Dimensional :** One of the most effective methods for separating organic and inorganic chemicals is two-dimensional chromatography. This form of chromatogram develops at right angles to each other, and the filter paper is dipped at right angles once the first chromatogram is finished. The second chromatogram appears at a right angle to the first.

Q.6. Explain briefly the components, advantages, disadvantages of affinity chromatography.

Ans.

Components of Affinity Chromatography

Matrix or support in this purification technique is any material to which a bispecific ligand covalently attached. The matrix must have the following characters in order to be effective: **Chemically** and **physically**, the matrix should be inert. It must be chemically and physically stable and insoluble in the solvents and buffers used in the procedure. It ought to be simple to connect to a ligand or a spacer arm to which the ligand can be attached. It needs to have good flow qualities as well as a big surface area for attachment.

Agarosegel and polyacrylamide are the most useful matrix materials. By overcoming the effects of steric hindrance, the spacer arm improves ligand-target molecule interaction. A ligand is a chemical molecule that binds to a specific target molecule in a reversible manner. It can be selected only when the type of the macromolecule to be separated has been determined. When affinity chromatography is used to purify a hormone receptor protein, the hormone is an excellent ligand candidate. A substrate analogue, cofactor, inhibitor or effector can be utilised as an immobilised ligand to purify an enzyme.

Advantages/Disadvantages

There are following advantages and disadvantages of affinity chromatography :

Affinity chromatographic technique is highly specific to the analyte in comparison to other purification technique. In comparison to other purification methods, affinity purification yields a significantly high level of purification fold. A typical affinity purification has a recovery rate of greater than 90%. It is repeatable and reliable in findings from one purification to the next as long as it is not affected by contaminating species. Affinity purification is very reliable, and it is based on the force that governs the formation of ligand-receptor complexes. In comparison to other procedures, affinity purification does not require column packing, a particular purification equipment, or sample preparation.

Apart from the benefits that affinity chromatography provides, it also has some drawbacks that should be considered if someone chooses to use it. Some main disadvantages of this techniques are sample volume limitations, skills necessity, sample or protein loss,

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interference with the structure, sometimes ligands leakage, non-specific leakage, degradation of solid support, costly ligands and limited life-time etc.

Q.7. Write a short note on the advantages and disadvantages of paper chromatography.

Ans.

Advantages/Disadvantages of Paper Chromatography

Advantages and disadvantages of paper chromatography are as follows :

Advantages : Paper chromatography's key advantages are its low cost, simplicity rapidity and unattended hassle-free operation. It can be used in a variety of ways, and quantitation can be done without the use of expensive equipment. The paper chromatography method can identify both unknown inorganic and organic substances and it has excellent resolving power.

Disadvantages : The main disadvantage of this method is that large quantity of sample cannot be applied on paper chromatography. It is not so effective in quantitative analysis. Paper chromatography cannot separate a complex combination. When compared to HPLC or HPTLC, this method is less accurate. Data cannot be saved for an extended period of time.

Q.8. Write a note on the gel filtrations chromatography and its application.

Ans.

Gel Filtrations or Size Exclusion or Gel Permeation Chromatography (GPC)

This chromatographic technique uses the molecular sieve capabilities of a range of porous materials for separating molecules based on their molecular size and shape. Exclusion chromatography works on a very basic basis. A stationary phase consists of a column matrix filled with porous gel beads made of a hydrated and insoluble polymer such as polyacrylamide agarose (sepharose), dextran (Sephadex), or (Sephacryl or BioGel P). GPC and gel filtration chromatography are two types of size exclusion chromatography. GPC employs an organic mobile solvent to separate and describe molecules, whereas gel filtration chromatography uses an aqueous mobile solvent. The molecules in the sample are pushed through microporous packing material in specialized columns (gel). The separation is based on the fact that molecules larger than a particular size are completely prohibited from the pores, while smaller molecules have partial or complete access to the interior of the pores. As a result of the mobile phase's movement, larger molecules will pass down the column unobstructed, without piercing the gel matrix, but smaller molecules will be delayed based on their gel penetration.

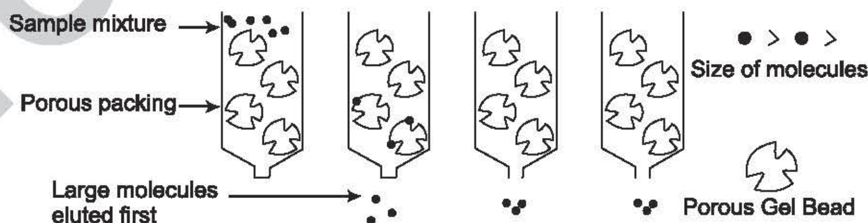

Fig. : Filteration chromatography

Application

GPC technique is widely used for the separation of proteins, enzymes, synthetic polymers and polysaccharides. It also determines the relative molecular weight of polymer samples. In the

separation and purification process of sugar, peptides, rubbers, proteins, and others on the basis of their size this chromatographic technique is very helpful. The quaternary structure of pure proteins can be determined using this method.

SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. What do you understand by centrifugation also explain the principles of centrifugation?

Ans.

Centrifugation

Centrifugation is a technique used for separating or concentrating the substances that are suspended in liquid medium with the involvement/ application of centrifugal force. The particles are separated from the liquid medium/solution according to their sedimentation rate which depends on a number of factors like, shape, density and size, rotor speed and viscosity of medium etc. Centrifuge is a device that produces centrifugal force and separates different components of mixture by spinning the fluid/medium at very high speed. Now-a-days modern centrifugation techniques represents a critical means for contemporary biochemistry and are working in nearly all invasive subcellular fields. Centrifugation technique is also used to measure the different physical properties like shape, molecular weight and density etc.

If centrifugation process is used to monitor the particle sedimentation behavior in order to characterize the diverse particles properties like shape, association and molecular weight etc., it is termed as "analytical centrifugation". On the other hand, if this process is used for separation and recovery of one or more components from the mixture sample then termed as "preparative centrifugation".

Principle of Centrifugation

The consequence of sedimentation due to the influence of Earth's gravitational force (g) versus the increased rate of sedimentation in centrifugal field is apparent in our day-to-day life. Earth's gravitational force pulls the particles downwards suspended in solution. It is well known that this force is depends only on the mass of the particles and not on chemical composition, charge and shape. A solution that contains particles of very small masses generally remains suspended owing to random thermal motion as Earth's gravitational field is very weak. Hence, forces greater than Earth's gravitational force are mandatory to cause significant sedimentation.

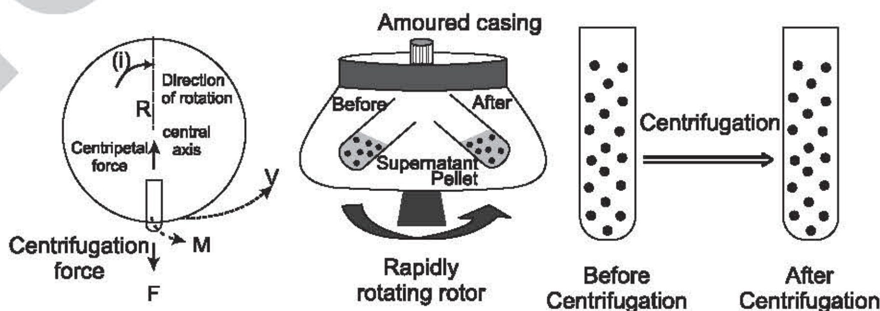

Fig. : Centrifugation

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In biological systems particles experience a frictional drag, moving through a viscous medium. By which the frictional force performances in the reverse direction to sedimentation and equals the velocity of the particle multiplied by the frictional coefficient. By way of the sample passages towards the bottom of a centrifuge tube its velocity increase owing to the increase in radial range. On the other hand, moving particles also experience a frictional drag that is directly proportional to their velocity. When particles moved through a viscous fluid its frictional force (acts in the reverse direction to sedimentation) is the product of frictional coefficient and velocity.

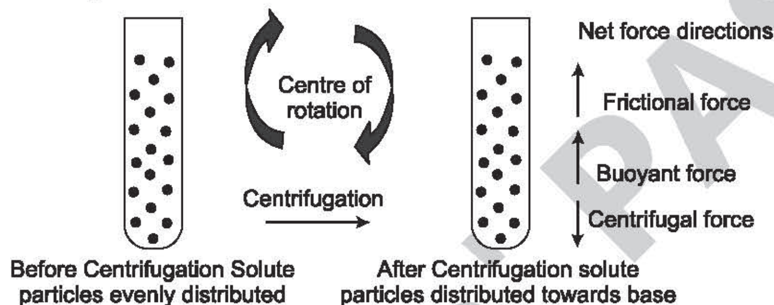

Fig. : During centrifugation, the direction of various forces and the distribution of solute particles.

Let us consider a solution being spinning in a centrifuge tube, the centrifugal force acting on the sample can be calculated by the formula :

$$G = \omega^2 r$$

Where, G is the centrifugal force measured in cm s^{-2}

ω is angular velocity of the rotor in radians per second.

r is the radial distance of the particle from the axis of rotation in cm.

However, it is more convenient to calculate or monitor the speed of centrifuge in terms of revolution per minute than in radian per second so relative centrifugal field (RCF) is calculated. The RCF or g-force is simply the ratio of centrifugal acceleration at a definite radius and the speed to the standard gravitational acceleration. The RCF (or g-force) can be calculated by the following formula :

$$\text{RCF} = G/g$$

$$\text{RCF} = \omega^2 r/g$$

$$\omega = 2\pi \times \text{rpm}/60 \text{ radians/sec}$$

$$\text{RCF} = r/\text{g} \times [2\pi \times \text{rpm}/60]^2$$

$$\text{RCF} = r \times (\text{rpm})^2 \times [4 \times 3.14 \times 3.14/60 \times 60 \times 980]$$

$$\text{RCF} = 1.12 \times r \times (\text{rpm})^2 \times 10^{-5}$$

The RCF compares and defines the field strength generated by the different size and operating speeds of rotors. For example, a centrifuge having higher radius of rotation definitely will spin the samples at higher RCF.

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For the sedimentation of the particle, equal volume of the solvent must be displaced from beneath it. This can only be accomplished by the centrifugation process if the mass of the moving particle is more than the mass of the solvent displaced.

Net Force = Centrifugal force – Buoyant force – friction

$$\text{Net Force} = m\omega^2 r - m_0\omega^2 r - fv$$

Where, m is mass of the moving particle.
m_0 is mass of the fluid displaced by the particle
$\omega^2 r$ is the centrifugal acceleration
f is the frictional coefficient in Nm-1sec
v is the sedimentation velocity.
The classification of particles is more convenient in terms of rate of sedimentation per unit centrifugal field which is known as **sedimentation coefficient** (s), has unit of **second.** It is usually expressed as Svedberg unit (S), $1S = 10^{-13}$ second. **Svedberg** was a Swedish chemist and pioneer in the field of centrifugation, developed first analytical centrifuge in late 1920s.

Q.2. Explain the various types of rotors.

Ans.

Types of Rotors

In all types of centrifuges there are two main components, the rotor assembly and the electrical motor. The rotor assembly is the part of the centrifuge that transfers the electrical motor's rotating motion. Centrifuge rotors are categories into three categories : **fixed angle rotors, swinging bucket rotors**, and **vertical rotors** depending upon the kind of centrifugation, centrifugation speed and sample volume. Some other commonly used rotors in laboratories are zonal rotors, continuous flow rotors and elutriator rotors. The fixed-angle and swinging-bucket rotors are the most prevalent for low-speed, table top and high-speed floor-model centrifuges, respectively. For ultracentrifugation vertical rotors are used steel, perspex or brass rotors are used for slow speed centrifugation. For high-speed centrifugation to endure more stress, rotors are made from a variety of materials such as carbon fibre, aluminium, and titanium.

1. **Vertical Rotors :** In vertical rotors tubes are held vertical during the centrifugation process. It provides the quickest run time, shortest path length and the best resolution

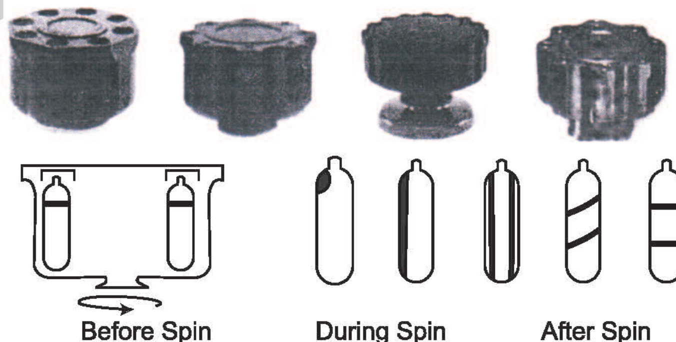

Fig. : Vertical Rotors

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than any other rotor. Because the tube in vertical rotors does not align with the direction of the centrifugal force, the rotor yield is less than optimum. As a result, rather than settling, particles incline to spread towards the tubes' outer walls. Density gradient and isopycnic centrifugation process often use this type of rotor. The main disadvantage of this type of rotor is that at the end of centrifugation, the pellet may fall back into the solution.

2. **Swing Out Rotors :** Swing out rotor also known as **bucket rotor** (rotor with variable angle) is a rotor with tubes situated in buckets (tube-holders) that are not securely attached to the rotor at a specific angle. The tubes of this rotor are suspended in racks that allow them to move enough to achieve a horizontal position.

   As the centrifugation process begins, swinging bucket rotors hold the tubes at a 90° angle as the rotor swings. The spinning particles are present along the direction or route of the force in this sort of rotor, allowing them to be transported away from the rotor and towards the bottom of the tubes. This rotor is especially beneficial when resolving samples in density gradients. Individual particle types can be better separated from a mixture due to the longer route length. However, for pelleting, this rotor is relatively inefficient.

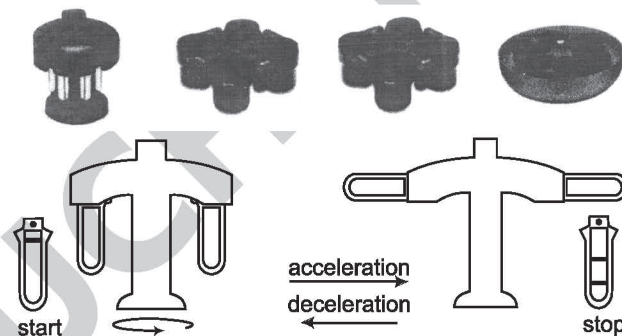

Fig. : Swing out rotors

3. **Fixed Angle Rotors :** A fixed angle rotor is one that keeps tubes at the same angle all of the time, generally 45°. The solution in the rotor tubes reorients when the rotor starts to rotate. As the path length of the tubes increases, these rotors become quicker than other variants. The particles strike the opposite side of the tube in this sort of rotor, where they eventually slide down and settle at the bottom. However, because the force's direction differs from the tube's position, some particles may linger on the tubes' sides. Pelletizing is the most prevalent application for this rotor type.

   Pelletizing bacteria, yeast, and various mammalian cells are examples. It can also be used to separate macromolecules like nucleic acids into isopycnic fractions. Fixed rotors can endure much higher gravitational forces due to the stiff design of the metal

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alloy material, which is important when separating biological macro molecules such as protein, RNA and DNA.

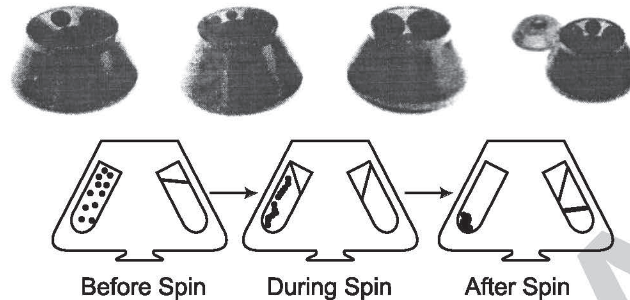

Fig. : Fixed angle rotor

Q.3. Describe in detail the types of centrifugation techniques and its applications.

Ans.

Types of Centrifugation Techniques

The centrifugation technique are classified into two types :

1. **Differential Centrifugation/Differential Pelleting :** It is the most common type of centrifugation process in which tissue, such as the liver, is homogenised in a sucrose solution including buffer at 32 degrees. The homogenate is then positioned in a centrifuge and spun at a consistent temperature and centrifugal force. A sediment called **pellet** and an overlaying solution called **supernatant** form at the bottom of a centrifuge after some time. The overlaying solution is then transferred to a new centrifuge tube, which is revolved at a faster speed in subsequent phases.

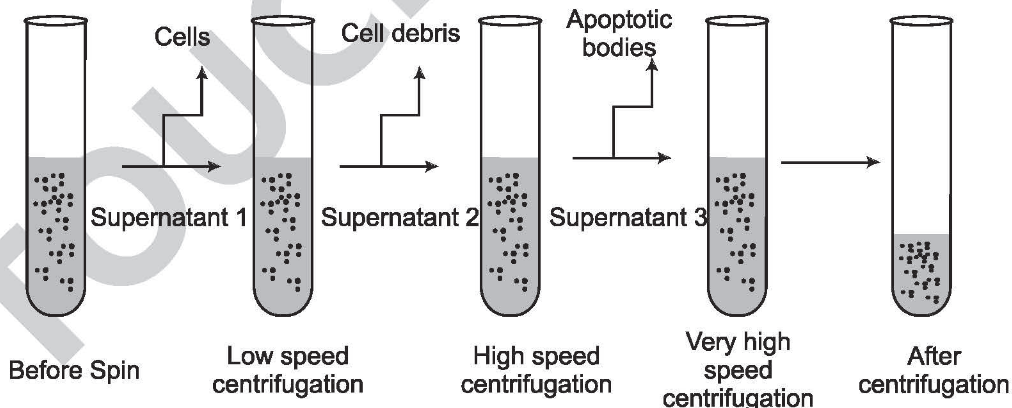

Fig. : Differential centrifugation

2. **Density Gradient Centrifugation :** It enables for the separation of many or all of the components in a mixture, as well as measurement. Density gradient centrifugation can be divided into two types : **rate zonal centrifugation** and **Isopycnic** or **sedimentation equilibrium centrifugation.**

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The solution exhibits a density gradient in rate zonal centrifugation. As a result, the sample has a higher density than the solution's layers. On a density gradient, the sample is applied in a thin zone at the top of the centrifuge tube. The particles will begin segmenting through the gradient due to centrifugal force. According on their shape, density and size, the particles will begin segmenting into different zones.

The solution comprises a wider variety of densities in this form of centrifugation. During **isopycnic** or **sedimentation equilibrium centrifugation**, particles are separated into zones depending on density differences.

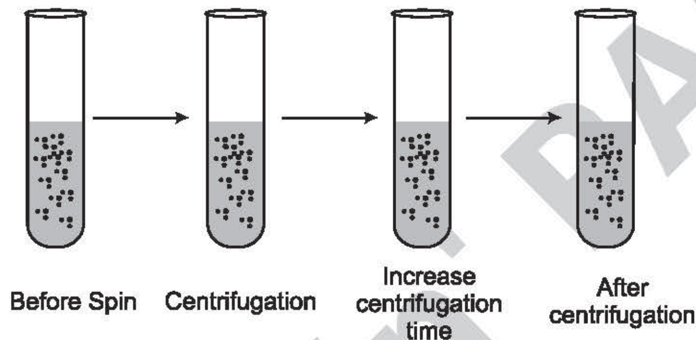

Fig. : Density gradient centrifugation

Application of Centrifugation

The application of centrifugation are as follows :

1. To separate two liquids that are miscible.
2. Bulk medication manufacturing.
3. Subcellular organelles fractionation including membranes/membrane fractions.
4. Separating chalk powder from water.
5. Mammalian cells purification.
6. Removing fat from milk to make skimmed milk.
7. Using cyclonic separation to separate particles from an airflow.
8. Assists in protein separation utilising purification processes like salting out, such as ammonium sulphate precipitation.
9. In forensic and research laboratories, the separation of urine and blood components.

Q.4. Explain the paper chromatography, it types and applications in detail.

Ans.

Paper Chromatography

Paper chromatography is a type of planar chromatography technique which runs on a piece of specialized paper through which a solution containing samples passes. Here, specialized paper, which is generally filter paper made of cellulose, acts as stationary phase on which the separation of compounds occurs. It is comparatively inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is the simplest and most widely used of the chromatographic techniques because of its

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applicability to isolation, identification and quantitative determination of organic and inorganic compounds. It was first introduced by German Scientist **Christian Friedrich Schonbein** in 1865.

Principle : Paper chromatography refers the involvement partition chromatography where in the substances are distributed or partitioned between liquid phases. One phase is the water, which is held in the pores of the filter paper used; and other phase is called the **mobile phase** that moves over the filter paper. The compounds in the mixture get separated due to differences in their affinity towards water (in **stationary phase**) and mobile phase solvents during the movement of mobile phase under the capillary action of pores in the paper.

The principle of paper chromatography also coincides with the principles of adsorption chromatography between solid and liquid phases, wherein the stationary phase is the solid surface of the paper and the liquid phase is of the mobile phase. But most of the applications of paper chromatography work on the principle of partition chromatography, *i.e.,* partitioned between to liquid phases as shown in fig. below.

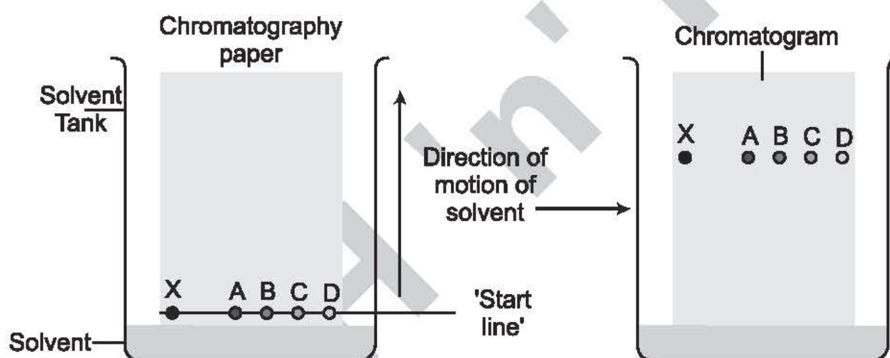

Fig. : Paper Chromatography

It is possible to determine the distinctive rate of movement of individual substance on the chromatographic paper as the moving phase moves at a certain temperature and for a specific solvent. This is represented by the *Rf* value, which stands for relative front or slowing factor. The ratio of the distance travelled by the substance and solvent front is known as *Rf*. *Rf* is the dimensionless expression for the ratio of the distance travelled by the substance and solvent front. Even when the mobile phase (solvent) is the same, the *Rf* values of various substances varies. Further more, the *Rf* value of a molecule might vary depending on the solvent.

The following formula can be used to compute *Rf* values :

Rf = Distance travelled by the substance from baseline/Distance travelled by the solvent front by baseline.

Types of Paper Chromatography

They are classified into two types :

1. **Paper adsorption chromatography :** In this type the adsorbent (stationary phase) is paper impregnated with silica or alumina, while the solvent is the mobile phase.

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2. **Paper partition chromatography** : In this type the stationary phase is moisture/water in the pores of cellulose fibers in filter paper, and the mobile phase is the solvent.

Applications of Paper Chromatography

The applications of paper chromatography are as follows :

1. It's used to separate colored material solutions.
2. To ensure that pharmaceutical purity is maintained.
3. It's utilized in forensic science to find and identify trace amounts of chemicals in the bladder and stomach contents.
4. Determination of food and drinks contaminants.
5. Cosmetics analysis.
6. In the study of ripening and fermentation process.
7. For detection of adulterants.
8. In biochemical laboratories, the reaction mixtures are analyzed by this technique.
9. To check the control of purity of pharmaceuticals.

Q.5. What is Ion-exchange chromatography? Also explain its procedure, applications advantages and disadvantages.

Ans.

Ion Exchange Chromatography

Ion chromatography (also known as **ion-exchange chromatography**) is a type of chromatography that separates ions and polar compounds according to their affinity for an ion exchanger. Ionic solutes interact with a charged stationary phase in this chromatographic process in a reversible electrostatic manner. The size of the charge and the charge density (amount of charge per unit volume) of the solute determine the strength of interactions. Neutral solutes have no or very little affinity for the stationary phase and elute with the eluting buffer. Because of its great resolving power and capacity, it is commonly used for the separation and purification of peptides, proteins, polynucleotides, nucleic acids, and other charged compounds. By eluting the column with a buffer with higher ionic strength or pH, the bound solutes can be freed. The displacement of bound solutes occurs when the buffer ionic strength is increased. If pH of buffer is increased, the strength of the interaction is decreases by reducing the charge on the resin or on the solute.

There are two types of ion exchangers in ion-exchange chromatography : **cation** and **anion exchangers**. Negatively charged groups in cation exchangers will attract positively charged cations. Because their negative charges are caused by the ionisation of acidic groups, these exchangers are also known as **acidic ion exchangers.** Positively charged groups in anion exchangers attract negatively charged anions. Positive charges are generated by the interaction of protons with basic groups; hence these exchangers are also known as **basic ion exchangers.**

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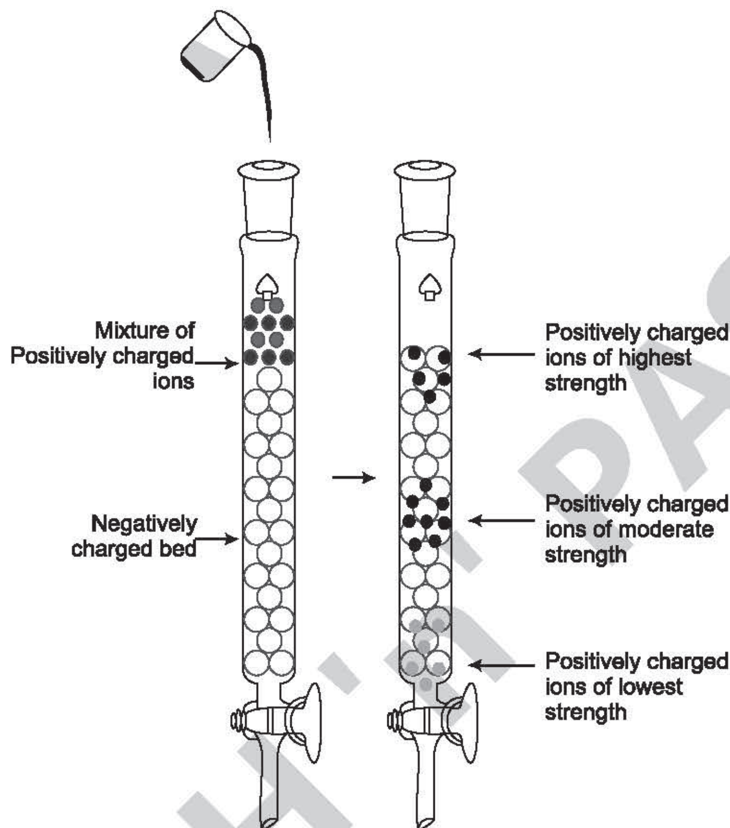

Fig. : Ion exchange chromatography

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Procedure

Ion exchange separations are typically performed in columns including an ion-exchanger. These ionic exchangers are constructed of styrene and divinylbenzene and are commercially accessible. An anionic-exchanger is DEAE-cellulose, while a cationic exchanger is CM-cellulose. The type of exchanger to use is determined by the charge of the particles to be separated. An Anionic Exchanger" is used to separate anions. "Cationic exchanger" is used for different cations. As soon as the column is packed, the solution to be determined is poured on top of it and allowed to travel across the ion exchanger's bed using a syringe or pipette. The chromatogram is created using various mobile phases such as acetate buffers, phosphate buffers, pyridine buffers, 1N NaOH, acetate buffers, and so on.

Ion exchange chromatography elution techniques are divided into two categories :

1. **Isocratic elution :** This method uses alkalis, acids or buffers of equivalent strength.
2. **Gradient elution :** At initially, a mobile phase that is less acidic or basic is used. Following then, there is an increase in acidity or basicity at regular intervals.

Different fractions of the elution are collected and tested for their contents based on volume or time. Several methods of analysis are used depending on the nature and quantity of the ionic species, such as : conductometric method, U.V. spectroscopy, polarography or flame photometric method. A conductivity detector is the most common and beneficial detector in

ion exchange chromatography. Finally, the obtained result is held in reserve. The ion exchange resin may not be used for the next separation experiment after separation; however, it is not supposed to be thrown away due to its high cost. As a result, it's critical to reactivate and regenerate used resins so that they're as effective as new resins.

Applications

The applications of ion exchange chromatography are as follows :

1. It's one of the most effective techniques for separating charged particles.
2. This is the most efficient way for purifying water. Exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions results in complete deionization of water (or) a non-electrolyte solution. This is commonly accomplished through the softening of drinking water approach.
3. Routine analysis of amino acid mixtures.
4. Analysis of products of hydrolysis of nucleic acids.
5. Analysis of lunar rocks and rare trace elements on earth.
6. Togather trace metals from saltwater, chelating resins are utilised.

Advantages/Disadvantages of Ion Exchange Chromatography

This technique has a wide range of advantages, several detection choices, well-developed hardware, reliability with good accuracy and precision, high speed, high selectivity, high separation efficiency, inexpensive consumable costs and good tolerance to sample matrices, large proteins, tiny nucleotides, and amino acids are all examples of charged molecules that can be employed. In the laboratory, ion exchange is utilised for both analytical and preparative objectives, with analytical applications being the most common.

The major disadvantage of this method is that only charged molecules can be separated and requirement of buffer.

Q.6. What is high performance liquid chromatography? Discuss its types, applications, advantages and disadvantages.

Ans.

High-Performance Liquid Chromatography (HPLC)

HPLC is a type of liquid column chromatography that has been greatly improved and widely used in analytical chemistry and biochemistry. It is used to separate a mixture of substances in order to identify, measure, or purify the different components of the mixture. In this technique, liquid is the mobile phase. Instead of allowing a solvent (mobile phase) to flow freely through a column, it is forced through at high pressure. Because the mobile phase is pushed at a high pressure, it produces superior performance and speed when compared to standard column chromatography.

HPLC includes injecting a small volume of liquid sample into a tube packed with tiny particles (3 to 5 microns) in diameter called the **stationary phase**, and moving individual components of the sample along the packed tube with a liquid (**mobile phase**) driven down the column by high pressure produced through a pump. The column packing technique is used to segregate the components. Chemical or physical interactions between segregating components and the packing particles are involved. The separated components are then detected by a detector that detects their amount at the column's exit. A "liquid chromatogram" is the output of this detector. The nature, kind, and size of the packing material, as well as the dimensions of the

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column, are the factors used to describe an HPLC column. Resolution is a metric for column efficiency in all types of chromatography. The separation of solutes is measured by resolution. When compared to traditional column chromatography, the higher resolution in HPLC is mostly due to adsorbents with very small particle sizes and huge surface areas. Because the smaller the particle size, the lower the flow rate, very small gel beads cannot be used in liquid column chromatography because low flow rates result in longer analysis times. Using a pressure difference across the column, greater flow rates can be achieved in HPLC. HPLC's great resolving power and quick analysis time are due to a combination of high pressure and small particle size adsorbents.

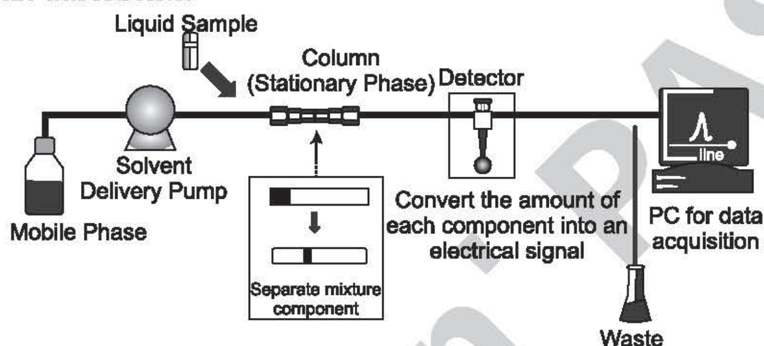

Fig. : Schematic diagram of HPLC

Types of HPLC

HPLC are classified into four types : Normal phase, reverse phase adsorption, partition, ion exchange, size exclusion, displacement, bioaffinity, aqueous normal phase are all chromatographic modes that the HPLC can use. In fact, when certain of these modes are used together, they appear to induce better and highly efficient separation.

1. **Normal Phase HPLC :** In normal phase, the stationary phase (column packing e.g silica) is polar, and the mobile phase is non-polar. Non-ionic and moderately polar chemicals, as well as lipophilic materials that are too strongly retained by reverse phase liquid chromatography, are best separated by this technique. Chiral compounds, cis-trans isomers, geometric isomers and water-sensitive compounds, all are also separated by this method.
2. **Reverse Phase HPLC :** Reverse-phase HPLC entails attaching an organic molecule to a stationary phase, often silica derivatized with alkyl chains, in a highly polar environment (the mobile phase), which may contain water, and then eluting the organic molecule with a gradient of a less polar organic solvent. The most frequent type of HPLC is reversed phase HPLC. The mobile phase is water along with miscible solvent, and the column packing is non-polar *e.g.,* C18, methanol. Non-polar, polar, ionizable, and ionic samples can all be employed.
3. **Ion Exchange HPLC :** The mobile phase is buffer, and the column packing comprises ionic groups. It is used for distinguishing anions and cations, water purification, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides ligand and ion-exchange chromatography of proteins.

4. **Size Exclusion HPLC :** In this technique, molecules diffuse into pores in a porous media and are segregated based on their size in comparison to the pore size. Large molecules elute first, followed by smaller molecules. The tertiary and quaternary structure of proteins and amino acids can be determined using this chromatography. It's also used to figure out what polysaccharide's molecular weight is.

Applications of HPLC

HPLC has evolved into a globally applicable approach, with applications in practically every field of chemistry, biochemistry, and pharmacology.

It is used for both qualitative and quantitative analysis, some of the key applications of HPLC include :

1. Analysis of drugs in pharmaceutical industry.
2. Water purification.
3. Synthetic polymers analysis.
4. Ligand-exchange chromatography.
5. Analysis of air and water pollutants.
6. To ensure the quality of raw materials, it is used in quality control.
7. Drug determination in biological matrices.
8. Non-volatile or thermally unstable chemicals separation and analysis.
9. Biopolymers such as enzymes and nucleic acids separation and purification.
10. Ion-exchange Chromatography of proteins.
11. Pre-concentration of trace components pre concentration.

Advantages, Disadvantages of HPLC

Compared to other chromatographic techniques, such as thin layer chromatography TLC, HPLC is extremely quick and efficient accurate, versatile. When it comes to recognising and quantifying chemical components, it's versatile and incredibly precise. Despite its benefits, HPLC can be expensive due to the massive volumes of pricey organics required.

The other main disadvantages of this technique are complexity, low sensitivity for certain compounds and some compounds are undetectable because they have been irreversibly adsorbed.

Q.7. What do you understand by affinity chromatography also discuss application?

Ans.

Affinity Chromatograhy

Affinity chromatography, unlike most other types of chromatography and procedures like centrifugation and electrophoresis, does not rely on variations in the physical properties of the analytes for separation and purification. To achieve separation and purification, it instead takes advantage of the unique property of extra ordinarily specialised biological interactions. A particular binding relationship between an immobilised ligand and its binding partner underpins this separation approach. Enzyme/inhibitor antibody/antigen, enzyme/substrate interactions are some examples of these types of relationship. As a result, affinity chromatography has the potential to provide absolute purification in a single procedure, even

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from complex mixtures. The method was originally designed to purify enzymes, but it has since been expanded to include nucleic acids, nucleotides, membrane receptors, immunoglobulins and even cell fragments and complete cells.

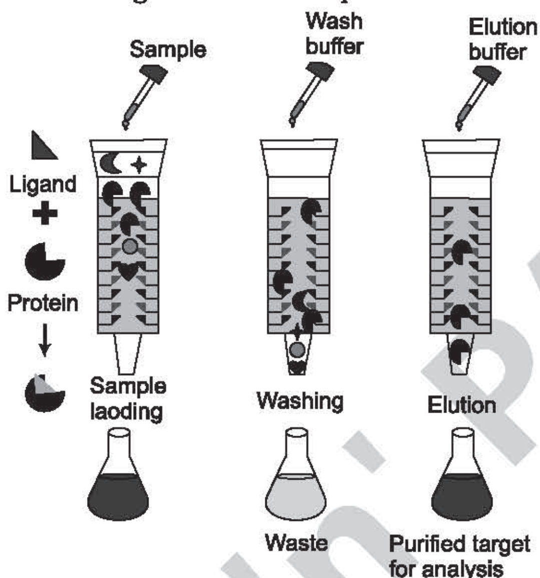

Fig. : Affinity Chromatography

The purified substance is selectively and reversibly absorbed to a ligand (binding substance), which is then immobilised by a covalent link to the chromatographic bed material (*i.e.,* matrix). For their particular binding to the ligand, samples are applied under favourable conditions. As a result, compounds of interest bind to the ligand, whereas unbound substances are washed away. A buffer with a high salt concentration, a pH change, or a competing ligand can then be used to release the molecule of interest from the resin.

Application

The following are some of the most important applications of affinity chromatography :

1. Nucleic acid purification, protein purification from cell free extracts.
2. It's employed in the purification of immunoglobulins.
3. Chiral compounds analysis.
4. By using recombinant tagged proteins in this technique, proteins can be purified.
5. It's also utilised to figure out how drugs and hormone proteins interact with each other.
6. Detection of substrates.
7. Binding sites of enzymes investigation.
8. Single nucleotide polymorphisms and mutations in nucleic acids detection.
9. In purification process or removal of impurities.
10. In in vitro antigen-antibody reactions.

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UNIT-VII

Spectrophotometry and Biochemical Techniques

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. What is pH and its importance?

Ans. pH is a measure of how acidic/basic water is. The range goes from 0 to 14, with 7 being neutral. pH of less than 7 indicate acidity, whereas a pH of greates than 7 indicates a base. pH is really a measure of the relative amount of free hydrogen and hydroxyl ions in the water. The pH can control the availability of nutrients, biological femetions, microbial activity and the behaviour of chemicals.

Q.2. Who discovered pH scale?

Ans. In 1909 Sorensen, a Danish chemist, introduced the concept of pH as a convenient way of expressing acidity.

Q.3. Why pH is not more than 14.

Ans. The general definition of a solution's pH value is the negative base –10 log of the solution's hydronium ion concentration. While there is no more than 1 M of hydroxide ions on the other hand, this results in a pH value not exceeding 14.

Q.4. What is a buffer solution?

Ans. Buffer solution is just like a water based solution that consists of a mixture that contains a weak and a conjugate acid of the weak base. They help to resist the change in pH on dilution or by addition of small amounts of acid or alkali to them.

Q.5. Why do we need to use buffers?

Ans. The purpose of a buffer in a biological system is to maintain intracellular and extracellular pH within a very narrow range and resist changes in pH in the preserve of internal and external influences.

Q.6. What is meant by colorimetry?

Ans. Colorimetry is a scientific technique that is used to determine the concentration of coloured compounds in solutions by the application of the Beer-Lambert law, which states that the concentration of a solute the proportional to the absorbance.

Q.7. Who introduced colorimeter?

Ans. Iules Duboseq (1817-86), a French optical instrument maker, invented this type of colorimeter in 1854.

Q.8. What are the units for Beer's law?

Ans. In UV spectroscopy, the concentration of the sample solution is measured in mole L^{-1} and the length of the light path in cm. Thus, given that absorbance is unitless, the units of molar absorptivity are $L\ mol^{-1} cm^{-1}$.

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Q.9. What are limitations of colorimeter?

Ans. One limitation of the chemical colorimeter is that some substances have variances that can cause an inaccurate test result. As these variance are different for every substance, the chemical colorimeter alone is not a completely fool proof testing device, according to global water instrumentation.

Q.10. What is the basic principle of spectrophotometry?

Ans. The basic principle of spectrophotometry is that it measures the concentration of substance by measuring the amount of absorption of transmission of light at a particular wavelength.

Q.11. What is the unit of spectrophotometer?

Ans. Most spectrophotometers have a scale that reads both in O.D. (absorbance) units, which is a logarithmic scale and in % transmittance, which is an arithmetic scale.

Q.12. What are the three main components of a spectrophotometer?

Ans. A spectrophotometer consist of three primary components : a light source, optics to deliver and collect the light and a detector.

Q.13. What is a radiotracer and how does it work?

Ans. Radioactive tracer are used in imaging test that help find problems inside the body. These tracers give off particles that can be detected and turned into a picture to help find problems in organs or other structures. The tracer is usually given through an intravenous (IV) line placed in a vein.

Q.14. What is an example of a traces used in medical tracer?

Ans. A good example of a ratioactive isolope used in a medical tracer is technetium –99 m. This excited state of the isotope technetium –99 unit low-energy gamma radiation and has a half-life of 6 hrs, making it excellent for use in a medical tracer.

SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. Write a short note on the pH scale?

Ans.

pH Scale

The degree of acidity or alkalinity of a solution is measured in pH, which is a unit of measurement. On a scale of 0 to 14, it is rated. **Soren Sorensen**, a Danish biochemist, used a logarithmic scale to express the concentration of H+ in 1909. This scale was known as pH, where, "p", which stands for power, and "H", stands for hydrogen ion concentration. He defined pH as the negative logarithm of hydrogen ion concentration (in moles/litre) in a solution. Thus,

$$pH = \log \frac{1}{[H^+]} = -\log [H^+]$$

By quantifying the degree of activity of an acid or base in terms of hydrogen ion activity, pH gives important quantitative information. The ratio of hydrogen ion (H^+) to hydroxyl ion $[OH^-]$ concentrations determine the pH value of a substance. The material is acidic if the H^+ concentration is more than the OH^- concentration, i.e. the pH value is less than 7. The material is basic if the OH concentration exceeds the H^+ concentration, resulting in a pH value greater

than 7. The material has a pH of 7 if there are equal numbers of H^+ and OH^- ions present then it will be neutral in nature. Free hydrogen and hydroxyl ions are present in acids and bases, respectively. Because the link between hydrogen ions and hydroxyl ions in a solution remains constant for a given set of conditions, knowing one can help you figure out the other. Even though pH is a selective assessment of hydrogen ion activity, it is a measurement of both acidity and alkalinity. Because pH is a logarithmic function, a ten-fold change in hydrogen ion concentration corresponds to a one-unit change in pH. Colorimetric methods, which use indicator fluids or papers, and electrochemical methods, which use electrodes and a millivoltmeter (pH meter), are the two methods for measuring pH.

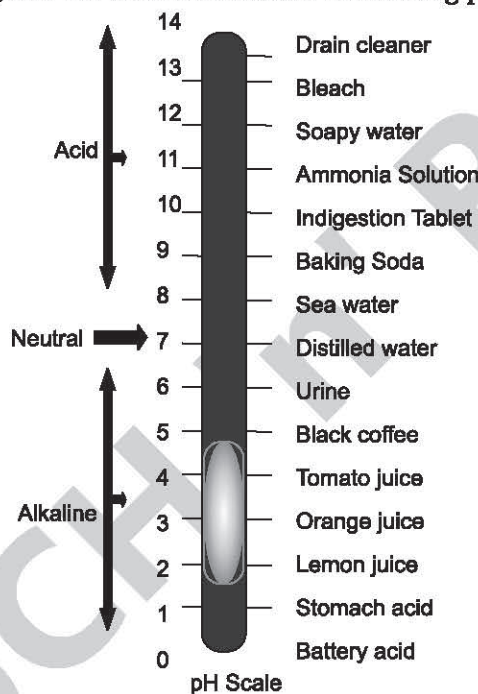

Fig. : pH Scale

Q.2. Write short note on the biological buffers with general criteria.

Ans. **Biological Buffers**

A biological buffer is an organic material that acts as a hydrogen ion neutralizer. A biological buffer works in this way to keep the body's pH stable so that biochemical processes can continue to run smoothly.

In a biological system, the aim of a buffer is to keep intracellular and extracellular pH within a small range and resist pH changes in the presence of internal and external forces. Biomolecules are kept in their ideal ionic condition by maintaining a precise and stable cytosolic pH, which is normally approximately pH 7. The pH of extracellular fluids (blood, for example) is similarly strictly regulated in multicelled organisms. Biological buffers, which are combinations of weak acids and their conjugate bases, are used to maintain pH consistency.

The buffer systems of bicarbonate, phosphate, protein, amino acids, and haemoglobin are some of the most essential buffer systems in body fluids that assist maintain pH. Many of these

are normally present in little amounts in the body and cellular fluids, therefore maintaining a steady pH requires a sophisticated mechanism.

Some Important Biological Buffer Solution

Body Fluid	Blood	Bicarbonate Buffer System Haemoglobin Buffer System, Protein Buffer System.
	Intracellular Fluid	Phosphate Buffer System, Protein Buffer System.
	Interstitial Fluid	Bicarbonate Buffer System.

General Criteria for Biological Buffers

The general criteria for biological buffers are as follows :

1. Their pKa value should be in the range of 6.0 to 8.0.
2. They must not pass-through cell membranes.
3. They should have a high solubility in water and a low solubility in organic solvents.
4. They should not be hazardous to cells in any way.
5. Although the salt effect should be low, salts can be added as needed.
6. They must not obstruct any biological processes.
7. Buffers should be unchanging and resistant to enzymatic degradation.
8. Buffer should not absorb in the visible or ultraviolet ranges.
9. Ionic composition of the medium and temperature should have negligible effect on buffering capacity.

Q.3. What are the differences between colorimeter and spectrophotometer?

Ans. Differences between Colorimeter and Spectrophotometer

Colorimetry and spectrophotometry are both quantitative methods for determining how much material is present in a sample. Colorimetry and spectrophotometry differ in that colorimetry employs fixed wavelengths that are only observable in the visible spectrum, whereas spectrophotometry can use wavelengths from a larger range.

Furthermore, a colorimeter quantifies colour by measuring the three major colour components of light (red, green and blue), but a spectrophotometer measures the accurate colour in human-visible light wavelengths. Furthermore, a colorimeter measures the quantity of light that passes through a sample, whereas a spectrophotometer measures the amount of light that goes through it. Colorimeter provides psychophysical analysis by measuring colour in the same way as the human eye and brain do when visualising things. While a spectrophotometer produces a physical analysis that can be used to acquire colorimetric data indirectly.

Q.4. Write a short note on the basic principle of colorimetry and spectrophotometry.

Ans. Basic Principle of Colorimetry/Spectrophotometry

When a beam of incident light goes through a solution, it is said to be passing through. It is possible for a ray of light to be absorbed by some materials while passing through unaffected by others. When a molecule absorbs light, the energy from the ray of light is transferred to the

molecule. Light will be absorbed if the frequency of the electrical and magnetic fields of a beam of light matches the frequency at which molecules vibrate; if the frequency does not match, the light will pass through unchanged. Because of how they affect light illuminating the object, inert molecules seem coloured. As a result, different objects absorb and reflect different wavelengths. When white light flows through a blue solution, for example, it absorbs all hues except blue.

Colorimeter's principle is based on the photometric approach, which claims that when an incident light of intensity (I_0) passes through a solution, then,

$$I_0 = I_r + I_a + I_t$$

where, I_0 is the intensity of incident light

I_r is the intensity of reflected light

I_t is the intensity of transmitted light

I_a is the intensity of absorbed light

Intensity of reflected light I_r is not measured in photometers (spectrophotometers and colorimeters) because the measurements of I_0 and It are adequate to determine the I_a. The amount of light reflected (I_r) is kept consistent for this purpose by using cells with identical characteristics. The values of (I_0) and (I_t) are then calculated.

The two fundamental Laws of photometry, on which the Spect, photometer is based, show the mathematical link between the amount of light absorbed and the concentration of the substance. These two fundamental laws of photometry are Lambert's Law and Beer's Law which observes quantitative photometric measurement of the absorption process. Lambert's law describes how much light is absorbed and how far it travels through an absorbing medium, while Beer's law describes how much light is absorbed and how concentrated the absorbing substance is.

Q.5. Describe briefly the radioisotope tracers and its application.

Ans.

Radioisotope Tracers

A radioactive tracer is a chemical molecule in which a radioisotope has replaced one or more atoms. In nature, there are two types of isotopes: stable isotopes and unstable isotopes (radioisotopes). Stable isotopes do not breakdown into other isotopic or elemental forms and keep their elemental integrity. eg., carbon-12, hydrogen-1, nitogen-15, oygen-16 etc. The biologically important stable isotopes are available in enhanced concentrations.

Unstable or radioactive isotopes disintegrate into other isotopes of the same or different elements by emitting nuclear particles (alpha particles, beta particles, X-rays, gamma rays,). Some examples of radioactive isotopes are carbon-14, hydrogen-3 (tritium), chlorine-36 etc.

Hydrogen, phosphorus, carbon, sulphur and iodine radioisotopes have all been employed to trace the path of biological reactions. A radioactive tracer can also be used to monitor the distribution of a material within a natural system, such as a tissue or cell, or as a flow tracer to monitor fluid movement. PET scans, SPECT scans, and technetium scans are all examples of imaging methods that use radioactive tracers. Radioactive tracers are also utilised in natural gas production to locate fractures caused by hydraulic fracturing. The naturally occurring carbon-14 isotope is used as an isotopic marker in radiocarbon dating.

Application of Radiotracer

The applications of radiotracer are as follows :

1. Radiotracer compounds of hydrogen, carbon, phosphorus, sulphur, and iodine have all been employed to chart the path of biological reactions.

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2. In the production of natural gas, radioactive tracers are also employed to pinpoint the location of fractures generated by hydraulic fracturing. For each stage of hydraulic fracturing, tracers of different half-lives of are utilised.
3. A radioactive tracer can also be used to follow the distribution of a substance within a natural system, such as a cell or tissue, or as a stream tracer to follow fluid flow.
4. Tracers are used in a variety of medical tests. PET (positron emission tomography) scans, SPECT (single-photon emission computed tomography) scans, and technetium scans are just a few of the imaging systems that use radioactive tracers. To identify helicobacter pylori infection, a dosage of 14C labelled urea was often employed in the urea breath test.
5. Tritium and 14C-labeled glucose are extensively employed in glucose clamps to assess glucose uptake, fatty acid synthesis, and other metabolic processes in metabolism research.
6. Great tool for genetic studies, microbial ecology, radiopharmaceutical research etc.

Viruses can be studied using radioimmunoelectro phoresis.

Q.6. Briefly explain preparation of some important buffers in biological science.

Ans.

Preparation of Some Important Buffers

The process of preparation of some important buffers is as follows :

Phosphate Buffer Solution : It is discussed in the following points :

1. **Reagents :** 0.1 M Sodium phosphate monobasic; 13.8 g/l (monohydrate, MW. 138.0) and 0.1M Sodium phosphate dibasic; 26.8 g/l (heptahydrate, M.W. 268.0)
2. pH range 5.8 to 8.0.
3. Adjust the final volume to 200 ml with deionized water after mixing sodium phosphate monobasic and dibasic solutions in the quantities indicated. Using a sensitive pH metre, adjust the ultimate pH.

Carbonate-Bicarbonate Buffer Solution : It is discussed in the following points :

1. **Reagents :** 0.1 M Sodium carbonate (anhydrous), 10.6 g/l (M.W.: 106.0 and 0.1 M Sodium bicarbonate, 8.4 g/l (M.W.: 84.0).
2. pH range 9.2 to 10.6.
3. Adjust the final volume to 200 ml with deionized water after mixing the sodium carbonate and sodium bicarbonate solutions in the quantities indicated. Using a sensitive pH metre, adjust the ultimate pH.

Hydrochloric Acid-Potassium Chloride Buffer Solution : It is discussed in the following points :

1. **Reagents :** 0.1 M Potassium chloride : 7.45 g/l (M.W.: 74.5) and 0.1 M Hydrochloric acid.
2. pH Range 1.0 to 2.2.
3. Combine 50 mL potassium chloride and the amount of hydrochloric acid specified. With deionized water, mix and adjust the final volume to 100 mL. Using a sensitive pH metre, adjust the ultimate pH.

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Citrate Buffer Solution : It is discussed in the following points :

1. **Reagents :** 0.1 M Citric acid: 19.21 g/l (M.W.: 192.1) (b) 0.1 M Sodium citrate dihydrate: 29.4 g/l (M.W.: 294.0).
2. pH range 3.0 to 6.2.
3. Adjust the final volume to 100 ml with deionized water after mixing citric acid and sodium citrate solutions in the quantities indicated. Using a sensitive pH metre, adjust the ultimate pH. The use of sodium citrate pentahydrate salt is not advised.

Glycine-Sodium Hydroxide Solution : It is discussed in the following points :

1. **Reagents :** 0.1 M Glycine; 7.5 g/l (M.W.: 75.0) and 0.1 M Sodium hydroxide; 4.0 g/l (M.W.: 40.0).
2. pH range 8.6 to 10.6.
3. Adjust the final volume to 200 ml with deionized water after mixing 50 ml of glycine and the required volume of sodium hydroxide solutions. Using a sensitive pH metre, adjust the ultimate pH.

Acetate Buffer Solution : It is discussed in the following points :

1. **Reagents :** 0.1 M Acetic acid (5.8 ml made to 1000 ml) and 0.1 M Sodium acetate; 8.2 g/l (anhydrous; M.W. 82.0) or 13.6 g/l (trihydrate; M.W. 136.0).
2. pH range 3.6 to 5.6.
3. Adjust the final volume to 100 ml with deionized water after mixing the acetic acid and sodium acetate solutions in the quantities indicated. Using a sensitive pH metre, adjust the ultimate pH.

Q.7. Discuss a brief description of safety measures of radiotracer techniques.

Ans.

Safety Measures of Radio-Tracer Techniques

Some safety measure of radio-tracer techniques are as follows :

1. The radioactive activity, energy and nature of emitted radiation, radiotoxicity and half-life should all be considered when choosing a radioisotope for any radiotracer investigation.
2. When possible, additional shielding should be employed to decrease the radiation worker's/medical practitioner's exposure.
3. In shipping containers, radiotracers are provided based on the radioisotope's characteristics.
4. During the tracer preparation stage and during the tracer injection, protective gear and equipment should be available.
5. At the workplace, monitoring and disinfection equipment should be provided.
6. Radiotracer studies can take many days to complete. A temporary storage facility for radioactive sources should be accessible near the field of operation as a result of this arrangement. The utilisation of regulated spaces for temporary storage of radiotracers is frequently required.

SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. What is pH meter? Explain in details the working principle, measurement, standardization and calibration process of pH meter.

Ans.

pH Meter

A pH metre is a precision tool that measures the hydrogen ions activity in water-based solutions and displays the acidity or alkalinity as pH. A pH metre is made up of a voltmeter

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connected to a pH-responsive electrode and a reference (constant) electrode. The pH-responsive electrode is normally made of glass, and the reference electrode is usually made of silver-silver chloride, however a mercury-mercurous chloride (calomel) electrode is also used occasionally. It is also called as "potentiometric pH meter" because it detects the difference in electrical potential between a pH electrode and a reference electrode. The glass electrode concept was first articulated in 1909 by Nobel Laureate Fritz Haber (1868-1934) and his pupil Zygmunt Klemensiewicz (1886-1963). Arnold Beckman (1900-2004), an American chemist, invented the current electronic pH metre in 1934.

Working Principle and Measurement of pH

The measuring electrode, reference electrode, and temperature sensor are all essential components of a pH metre. The pH Meter calculates the voltage of an electrochemical cell and determines the pH of a suspension using the temperature sensor. The majority of pH metres use combination electrodes, which combine the electrodes and the temperature sensor into a single frame. The total of the potentials of the measuring electrode, reference electrode, and liquid junction is the overall potential or voltage. The reference electrode has a fixed concentration of a neutral solution, such as potassium chloride solution. It produces a constant voltage.

On the other hand, the measuring electrode's potential is entirely determined by the pH of the suspension. The potential difference (voltage) between a measuring electrode glass membrane and a reference electrode immersed in the sample liquid to be studied is calculated. When the two electrodes are immersed in the sample suspension, an ion-exchange process occurs, in which some hydrogen ions flow towards the outside surface of the measuring electrode, displacing some of the metal ions within it. Some metal ions travel from the glass electrode to the sample suspension in the same way. The reference electrode potential is either unaffected by pH variations or has a minor response to pH variations, resulting in a steady voltage.

Ion-exchange processes from the sample suspension also occur on the inside surface of the glass electrode, resulting in a potential difference (Hydrogen-ion activity) between them. The liquid junction potential is generally minute and relatively constant, and it is mostly determined by the strength of the ions in the sample solution. The high impedance voltmeter adds up and ranks each of the three potentials. Temperature affects the potential voltage generated beyond the glass electrode membrane. Automatic temperature compensation is a feature of pH metres that allows them to improve pH readings as the temperature changes. The impedance voltmeter's output is voltage studies, and it must be calibrated in order to prepare correct pH measurements. The measuring electrode is calibrated by immersing it in a buffer liquid with a known pH, which aids in comprehending millivolt readings as pH measurements of the sample suspension at the delivered temperature.

Standard Procedure and Calibration of pH Meter

By pressing the ON button on the pH metre, it will turn on the pH meter. The MEAS annunciator and automatic temperature compensation indicator will appear on the LCD after turning on the pH metre. After that, the electrodes should be cleaned with distilled water. Maintain a temperature of 25°C for the sample. The electrodes were then immersed in the sample and stirred to achieve a homogeneous sample. Make sure the electrode's tip is completely immersed in the sample. Wait for the reading to stabilise. The READY indicator will turn on once the reading has stabilised. After that, press the HOLD key to freeze the

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reading and then the ENTER key to store it. Make a note of the pH and temperature. Finally, rinse the electrodes in distilled water before storing them in the buffer solution.

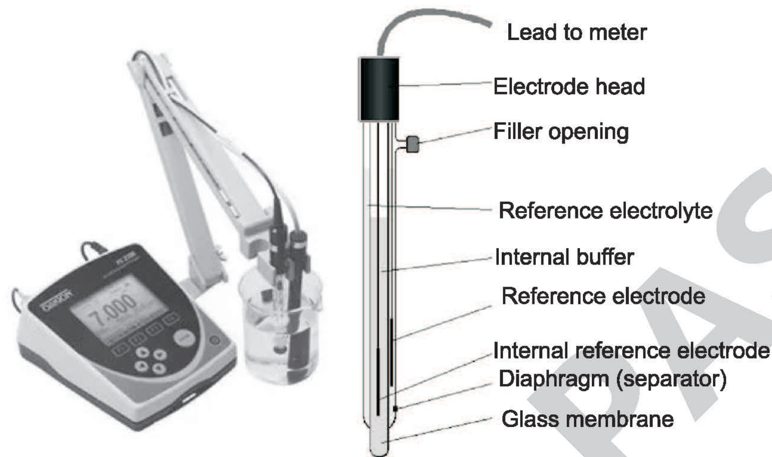

Fig. : pH meter and diagram of pH combination electrode

The pH metre must be calibrated before each test in order to produce very accurate results. Calibration is also performed once per day of work. Because the glass electrode does not give repeatable electrostatic potentials over prolonged periods of time, calibration is required. Check that the pH metre is set to the correct measurement mode. Then, using distilled water, wash the electrodes without wiping them; otherwise, an electrostatic charge will form on the electrodes. Submerge the electrodes in the standard buffer or calibration solution of pH 7/pH 10. Make sure the electrodes' endpoints are completely immersed in the buffer solution. Stir the electrodes in the buffer to make a homogeneous sample. To enter pH calibration mode, press the CAL/MEAS key. The CAL symbol will be shown. The primary screen will display the measured value, while the secondary screen will display the pH standard buffer solution reading. Wait until the pH levels are stable. After stabilisation, use the HOLD/ENTER key to validate the calibration. The pH metre is now calibrated to the buffer solution in use. Lastly, rinse the electrodes in pure water before placing them in the buffer solution.

Q.2. What is buffer solution? Explain the Henderson-Hassel batch equation also mention the term buffer capacity.

Ans.

Buffer Solution

A buffer solution is one that does not change pH when an acid or base is added. Many biological substances are weak electrolytes whose ionic status varies with pH, therefore they are crucial in actual biochemical operations. During the course of a practical experiment, the ionic state must be stabilised in order to maintain stable environmental conditions. A buffer solution is an aqueous mixture of a weak acid and its conjugate base in practise. Any hydrogen ions formed during an experiment will be neutralised by the conjugate base component, whereas any base generated will be neutralised by the unionised acid. The Henderson-Hasselbalch equation is crucial in buffer solution preparation since it allows for the estimate of the pH of a buffer solution using the acid dissociation constant Ka and the molar concentrations of the buffer pair's components :

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$$pH = pKa + \log \frac{[\text{conjugate base}]}{[\text{weak acid}]}$$

For a buffer based on a weak base's conjugated acid

$$pH = pKa + \log \frac{[\text{weak acid}]}{[\text{conjugate base}]}$$

A buffer solution resists a change in pH more successfully than an equal volume of water when acid (H^+) or base (OH^-) is added. The buffer solution is often made up of a weak bronsted acid and any conjugate base; for example, buffer solutions can be made up of acetic acid and sodium acetate or ammonium hydroxide and ammonium chloride. Almost every biological function is pH-dependent; a little change in pH causes the pace of the process to shift dramatically. This is true not just for the numerous reactions in which the H^+ ion plays a direct function, but also for those in which H^+ ions appear to play no role at all. Ionizable groups with certain pk values are found in enzymes and many of the compounds on which they function. Protonated amino ($-NH_3^+$) and carboxylic groups of amino acids, as well as the phosphate group of nucleotides, are weak acids whose ionic state is determined by the pH of the solution in which they are dissolved.

The Buffer Capacity

The Henderson-Hasselbalch equations show that when the concentrations (or, more precisely, activities) of the weak acid and base are equal, the ratio is 1 and the logarithm is 0, resulting in pH = pKa. Buffer capacity measures a solution's ability to tolerate pH fluctuations by absorbing or desorbing H^+ and OH^- ions. The effect of adding an acid or base to a buffer system on pH change can be considerable or modest. depending on both the starting pH and the buffer's ability to resist change in pH. Buffer capacity (B) is a unitless number defined as the number of moles of acid or base required to change the pH of a solution by one, divided by the pH change and the volume of buffer in litres. Through buffer consumption, a buffer resists pH changes caused by the addition of an acid or base. The pH will not change significantly if the buffer has not been thoroughly reacted. As the buffer is depleted, the pH change will become more pronounced (or less pronounced): it will be less resistant to change.

The pKa values of some acids/bases and their conjugate base/acid used as buffer solutions

Acid/Base Name and formula	pKa	Conujugate base/acid and formula	Uses
Acetic acid (CH_3COOH)	4.8	Acetate ion (CH_3COO)	In the purification and precipitation of nucleic acids, protein crystalization. Staining of gels in protein gel electrophoresis and HPLC.
Barbituric acid ($C_4H_4N_2O_3$)	4.0	Barbiturate ($C_4H_3N_2O_3$)	Solutions of sodium barbital used in immune-electrophoresis or in fixative solutions.
Carbonic acid (H_2CO_3)	6.1, 10.2	Bicarbonate (HCO_3)	Carbonic acid- bicarbonate buffer system is the most important buffer for maintaining the pH homeostasis of blood.

TOUCH in PASS

CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) ($C_9H_{19}NO_3S$)	10.4	2-(Cycloheptylamino) ethanesulfonate ($C_9H_{18}NO_3S$)	Capillary electrophoresis, diffusion blotting, electroblotting, western blot, identification and sequencing of peptides and proteins with high isoelectric points.
MES (2-N-morpholino) ethanesul fonic acid ($C_6H_{13}NO_4S$)	6.1	2-(N-morpholino) ethanesulfonate ($C_6H_{12}NO_4S$)	Zwitter ionic, morpholinic buffer used in cell culture media, as a running buffer in electrophoresis, and for protein purification in chromatography.
MOPS (3-N-morpholino propanesulfonic acid) ($C_7H_{15}NO_4S$)	7.1	3-(N-morpholino) propanesulfonate ($C_7H_{14}NO_4S$)	Used in polyacrylamide gel electrophoresis.
PIPES [piperazine-N, N'-bis (2-ethanes ulfonic acid)] ($C_8H_{18}N_2O_6S_2$)	6.8	2-[4-(2-sulfoethyl) piperazin-1-yl] ethanesulfonate ($C_8H_{17}N_2O_6S_2$)	Cell culture, cosmetics, chromatography, diagnostic tests, electron microscopic study and protein purification.
TAPS [Tris hydroxy methyl) methylamino] propanes ulfonic acid ($C_7H_{17}NO_6S$)	8.4	TAPS(1-) ($C_7H_{16}NO_6S$)	Used in capillary electrophoresis to analyze DNA, in planar chromatography to separate dyes and in culture media.

Q.3. What do you understand by colorimetry? Describe in detail the instrumentation, working and application of colorimeter.

Ans.

Colorimetery

Colorimetry is a science that uses numerical measures rather than subjective responses to determine colour. It has a significant impact on colour conception, interpretation, and perception in all domains. This technique is widely used for determination of the wavelength and intensity of electromagnetic radiation in the visible spectrum. It's a popular method for detecting and quantifying light-absorbing substances. This is accomplished by sending visible spectrum light of a specified wavelength through the solution in a photoelectric colorimeter equipment and observing the galvanometric reading of reflection, which sensitises the amount of light absorbed.

A colorimeter is a light-sensitive instrument that measures the amount of light that passes through a liquid sample and its transmittance and absorption. When a specific reagent is introduced into a solution, the colorimeter instrument also measures the colour concentration or intensity that develops.

Instrumentation of Colorimeter

Any colorimeter, simple or complex, is made up of five essential parts :

1. **Light source :** Tungsten filaments are the most frequent light source in colorimeters.
2. **Monochromator :** Filters or monochromators are used to separate the light from the light source to pick a specific wavelength.
3. **Sample holder :** Color solutions are held in test tubes or cuvettes, which are composed of glass at visible wavelengths.
4. **Photo Detector System :** An electric current is generated when light falls on the detection system, which reflects the Galvanometer reading.
5. **Measuring device :** The current from the detector is routed into the Galvanometer, which displays a metre reading that is directly proportional to light intensity.

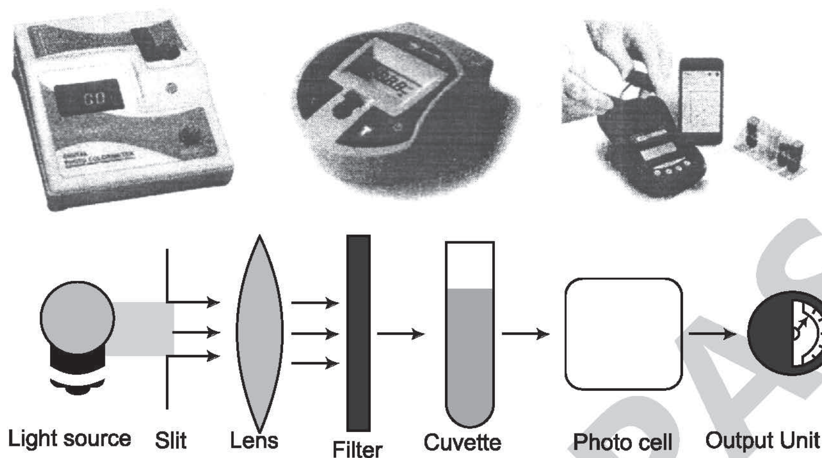

Fig. : Colorimeter

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Working Process of Colorimeter

A ray of light with a specific wavelength is directed towards a solution when a colorimeter is used. The ray of light goes through a succession of lenses before reaching the solution. These lenses are employed in the colorimeter to navigate coloured light. The colorimeter examines reflected light and compares it to a set of standards. The absorbance of light by the solution is then calculated using a microprocessor integrated in the device. More light will be absorbed by the solution if the absorption of the solution is higher, and more light will be transmitted through the solution if the concentration of the solution is low.

As previously stated, the colorimeter is based on **Beer-law, Lambert's** which states that the absorption of light passed through a medium is proportional to the concentration of the medium.

$$A = \in c\ I$$

Where A is the absorbance (no units), ismolar extinction co-efficient in L/mol/cm, c is the concentration of the compound in solution (expressed in mol $L-1$) and l is the length that the light passes through (expressed in centimeters).

In colorimeter if the solution is constantly cnanging, the percentage of transmittance, against time is commonly utilised. The amount of light absorbed to determine concentrations is proportional to the amount of solute. A higher concentration of dissolved solute in a solution means more light is absorbed, and *vice-versa*; thus, the concentration can be deduced from the absorption of specific wavelengths. Several sample solutions of known concentration are made and evaluated before determining the concentration of an unknown sample. After that, the concentrations are plotted against. absorbance on a graph, yielding a calibration curve. To calculate the concentration, the results of the unknown sample are compared to those of the known sample on the curve.

Applications of Colorimeter

The following are some of the most important applications colorimeter :

1. A colorimeter is a device that measures the optical density or absorbance of a coloured chemical to determine its concentration.

2. It screens substances including chlorine, fluoride, cyanide, dissolved oxygen, zinc, iron, and molybdenum and hydrazine to determine water quality.
3. It's used to analyse proteins, glucose, and other biological substances quantitatively.
4. It can also be used to determine the levels of plant nutrients in the soil (such as phosphate, ammonia and nitrate,) or haemoglobin in the blood.
5. It's used to make paints.
6. It's utilised in the textile and food industries.
7. It's used to calculate biochemical samples including urine, cerebrospinal fluid, plasma, and serum in laboratories and hospitals.
8. It can also be used to determine the reaction's progress by observing the rate of production and disappearance of the light-absorbing compound in the visible spectrum of light.

Q.4. What is spectrophotometer? Explain its instrumentation, working process and applications.

Ans.

Spectrophotometry

The quantitative measurement of a material's reflection or transmission qualities as a function of wavelength is referred to as spectrophotometry, which is a branch of electromagnetic spectroscopy modern spectrum, including ultraviolet, x-ray, visible, microwave and infrared wavelengths.

The instrument used in this technique is called spectrophotometer. It is made up of two instruments : a spectrometer and a photometer. The spectrometer produces the desired wavelength of light because different compounds absorb best at different wavelengths. For example, *p*-nitrophenol (acid form) has maximum absorbance of approximately 320 nm and *p*-nitrophenolate (basic form) absorbs best at 400 nm. The photometer detects the amount of photons that is absorbed after the desired wavelength of light passes through the sample solution in the cuvette.

Instrumentation of Spectrophotometer

There are two types of Spectrophotometers; **single beam** and **double beam** spectrophotometer. A single beam spectrophotometer uses a single beam of light to operate between the wavelengths of 325 nm and 1000 nm. The test solution and blank are both read in the same direction as the light. The wavelength range of a double beam spectrophotometer is 185 nm to 1000 nm. It is equipped with two photocells. The light from the monochromator is separated into two beams by this device. One beam serves as a reference, while the other is used to read samples. It eliminates the inaccuracy caused by fluctuations in the light output and the detector's sensitivity.

All spectrophotometers consist of following basic parts :

1. **Light source :** Three distinct light sources are often employed in spectrophotometers to create light of different wavelengths. A tungsten lamp is the most frequent light source used in spectrophotometers for the visible spectrum. The hydrogen lamp and the deuterium lamp are popular sources of ultraviolet radiation. The most suitable sources of *IR* (Infrared) radiation are Nernst filament or globar.

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2. **Monochromator :** A prism or diffraction grating splits the light from the light source to pick a specific wavelength.
3. **Sample holder :** The coloured solutions are held in test tubes or cuvettes. At a visible wavelength, they are made of glass.
4. **Beam splitter :** It's only found in spectrophotometers with two beams. It is used to divide a single light beam from a light source into two beams.
5. **Mirror :** It's also only found in spectrophotometers with two beams. It is utilised to steer the splitted light from the beam splitter in the proper direction.
6. **Photodetector system :** When light strikes the detection system, it generates an electric current, which reflects the Galvanometer reading.
7. **Measuring device :** The current from the detector is supplied into the Galvanometer, which acts as a measuring instrument. The metre reading is related to the amount of light present.

Working Process of Spectrophotometer

A spectrophotometer must first be calibrated before it can be used. This is accomplished by employing standard solutions containing the known concentration of the solute to be determined in the test solution. The standard solutions are put into cuvettes and placed in the cuvette holder of a spectrophotometer comparable to a colorimeter for this. A ray of light with a specified wavelength for the test is aimed at the solution. The ray of light goes through a succession of diffraction gratings, prisms, and mirrors before reaching the solution. The prism splits the beam of light into multiple wavelengths, and the diffraction grating allows the desired wavelength to pass through it and reach the cuvette containing the standard or test solutions, and these mirrors are utilised for light navigation in the spectrophotometer. It examines the reflected light and compares it to a set of established standards. When monochromatic light (light with a single wavelength) strikes the cuvette, some of it is reflected, some is absorbed by the solution, and the rest is transmitted through the solution and lands on the photodetector system.

The photodetector system converts the intensity of transmitted light into electrical impulses for transmission to the galvanometer. The Galvanometer detects electrical signals and converts them to digital data. The absorbance or optical density of the investigated solution is the digital representation of the electrical signals. If the solution absorbs more light, more light is absorbed by the solution, and if the solution absorbs less light, more light is transmitted through the solution, affecting the Galvanometer reading and corresponding to the concentration of the solute in the solution. The concentration of the solution can be easily determined by plugging all of the data into the formula given in the following section. Beam splitters are used in double beam spectrophotometers to split monochromatic light into two beams, one for the standard solution and the other for the test solution. The absorbance of standard can be compared to a single standard in this way. It produces more exact and accurate findings, and it reduces errors caused by changes in the and the any number of test solutions light output and the sensitivity of the detector can be measured at the same time.

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Applications of Spectrophotometer

Some of the most important applications of spectrophotometer are as follows :

1. A spectrophotometer is a device that measures the concentration of colourless or coloured solutes in a solution.
2. In the pharmaceutical business, UV-spectrophotometers are used to determine drug composition.
3. It is used to calculate the rate of reaction by monitoring the formation and disappearance rates.
4. Impurities detection.
5. In hospitals for respiratory gas analysis spectrophotometer is used.
6. In both the pure state and biological preparations, the visible and UV-spectrophotometer can be utilised to identify classes of chemicals.
7. Organic compound's structure elucidation.
8. In forensic sciences.
9. A spectrophotometer is used to examine the blood.
10. In the foods and paints industry it is also used.
11. Determining the amount of dissolved oxygen in a body of water.
12. In molecular biology spectrophotometer can be used to measure the development of microorganisms such as bacteria.

Q.5. What is Beer-Lambert's law? Explain the derivation, applications and limitations of this law.

Ans.

Beer-Lambert's Law

Following are the major definitions of Beer-Lambert's Law :

Lambert's Law or Bouguer's Law : Bouguer (1729) and **Lambert** (1760) originally developed the relationship between radiation absorption and the path length of the absorbing medium. This law states that when a monochromatic light beam passes through a homogeneous absorbing medium, the rate of decrease in radiation intensity with absorbing medium thickness is proportional to the incident medium intensity.

Beer's Law : August Beer, a German mathematician and scientist, devised this law in 1852 to describe the link between radiant energy absorption by an absorbing media. This law states that the amount of light absorbed is directly proportional to the concentration of the solute in the solution.

Derivation of Beer-Lambert Law

When material things are subjected to radiation, a portion of it is absorbed, a portion is scattered, and a portion is transmitted. The intensity of light travelling through material bodies, i.e. the intensity of transmitted light, diminishes as a result of absorption. The thickness of the absorbing media determines how much of the incident light is absorbed. Lambert discovered a quantitative relationship between the intensity of light I and the decrease in intensity of monochromatic light owing to passage through a homogeneous medium of thickness dx. The decrease in intensity of light with thickness of the absorbing

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media at any location is directly proportional to the intensity of light, according to Lamberts law.

Beer lambert law can be represented mathematically as :

$$-\frac{dI}{dx} \alpha I \quad ...(1)$$

Where dI is the intensity of monochromatic light immediately before entering the medium, and I is the intensity of monochromatic light just before entering the medium.

Equation (1) may be written as :

$$-\frac{dI}{dx} = \alpha I \quad ...(2)$$

In equation (2), – ••••/••••is the rate of decrease of intensity with thickness dx and a is called the absorption co-efficient. After rearrangement, the integration of equation (2) yields,

$$-\ln I = ax + C \quad ...(3)$$

where C is a constant of integration. At $x = 0, I = I_0$. So, $C = -\ln I_0$. Introducing this in equation (3) we get,

$$\ln \frac{I}{I_0} = -ax \quad ...(4)$$

After rewriting equation (4), we get

$$I = I_0 e^{-ax} \quad ...(5)$$

or we can write

$$\log \frac{I}{I_0} = -\frac{a}{2.303} x \quad ...(6)$$

or,

$$\log \frac{I}{I_0} = -a' x \quad ...(7)$$

where a' $(= a/2.303)$ denotes the extinction co-efficient and $-\ln I/I_0$ denotes the medium's absorbance. The letter A stands for absorbance.

Beer extended Lambert's law by demonstrating that, "The fraction of incident light absorbed depends not only on the intensity I of light but also on the concentration c of the solution when light travels through a solution of a particular thickness" can be represented mathematically as :

$$-\frac{dI}{dx} \alpha\, c \quad ...(8)$$

The two laws can be joined to form a new legislation.

$$-\frac{dI}{dx} \alpha\, I \,\alpha\, c \quad ...(9)$$

or

$$-\frac{dI}{dx} = b \times I \times c \quad ...(10)$$

The molar absorption co-efficient, b, is defined as the concentration, c, given in mol/L. Equation (9) can be translated into, as in the case of Lambert's law.

$$\log \frac{I}{I_0} = -\frac{b}{I_0} = -\frac{b}{2.303} X_c X_x \quad ...(11)$$

TOUCH 'n' PASS

$$\log \frac{I}{I_0} = - \in X_c X_x \quad ...(12)$$

The molar extinction co-efficient is stated in L/mol/cm and is equal to (=b/2.303). The nature of the absorbing solute as well as the wave length of the incident light employed determine the molar extinction co-efficient. Beer-Lambert's law is the name given to the expression (equation 11).

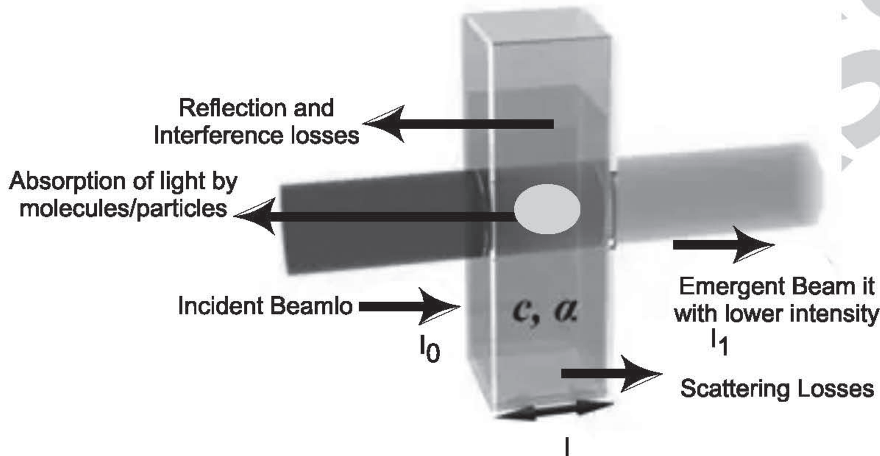

Fig. : Beer-Lambert's law

The amount of light that is transferred to the detector after passing through the sample (I) and the initial amount of light (I_0) is known as the transmittance (T). This is described in the formula given below :

Where I_0 represents the intensity of the light beam that is incident and I represent the intensity of the light that is emitted by the sample. The proportional percentage of light that passes through the sample is known as transmittance. So, if just half of the light is transmitted, the solution has a 50% transmittance.

$$T\% = (I/I_0) \times 100\%$$

The following formulas can be used to express the relationship between transmittance (T) and absorbance (A).

Applications

The following are some of the most important applications of Beer-Lambert's law :

1. With the help of this law the concentration of an unknown solution can be measured by comparing it to a known concentration solution using a spectrophotometer.
2. This law can be applied to the atmosphere to characterise solar or stellar radiation.
3. Using the Beer Lambert Law, we may determine the concentration of various compounds in cell structures by studying their absorption spectra.
4. A spectrophotometer can be used to check the purity of a substance by measuring the absorbance of a chemical.

Limitations

The limitations of Beer-Lambert's law are as follows :

1. Light scattering caused by particles in the sample.
2. Electrostatic interactions between molecules in close proximity cause changes in absorptivity coefficients at high concentrations (>0.01M).
3. The sample's fluorescence or phosphorescence.
4. When very high-intensity radiations are utilised, this law is also invalid.
5. Chemical equilibrium shifts as a function of concentration.
6. Deviations in non-monochromatic radiation can be reduced by using a reasonably flat section of the absorption spectrum, such as the absorption band maximum.
7. A level of 0.1 percent stray light at any wavelength will impede proper absorption measurements, causing apparent negative deviations from this law.

Q.6. What are radiotracer technique? Explain in detail the different types of detectors used for identification and measurement of radiotracer.

Ans.

Radiotracer Techniques

In radioactive tracer technique, radioactive nuclides are used to trail the behaviour of elements or chemical species in chemical and other processes. The use of radioactivity measurements is used to do this. This is accomplished through the use of radioactivity measurements. A radioactive tracer, also known as a radioactive label or radiotracer, is a chemical molecule in which one or more atoms have been replaced by a radionuclide, which can be used to investigate the mechanism of chemical processes by following the radioisotope's passage from reactants to products. The radioactive version of isotopic labelling is hence radio labelling or radiotracing. In biological situations, radio isotope tracers are sometimes known as radioisotope feeding experiments.

Hevesy and **Paneth** used naturally available 210Pb (a radioactive isotope in the 238U decay chain) as a radioactive tracer to determine the exceedingly low solubility of lead salts in 1913. This technique has been frequently used in chemical equilibrium and reaction investigations, as well as chemical analysis, since numerous radioactive nuclides were made available. It's also used in biological, biochemical, geological, environmental and medicinal, research. Its most major practical applications are industrial process control and medical diagnostics.

Detectors used for Identification and Measurement of Radiotracer

Because the analytical methods for measuring radioactive isotopes are exceedingly sensitive, they are typically more useful as tracers than stable isotopes. There are several methods/techniques used for identification and measurement of radiotracer like Geiger muller counter, Auto radiography, Scintillation counter, Gas ionization chamber, Mass spectrometer, Bernstein ballentine counter and NMR (Nuclear magnetic resonance) spectrometer etc.

1. **Geiger Mullar Counters :** The Geiger counter is an instrument used to detect and measure particles in ionised gases. When radioactive isotopes are utilised in human medical research, it is critical that the amount of radioactive material provided to human participants is kept to a minimum. The radioactivity of materials must be measured with an extremely sensitive device in order to achieve this. In the year 1928, Geiger and Muller created a 'particle detector to measure ionising radiation, which

they dubbed the 'Geiger Muller Counter,' or simply the 'GM counter.' Only a few of the applications include radiological protection, radiation dosimetry and experimental physics. It is constructed composed of a metallic tube filled with gas that is subjected to a high voltage range of multiples of 100V. The purpose of this equipment is to detect and measure ionising radiation such as alpha particles, beta particles, and gamma rays.

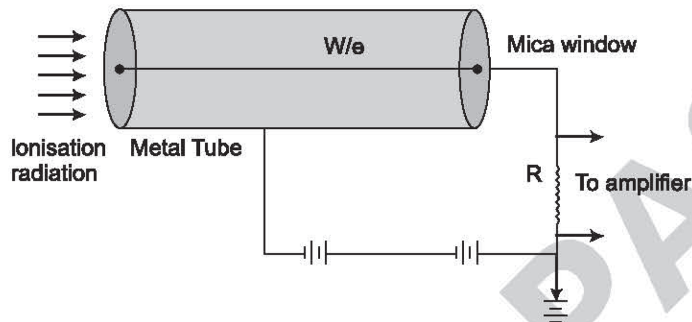

Fig. : Geiger-Muller Counter

It is made up of a huge, circular tube that serves as the cathode and a fine wine that serves as the anode. In relation to the outer cathode, the fine wire is kept at a high potential (1,000-2.500 vol). The tube is filled with a readily ionised gas like neon, argon or helium, as well as an organic quenching agent like ethanol. The voltage and gas fling have been adjusted to the point where no current flows naturally. An extremely thin window of synthetic plastic or mica is used to cover the tube's open end. Under this window, the radioactive substance, which is usually a solid, is deposited. As the radioactive particle enters the tube, they ionise the gas molecules, causing a shower of electrons to be released. Following that, the liberated electrons are driven to the positive wire. Additional molecules are ionised as they move through the gas. The tube briefly turns conductive. The resulting electrical pulse is gathered in a scaler, an electric computing machine that records the number of such pulses in a set amount of time.

In a Geiger Mullar counter, there are two sorts of displays : **detected radiation readouts** and **counts**, and **radiation dose**. Counts are the most basic readout, displaying the number of counts per unit of time, such as counts per minute.

2. **Scintillation Counter :** A scintillation counter detects and measures ionising radiation by using the excitation impact of incident radiation on a scintillating substance and detecting the light pulses that arise. When a scintillation detector is connected to an electronic light sensor, such as a photomultiplier tube (PMT) or a photodiode, a scintillation detector or scintillation counter is created. A scintillator is a substance that shows scintillation, or light induced by ionising radiation. Samples must be suspended or dissolved in a "cocktail" that includes a solvent (aromatic organics like toluene or benzene), small amounts of scintillators and a surfactant. The excitation takes place in an organic solvent, and the scintillation medium consists of a fluorescent solute and an organic solvent such as benzene or toluene. In the scintillation medium, dissolve the radiolabelled metabolite sample. A specific amount of the resulting solution must be placed in small, transparent glass vessels. Following that, the vessels

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are placed in a liquid scintillation counter with two image multiplier tubes. A solution containing radiolabelled metabolites emits beta particles. The beta particle's energy is first transferred to the molecules of the solution, then to the molecules of the solute. When a solute is excited, it emits light, which disperses the energy. A single light pulse corresponds to one beta particle. Photomultiplier tubes are used to measure the pulse.

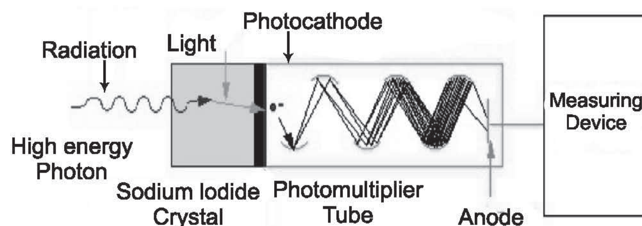

Fig. : Scintillation Counter

3. **Autoradiography :** This has evolved into a set of techniques for imaging radioactive tracers within a sample in close proximity to a detection medium. Autoradiography is a bio-analytical technique that uses radioisotopes to see the distribution of radioactively labelled compounds in biological samples. It's a technique for locating radioactive materials within a certain cell, tissue, cell organelles, or even biomolecules. It's a highly sensitive approach that's employed in a wide range of biological studies. Autoradiography can be utilised for quantitative estimation using a densitometer, in addition to locating radioactive compounds.

The first autoradiography was discovered by chance in 1867, when uranium salts caused a blackening on silver chloride and iodide emulsions noticed by Niepce de St. Victor. The distribution of polonium in biological material was traced using autoradiography for the first time in 1924.

The basic principle of autoradiography is based on a radioactive substance's capacity to ionise photographic film and expose it. A radioactive substance is placed in direct contact with a thick layer of photographic emulsion containing gelatin and silver halide crystals in this process. This emulsion is distinguished from normal photographic film by its greater silver halide to gelatin ratio and small grain size. After that, it's put in the dark for a few days to ensure optimum exposure. The silver halide crystals are subjected to radiation, which chemically changes silver halide to metallic silver (reduced) and produces a dark colour band. Electron microscope, intensifying screen, preflashed screen, digital scanners, electrophoresis and other methods are used to examine the resulting radiography. At the last records in the form of autoradiographs are created. ●

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UNIT-VIII

Molecular Techniques

SECTION-A VERY SHORT ANSWER TYPE QUESTIONS

Q.1. What is electrophoresis?

Ans. Electrophoresis is a laboratory technique used to separate DNA, RNA and protein molecules based on their size and electrical charge. An electric current is used to move the molecules through a gel or other matrix.

Q.2. Who discovered electrophoresis?

Ans. During the 1930s Arne Tiselius developed a method called electrophoresis, which makes use of this phenomenon to separate different substances from one another.

Q.3. What are the type of gel electrophoresis?

Ans. The following types of gel electrophoresis : (i) Starch gel electrophoresis, (ii) Agrose gel electrophoresis, and (iii) polyacrylamide gel electrophoresis.

Q.4. What are the media used in electrophoresis?

Ans. Polyacrylamide and agarose are two support matrices commonly used in electrophoresis. These matrices serve as porous media and behave like a molecular siene.

Q.5. Is PCR used in DNA sequencing?

Ans. Polymerase chain reaction (PCR) is a laboratory technique that use selective primers to copy specific selective primers of a DNA sequence.

Q.6. What is the difference between PCR and sequencing?

Ans. PCR is the process which creates a large number of copies of a DNA fragment DNA sequencing is the technique which results in the precise order of the nucleotides of given DNA fragment. This is the key difference between PCR and DNA sequencing.

Q.7. What is Sanger DNA sequencing?

Ans. Sanger sequencing also known as the 'Chain termination method' is a method for determining the nucleotide sequence of DNA. The methods was developed by two time Nobel Laureate Frederick Sanger and his colleagues in 1977, hence the name the Sanger sequencing.

Q.8. What can DNA be used for?

Ans. DNA can be used to identify criminals with incredible accuracy when biological evidence exists. By the same taken, DNA can be used to clear suspects and exonerate persons mistakenly accused or convicted of crimes.

Q.9. What is the principle of DNA fingerprinting?

Ans. The most important requirement for DNA fingerprinting is short nucleotide repeats that vary in number from person to person but are inherited. These are called variable number tendem repeats or VNTRs and this is the main principle of DNA fingerprinting.

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Q.10. Who is the father of fingerprint?

Ans. In 1901, Sir Edward Richard Henry was appointed assistant commissioner at Scotland yard. His system was so applicable that Henry energed as the 'father of fingerprinting' at least as the first man to successfully apply fingerprints for identification.

Q.11. What is RELP and how it is used?

Ans. RELP stands for Restriction Fragment length polymorphism such variation results in different size (or length) DNA fragments produced by digesting the DNA with a restriction enzyme, RELPs can be used as genetic markers, which are often used to follow the interitance of DNA through families.

Q.12. What is the difference RELP and RAPD?

Ans. RAPD (random amplified polymorphic DNA) refers to a PCR-based technique for identifying genetic variation while RELP (restriction fragment length polymorphism) refers to a molecular method of genetic analysis, which allows individuals to be identified based on unique patterns of restriction enzyme cutting in specific regions of DNA.

Q.13. What is the basic principle of PCR?

Ans. Its principle is based on the use of DNA polymerase which is an *invitro* replication of specific DNA sequences. This method can generate tens of billions of copies of a particular DNA fragment (the sequence of interests, DNA of interest, or target DNA) from a DNA-extract (DNA template).

Q.14. Which enzyme is used in PCR?

Ans. Taq polymerase is the most common enzyme used for PCR amplification. This enzyme is extremely heat resistant with a half-life of 40 minute at 95°C.

Q.15. What is ELISA and why is it used?

Ans. ELISA stands for enzyme-linked immunoassay. It is a commonly used laboratory test to detect antibodies in the blood. An antibody is a protein produced by the body's immune system, when it detects harmful substances, called antigens.

Q.16. Which enzyme is used in ELISA test?

Ans. There are many substractes available for use in ELISA detection. However, the most commonly used horseradish peroxidase (HRP) and alkaline phosphates (ALP).

Q.17. What is control in ELISA?

Ans. A positive ELISA control can be a recombinant or natural sample that you know will be detectable in assay. Positive controls help to show that a negative sample is truly negative.

Q.18. What is the western blot test used for?

Ans. The western blot test separates the blood proteins and elects the specific protein (called HIV antibodies) that indicate an HIV infection. The western blot is used to confirm a positive ELISA and the combined tests are 99.9% accurate.

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SECTION-B SHORT ANSWER TYPE QUESTIONS

Q.1. Write about the polymerase chain reaction also give it principle.

Ans. **Polymerase Chain Reaction (PCR)**

Polymerase chain reaction (PCR) is one of the revolutionary techniques of molecular biology which was developed by **Kary Mullis** in 1980 to amplify DNA fragments. It is basically used to amplify/multiply a specific region of DNA to generate many to millions of copies of a selected particular DNA sequence. PCR is the most widely used method for the amplification of targeted nucleic acid. This techniqueis mainly based on the ability of DNA polymerase to synthesise of a new DNA strand that is complementary to the selected template DNA strand. PCR employs the principles of complementary nucleic acid hybridization and nucleic acid replication in a series of cycles. As a result, there are plenty of targets that can be easily detected by a variety of methods.

Principle of PCR : The synthesis of new DNA on a template strand requires primer RNAs which can be extended by DNA polymerase. In the presence of stable polymerases and suitable RNA primers, DNA synthesis can be done repeatedly in multiple cycles if the *ds*DNA formed in every cycle is quickly denatured into *ss*DNA to start new cycles of replication.

Thus, in this technique nucleic acid target sequence is denatured to single strands, specific primers for each target strand sequence are further added and the DNA polymerase catalyses the addition of deoxyribonucleotides to lengthen and create new complementary strands to each of the target sequence strands (cycle one). Cycle two denatures both double-stranded products of cycle first which then serves as a target for further primer annealing and extension by the DNA polymerase. This thermal cycling may produce at least 107 copies of target DNA after 25-30 cycles.

Q.2. What do you mean by polyacrylamide gel electrophoresis? Also write its types and principle.

Ans. **Polyacrylamide Gel Electrophoresis (PAGE)**

Gel electrophoresis is the type of electrophoresis that involves movement of particles through the porous gels under electric field. This is an extensively used technique for separation of biomolecules. Of all the gels used in electrophoresis, agarose and polyacrylamide gels are most common. While agarose gels are more suitable for separation of large biomolecules such as DNA, polyacrylamide gels are usually employed for separating small molecules of proteins and ucleic acids. Unlike agarose gel electrophoresis which is run in a horizontal submarine gel apparatus, polyacrylamide gels are run in a vertical set up.

Types of PAGE : There are two types of ways in which polyacrylamide gels can be run :

1. **Native PAGE :** In this version, molecules are preserved in their native form and their higher order structure is not altered as they run through the gel.
2. **SDS-PAGE :** It involves denaturation of protein molecules by a detergent such as SDS (sodium dodecyl sulphate) so that their higher order structure (tertiary and secondary) is disrupted and they become linear and their mobility depends on their size only.

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Principle : Electrophoresis is the migration of particles under electric field. Electrophoretic mobility (v) of particles depends on the charge of particles (q), strength of electric field (E), and frictional drag (f) which are related through the following equation :

$$Eq = fv$$

Thus, it appears that molecules should be separated according to their charge under an applied electric field. However, it is more often required to separate molecules on the basis of their size. Thus, the basic technique of electrophoresis has to be modified in such a way that the movement of particles is dependent on their size. One way of achieving this goal is to impart uniform charge to all the particles so that their charge becomes proportional to their size. In PAGE, this can be done by treating proteins with a detergent molecule. Sodium Dodecyl Sulphate (SDS) is a detergent that destroys tertiary and secondary structure of proteinsso that their mobility depends on their length. SDS-PAGE is carried out at such pH that SDS molecules are negatively charged and bind to proteins in a ratio which imparts uniform charge. This allows the separation of molecules on the basis of their size.

The proteins being covered by SDS are negatively charged and when loaded onto a gel. Thus when a negatively charged protein molecule is placed in an electric field, it will migrate towards the anode (positively charged electrode) and will be separated by a molecular sieving effect based on size. After the visualization by a staining (protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker).

Q.3. Briefly describe the principle of Enzyme-Linked Immuno Sorbent Assay (ELISA). Also discuss the procedure and advantages.

Ans.

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

ELISA stands for 'enzyme-linked immunosorbent assay' which is a type of immunological assay techniques first described in 1971 by **Eva Engvall** and **Peter Perlmann**. It is based on antigen-antibody binding and is widely used for detecting and quantifying the presence of analytes (antigens, antibodies, proteins and glycoproteins etc) in biological samples.

In this technique, 96-well microtiter plates are used to immobilize the antigen or antibody in fluid and allow it to bind to a specific antibody that is there after detected by using a peripheral, enzyme-coupled antibody. The enzymes commonly used to produce a signal in ELISA are alkaline phosphatase and glucose oxidase which transform a pale substrate (chromogen) to a visible colored product that indicates the presence of analyte. Finally, various qualitative and quantitative measures can be used that depend on the colorimetric reading for sensitivity and concentration levels of analytes in the sample.

Although there are several variants of ELISA but all of these involve the following essential steps :

1. **Coating or capture :** It involves immobilization (direct or indirect) of antigens to microtiter plate.
2. **Plate Blocking :** All the unoccupied sites on the microtiter plate are blocked by adding some unreactive and irrelevant molecule.
3. **Probing :** Probing or detection is done by incubating the plate with antigen-specific antibodies.
4. **Signal quantification :** In this step the coloured signal generated by the enzymatic reaction of the substrate is measured.

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Advantages of ELISA

The ELISA technique has the following advantages :

1. Unlike RIA which involves use of radioactive labeling and is associated with health hazards, ELISA uses enzyme-catalyzed colour reactions which is much safer.
2. The use of two antibodies (primary and secondary) makes the diagnosis more accurate and reliable.
3. ELISA can be performed on complex samples because the antigen does not need to be purified to be detected.
4. It is highly responsive because both direct and indirect analysis methods can be used.
5. It is a quick test with immediate results.
6. ELISA detection models include quantitative, semi-quantitative, standard curve, qualitative, and calibration curve models, among others.

Q.4. Write a short note on the application of RELP test.

Ans.

Application of RFLP Test

This technique finds a number of uses in molecular research and diagnostics. Some of its applications are as follows :

1. **Genome mapping :** RFLP helps in analysis of unique pattern in genome for organism identification and differentiation. It also helps in determining recombination rate in the loci between restriction sites.
2. **Genetic disease analysis :** RFLP is of great help in diagnosis of genetic diseases. After identification of gene for particular genetic or hereditary disease, that gene can be analysed among other family members.
3. **Detection of mutations :** The technique of RFLP makes use of gene polymorphisms which arise due to mutations. It is also used to detect mutated genes.
4. **DNA fingerprinting :** It is the basis of DNA finger-printing for paternity test and criminal identification which is of great use in forensic science.

Q.5. Briefly explain the DNA sequencing their applications.

Ans.

DNA Sequencing

DNA is a polynucleotide made up of four types of nucleotides which differ with respect to the nitrogenous bases (purines and pyrimidines) contained in their structure. Adenine (A) and thymine (T) are the two purines present in DNA and they bind to two different pyrimidines -cytosine (C) and guanine (G) -- through hydrogen bonds. Adjacent nucleotides in the DNA are linked through phosphodiester bonds between deoxyribose sugars, Thus, every segment of DNA is a specific sequence of these nucleotides. The process of determining the sequence of nucleotides (As, Ts, Cs, and Gs) in a piece of DNA is called **DNA sequencing**. It is one of the most frequently used techniques in life sciences today and has revolutionized the fields of biology and medicine.

The history of nucleotide sequencing starts with the sequencing of RNA molecules. **Robert Holley** was the first to sequence the yeast alanyl *t*RNA containing a sequence of 77 bases which was published by him in 1965. This was followed by determination of the 120 bases-long sequence of 5S *r*RNA of *E. coli* by **Frederick Sanger** in 1967. **Ray Wu** was the pioneer in developing a DNA sequencing strategy based on location-specific primer extension.

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This strategy was used by Frederick Sanger in developing his chain-termination method of DNA sequencing in 1977. Around the same time (1976-1977), **Allan Maxam** and **Walter Gilbert** developed their chemical degradation method which was initially very popular but later fell out of favour due to improvements in Sanger's method.

The early methods of DNA sequencing which were slow and labour-intensive soon became replaced by methods of High Throughput Sequencing (HTS). These are also called Next Generation Sequencing (NGS) methods and several such methods appeared in the 1990s. Modern methods of sequencing which are extremely fast and cost-effective enable us to sequence whole genomes in a very short time.

Applications of DNA Sequencing

Today, traditional, chain-termination technology, and HTS methods are used for a variety of applications. Sanger sequencing is now mostly used for *de novo* DNA molecule sequencing to obtain the primary sequence data for a gene or organism.

Some of the more important applications of DNA sequencing are as follows :

1. It is used to compare the genesequences in DNA fingerprinting.
2. Sequencing enables the detection of single-nucleotide polymorphisms (SNPs), which are amongst the most common types of the genetic variation within a population.
3. Sequencing is useful in phylogenetic studies because sequence variations indicate evolutionary relationships.
4. Sequencing is a useful tool in the detection of mutated genes that can cause disease. For example, sequence variations in lung adenocarcinoma samples endorsed the detection of the rare mutations related with the disease.
5. These methods can also be used to precisely identify the chromatin binding positions for the specific nuclear proteins.
6. In diagnostics, the genome sequencing is especially helpful in determining the causes of rare genetic disorders. Furthermore, HTS has played an important role in developing a better understanding of tumours and cancers.

Q.6. Explain differences between native PAGE and SDS PAGE?

Ans. Differences Between Native PAGE and SDS PAGE

S.No.	Items	Native PAGE	SDS PAGE
1.	Description	Native PAGE is an electrophoretic technique that separates proteins on the basis of their size and charge.	SDS PAGE is a separation technique that separates proteins on the basis of their mass.
2.	Nature of Gel	The gel is not denatured.	The gel is denatured.
3.	Denaturation	No such activity is required.	SDS is added to the gel to impart a negative charge on the protein samples.
4.	Basis of Separation	The proteins are separated on the basis of size and charge.	The proteins are separated on the basis of mass.
5.	Protein Stability and Recovery	The proteins are stable in a Native PAGE, and can be recovered later.	The proteins are not stable in the SDS PAGE, and hence cannot be recovered.

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Q.7. What are the differences between ELISA and RIA?

Ans. Some of the differences that are shared between ELISA and RIA are listed below :

1. ELISA detects antibody-antigen complex using enzymes whereas RIA detects antibody antigen complex using radioisotopes.
2. ELISA involves labelling the antibody whereas RIA involves labelling the antigen.
3. ELISA assay is less sensitive when compared to RIA assay.
4. ELISA procedure does not require a specific laboratory or trained staff whereas RIA will require a specific laboratory area to handle the radioactive material and specially trained staff.
5. ELISA does not require special arrangements whereas RIA requires special arrangements for requisition, storage and the disposal of the radioactive material.
6. ELISA does not involve the use of any radiation hazards whereas for RIA any radiation hazards will need to be documented and reported.

Q.8. Write about the enzyme kinetic of *Taq polymerase*.

Ans.

The Enzyme Kinetics of *Taq Polymerase*

Taq polymerase demonstrates substantial enzymatic activity at 37°C. However, it operates optimally at a much higher temperature (~72°C). Nucleotides are incorporated at a rate of two to four kilobases per minute. However, functioning at this temperature allows for non-specific amplification that is associated with mispriming events that occur during the initial phase of the PCR reaction. The extension can occur from oligodeoxynucleotide primers that bind non-specifically to the template DNA before the first denaturation step, which occurs at 93-95°C.

Mechanisms to circumvent this include the use of a thermolabile inhibitor that blocks the activity of the *Taq polymerase* until it is heat-inactivated. Consequently, the *Taq polymerase* only becomes active after the temperature destroys the monoclonal antibody during the initial denaturation of the PCR reaction. This means of antibody-mediated inhibition of *Taq polymerase* allows room temperature assembly of the PCR reaction mixture. As such, nonspecific amplification about arises from mispriming events is eliminated or reduced.

Q.9. Write a note on the Western blotting with principle.

Ans.

Western Blotting

Western blot or immunoblotting is an analytical technique often used in analytical research for the **separation of proteins** and their identification in an unknown complex biological sample.

The process of western blotting involves following steps :

1. Separation of proteins molecules on polyacrylamide gel.
2. Transfer of protein bands from gel to nitrocellulose membrane.
3. Detection of protein bands with suitable probes (antibodies).

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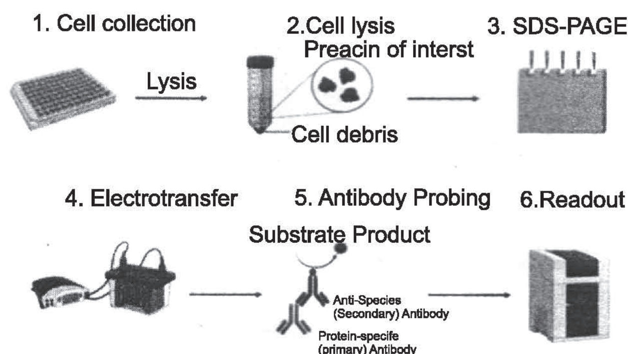

Fig. : Diagram of Western blotting

Principle of Western Blotting

The technique of Western blotting exploits the advantage of transferring protein bands from polyacrylamide gels to nitrocellulose membranes where they can be made to bind suitable probes. The fact that the interaction between proteins and their probe scan be used to detect and identify the desired proteins is the basic principle of Western blotting. The proteins used in Western blotting are separated on a gel matrix using gel electrophoresis. They are then transferred to nitrocellulose membranes where they are detected by binding them with antibodies (primary and secondary). A reporter-labelled primary antibody directed against the protein or a reporter-labelled secondary antibody directed at the primary antibody can be used to detect the protein on the membrane. Detection antibody (reporter-labelled primary or secondary antibody) is coupled to an enzyme that produces colour reaction on coming in contact with its substrate. The colour signal can then be visualized and even quantified. The signal or colour produced by the probe necessitates the use of a detection system appropriate for the signal or intensity produced.

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SECTION-C LONG ANSWER TYPE QUESTIONS

Q.1. What is electrophoresis? Explain its principle, types and applications.

Ans. **Electrophoresis**

Electrophoresis is the motion of dispersed molecules relative to a fluid under the influence of uniform electric field. The basic technique of electrophoresis has evolved into a number of useful variants which find extensive applications in analytical biochemistry. Electrophoretic methods are indispensable tools of molecular biology today and are used for separation of biomolecules such as proteins and nucleic acids.

Peter Strakhov and **Ferdinand Reuss** (1807) were the first to observe that clay particles dispersed in water migrate under the influence of electric field and the basis of this movement is the charged interface between the particle surface and the surrounding fluid. The application of electrophoresis for separation of biomolecules began with the work of **Alex Tiselius** (1931) who developed **Tiselius apparatus** for moving boundary electrophoresis. This was followed by the development of zone electrophoresis in 1940s and finally the advent of gel electrophoresis in 1960s. Gel electrophoresis has now become an integral part of

several important molecular techniques such as Polymerase Chain Reaction (PCR). DNA sequencing, fingerprinting, and blotting methods.

Principle of Electrophoresis

Electrophoresis is an electrokinetic phenomenon involving migration of charged particles under the influence of electric field. Charged particles move under electric field and the equation that describes their movement is as follows :

$$Eq = fv$$

Where, E = strength of electric field

Q = Charge on a particle

f = frictional drag

v = velocity

If the particles are coated uniformly by a particular charge then the charge of particles becomes proportional to their size and hence their separation under electric field takes place according to their charge. Paper and gel are two important media used for separation of molecules through electrophoresis.

Gel Electrophoresis

In gel electrophoresis, biomolecules are separated on a gel which is a matrix of cross-linked polymers having a specific porosity and allows separation of molecules falling within a particular range of sizes. The two most commonly used gels in electrophoresis are agarose and polyacrylamide.

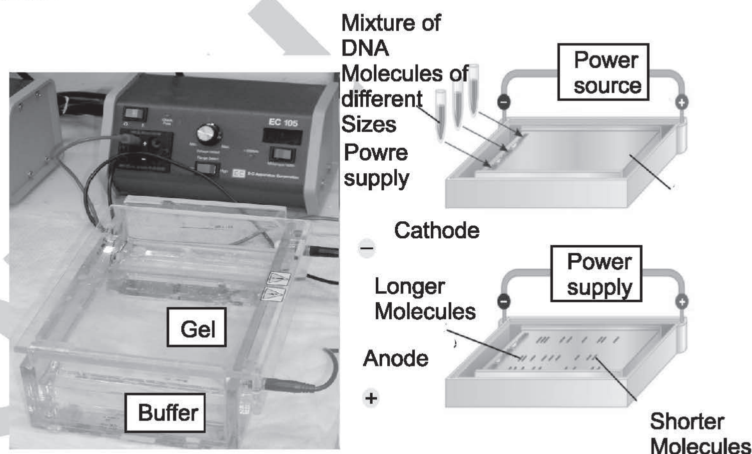

Fig. : Gel electrophoresis apparatus

1. **Agarose gel Electrophoresis :** It is the most commonly used method for the size- and shape-based separation of DNA molecules comprising several hundred or more base pairs, including plasmid DNA molecules. Agarose is one of the main components of agar extracted from the cell wall of red algae. Agarose is a linear polysaccharide composed of galactose and anhydrogalactose units. The Agarose gel possesses a number of features that make it especially advantageous for the purposes of gel electrophoresis. The gel is hydrophilic, chemically inert and stable. It does not bind the dye molecules used to visualize DNA molecules that are separated in the gel. The three

dimensional matrix of the Agarose gel is brought about by non-covalent bonds formed between polysaccharide units.

As the structure of the Agarose gel is held together by non-covalent bonds, the gel undergoes a phase transition at elevated temperature, and forms a sol state. The gel is prepared by mixing Agarose powder into a running buffer, with subsequent formation of the sol state at high temperature, casting and subsequent cooling. The pore size of the resulting gel depends on Agarose concentration. The pore size determines the size range of DNA molecules that can be efficiently separated in the gel. For Agarose gel electrophoresis, gels with Agarose concentrations of 0.5-3 w/v % are generally used. The lower and higher ends of this concentration range are applicable in the case of larger and smaller DNA molecules, respectively.

DNA is a negatively charged molecule. Therefore, the electric field applied during electrophoresis will cause migration of DNA molecules towards the positive pole, i.e. the anode. Therefore, the gel is placed into the electrophoresis tank in an orientation that the sample loading wells will be towards the negative pole (*i.e.,* the cathode).

2. **Polyacrylamide Gel :** It is made by mixing acrylamide and bis-acrylamidewhich crosslink together so as to give a mixture of uniform porosity. The pore size depends on the concentration of acrylamide and bis-acrylamide. Nowadays, polyacrylamide gels are mostly used for separating proteins having a size in the range of 5-2000 kDa. However, the early techniques of DNA sequencing (e.g., Maxam-Gilbert & Sanger methods) used polyacrylamide gels for resolving DNA fragments that differed by a single base pair in their length.

Applications

Agarose gel electrophoresis is a basic procedure routinely performed in molecular biology labs. It finds many applications, some of which are as follows :

1. Agarose gel electrophoresis is use for separation of DNA fragments from various sources.
2. It is used for estimation of size and Mol wt of DNA fragments obtained in restriction digestion *i.e.*, restriction mapping of cloned DNA.
3. It can also be used to determine concentration and purity of DNA in unknown samples.
4. This process is a prerequisite for Southern and Northern blotting because the fragments of DNA/RNA have to be separated on gels before being transferred to nitrocellulose membrane.
5. PCR and genetic Fingerprinting protocols also depend on agarose gel separation of DNA.

Q.2. Describe in detail the components, procedure of PAGE. Discuss the applications of PAGE in biomedical research.

Ans.

Components of PAGE

Polyacrylamide gels are run in vertical slab gel apparatus which consists of following components :

1. **Power Supply :** This maintains the desired conditions of voltage and current during electrophoresis and prevents fluctuations.

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2. **Electrophoresis chamber :** This is the main part of the electrophoretic apparatus where gels are placed. It has a lower and an upper buffer chamber. These chambers remain filled with buffers and are in contact with the slab gel.
3. **Glass plates :** Glass plates are used to cast the slab gel and hold it between them. One of the two plates bears a notch and the other is rectangular.
4. **Gel casting apparatus :** The sides of glass plates are separated by spacers of desired thickness while casting the gel. After sealing the sides of the glass plates, they are placed in a gel casting stand and a comb is placed on the top of the slab to make wells.
5. **Reagents needed :** Running a polyacrylamide gel requires acrylamide ($CH_2 = CH{\cdot}CONH_2$) solutions for making stacking and running gels. Buffers (Gel loading buffer and running buffer) are needed to fill the tanks and keep the gels submerged. Besides, protein samples and molecular weight markers are required to be loaded in sample wells. Dyes are mixed in samples to track the progress of gels. Finally, stains are used to colour the protein bands in the gel.

Procedure of PAGE

The process of running a polyacrylamide gel is quite elaborate and consists of the following steps :

1. **Sample preparation :** Samples for PAGE may be proteins or nucleic acids. The sample is optionally mixed with a chemical denaturant which is usually SDS for proteins or urea for nucleic acids. SDS is an anionic detergent that denatures secondary and non-disulfide-linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Urea breaks the hydrogen bonds between the base pairs of the nucleic acid, causing the constituent strands to anneal. Heating the samples to at least 60 °C further promotes denaturation.

   A tracking dye is added to the solution. This dye should have higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the sample through the gel during the electrophoretic run.
2. **Preparation of polyacrylamide gels :** The gels typically consist of acrylamide ($CH_2 = CH{\cdot}CONH_2$), bisacrylamide [$(CH_2 = CH{\cdot}CONH)_2CH_2$)], the optional denaturant (SDS or urea), and a buffer with an adjusted pH.The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also be varied (generally from 5% to 25%) in order to change the porosity of the gel. Gels of lower percentage have large pore size and are suitable for resolving large molecules, while much higher percentages of acrylamide are needed to resolve smaller proteins.

   Gels are usually polymerized by mixing acrylamide and bisacrylamide and pouring the mixture between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells. At first the running gel (of high porosity) is poured and then a stacking gel (of low porosity) is layered above it. After the gel is polymerized, the comb can be removed and the gel is ready for electrophoresis.
3. **Electrophoresis of PAGE :** For running the electrophoresis, the gel cast between glass plates has to be kept in the electrophoretic apparatus such that the sample wells lie towards the top. The two tank buffers remain in contact with the gel slab. Various

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buffer systems are used in PAGE depending on the nature of the sample and the experimental objective. The buffers used at the anode and cathode may be the same or different. Of the two electrodes, the cathode lies above and is in contact with upper tank buffer whereas the anode is connected to the lower tank.

After placing the gel in the apparatus, an electric field is applied across the gel. Since the gels are run in a buffer which has alkaline pH, the analytes (proteins and nucleic acids) are negatively charged. Thus, the electric field causes the negatively charged proteins or nucleic acids to migrate across the gel towards the positive electrode (the anode).

Under the influence of electric field the biomolecules move through the gel at different rates such that the distance migrated through the gel is inversely proportional to their size. Thus, small molecules move farther from the sample wells compared to large molecules. The gel is run usually for a few hours, though this depends on the voltage applied to the gel.

4. **Detection :** Following electrophoresis, the gels may be stained for visualization. Proteins are commonly stained with Coomassie Brilliant Blue whereas nucleic-acids are stained with ethidium bromide or silver stain. If the protein gels are to be processed further (*e.g.,* Western blot) they are left unstained.

After staining, the separated biomolecules appear as distinct bands within the gel. It is common to run markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the molecular mass of unknown biomolecules by comparing the distance travelled relative to the marker.

Applications of PAGE

This is a routine exercise in molecular biology laboratories because it is needed in several other processes (*e.g.,* western blotting). It has following important applications :

1. It is used for measuring the molecular weight and for estimation of protein size.
2. PAGE can be helpful in protein quantitation, in determination of protein subunit structure and in peptide mapping.
3. Protein purity can be estimated and protein integrity can be monitored through PAGE.
4. Comparison of the polypeptide composition of different samples.
5. Analysis of the number and size of polypeptide subunits can be done by comparing the bands obtained on native PAGE and SDS-PAGE.
6. Post-electrophoresis applications, such as Western blotting.
7. Selective labelling of cell surface protein and detection of protein ubiquitination is also possible through PAGE.

Q.3. What are various methods of DNA sequencing? Also describe the significance of genome sequencing.

Ans.

Basic Methods of Sequencing DNA

The two most basic methods of sequencing DNA are : **Maxam** and **Gilbert's chemical degradation** method and **Sanger's chain termination method**. Both these are described in detail here.

1. **Maxam & Gilbert's Chemical Degradation Method :** This method is based on chemical modification of DNA followed by its cleavage at specific bases. It allows the

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use of purified *ds*DNA without further cloning. The method requires radioactive labeling at the 5'-end of DNA by a kinase reaction involving? T-32P ATP. This is followed by purification of the DNA which is to be sequenced. Thereafter, the fragment to be sequenced is subjected to chemical degradation by setting up four different reactions :

(i) Reaction in which break is generated towards the 5-end of base G.

(ii) Reaction in which break is generated at the 5-end of bases A or G (A+G).

(iii) Reaction in which break is generated towards the 5'-end of base C.

(iv) Reaction in which break is generated at the 5-end of bases C or T (C+T).

The four reactions are set up and regulated in such a manner that only one modification occurs per DNA molecule. It yields a series of labelled fragments from the radiolabeled 5'-end to the first cut site on 3'-end in each molecule. The radiolabeled fragments obtained in all the four reaction mixtures are kept side by side and separated by acrylamide gel electrophoresis on the basis of size. Autoradiography of the gel is done by exposing it to X-ray films which yields a pattern of dark bands where each band corresponds to a radiolabelled DNA fragment. Thereading of bands from one end to the other gives the sequence of the DNA.

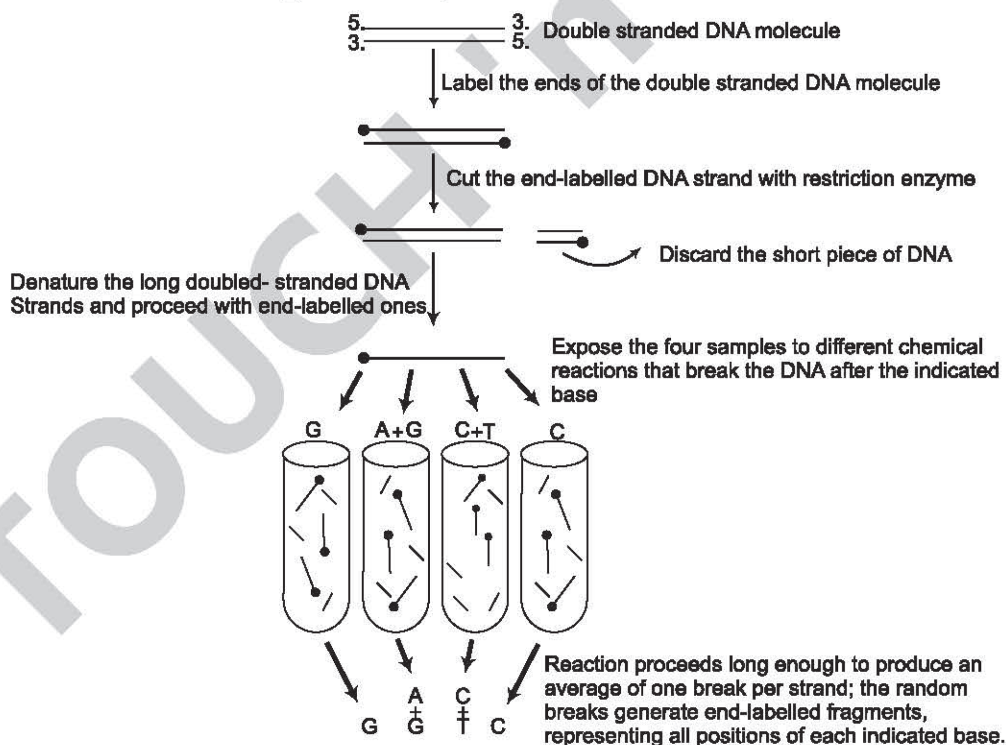

Fig. : Maxam and Gilbert technique of DNA sequencing

2. **Sanger's chain termination method (dideoxy method) :** This method has been one of the most frequently used methods of DNA sequencing in the past and is still being used as a cheap and accurate way of sequencing in some situations. This method

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requires a single strand of DNA template, a DNA polymerase, a DNA primer, normal deoxyribonucletide triphosphates (*d*NTPs), and modified dideoxyribonycleotide triphosphates (*dd*NTPs). These *dd*NTPs cause chain termination because they can't furnish 3-OH group which is needed for linking adjacent sugars through phosphodiester bond.

The process involves setting up of 4 separate reactions such that all of them contain all the 4 normal *d*NTPs but each of the reactions has a particular *dd*NTP in a high concentration. This causes chain termination at a specific nucleotide in each of the 4 reactions. Thus, when DNA synthesis is allowed in the 4 reactions by adding DNA template, DNA polymerase and dNTPs, the synthesis of DNA fragment stops at a particular nucleotide in all the four reactions. Consequently, several fragments of varying lengths are formed in each well. These fragments are separated by electrophoresis in four separate lanes corresponding with four reactions. Finally, autoradiography is performed and the DNA sequence is inferred by reading the order of nucleotide bases in four lanes.

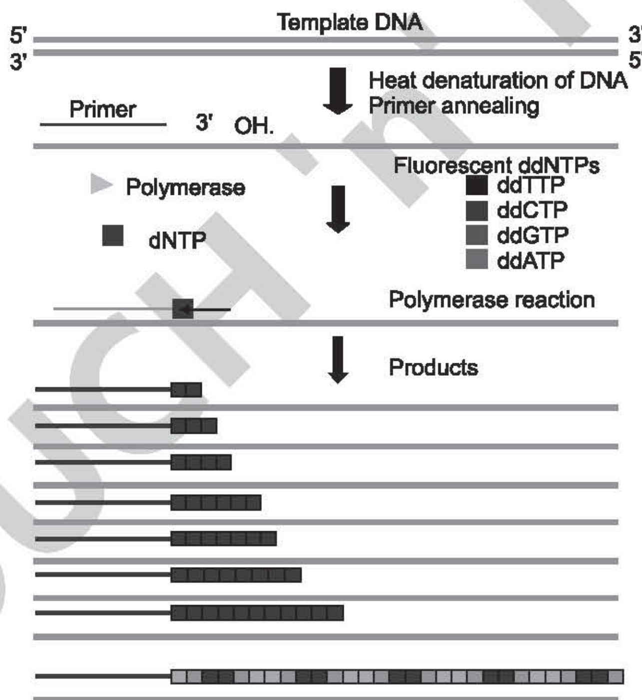

Fig. : Enzymatic procedure of DNA sequencing

3. **High Throughput Sequencing (Next Generation Sequencing) :** This is an advancement over traditional methods of sequencing. The high throughput methods were initially introduced during 1990s and soon became immensely popular. They have considerably increased the efficiency of sequencing and have reduced the time needed to sequence DNA. Thus, these methods have made it possible to sequence whole genomes of organisms in a very short time and at lower cost. Some of the popular methods of high throughput sequencing are as follows :

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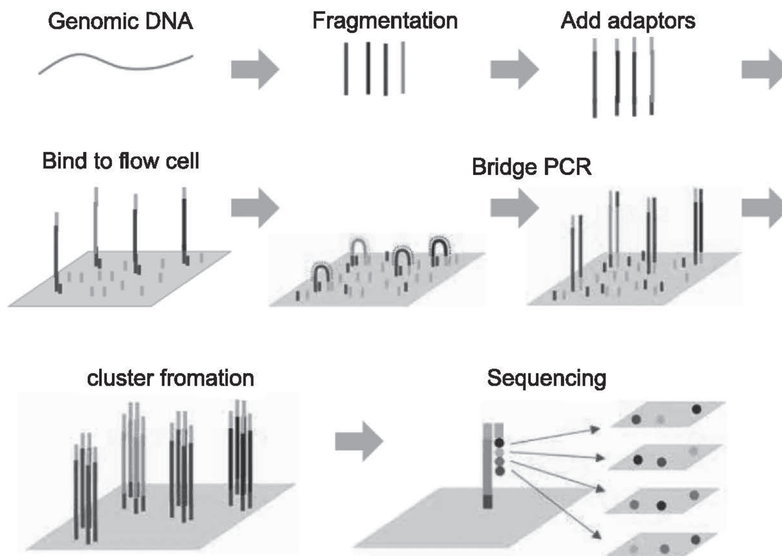
Illumina Sequencing Technology leverages clonal array formation and proprietary reversible terminator technology for rapid and accurate large-scale sequencing. The innovative and flexible sequencing system enables a broad array of applications in genomics, transcriptomics, and epigenomics.

The illumina sequencing platforms generate up to 100 gigabases of high squality sequence data per lane (HiSeq 4000) or up to 15Gb (MiSeq), using a massively parallel sequencing approach. The illumine instruments provide currently the highest yields as well as the highest quality data.



Multi-locus sequence typing (MLST) compares the DNA sequences of several housekeeping genes to classify strains of a particular species. Although previously commonly used, MLST does not discriminate as well as more modern techniques between closely related strains of the same species.

Whole Genome Sequencing (WGS) : This is a very powerful tool in the detection of antimicrobial resistance. In addition to identification of the species. WGS can also be used to identify resistant genes and to identify resistant genes and to identify transmission in outbreak situations. Although WGS sequencing has advanced rapidly in the last couple of decades, it is still expensive to set up and run, and requires expertise for analysis and interpretation, although new automated tools are being developed.

Single Molecule, Real-Time (SMRT) Sequencing : SMRT sequencing by pacific Biosciences (PacBio) is another example of third generation long read sequencing technology. SMRT sequencing is capable pf generating reads tens of kilobases in length.

Adapters are ligated to double-stranded DNA to form a circular template. Primers and polymerase are added to the library and the sample loaded onto the instrument. The

sequencing occurs on a SMRT cell which contains millions of tiny wells called Zero-Mode Waveguides (ZMWs).

Nanopore Sequencing is a third-generation sequencing platform developed by oxford nanopore technologies. Nanopore sequencing of DNA became available to interested researchers when the MiniON device was delivered to early access users in 2014. Over the nest five years, the long reads, portability, rapid throughput and low cost of the MiniON has allowed unprecedented access to DNA and RNA has sequences in a varisting of environments from remote African villages during the Ebola epidemic.

Significances of Whole-Genome Sequencing

1. Provides a high-resolution, base-by-base view of the genome.
2. Captures both large and small variants that might be missed with targeted approaches.
3. Identifies potential causative variants for further follow-up studies of gene expression and regulation mechanisms.
4. Delivers large volumes of data in a short amount of time to support assembly of novel genomes.

Q.4. What is restriction fragment length polymorphism? Describe its principle, process steps and disadvantages.

Ans.

Restriction Fragment Length Polymorphism (RFLP)

RFLP stands for **Restriction Fragment Length Polymorphism** which is a technique developed by **Alec Jeffreys** in 1984. It exploits variations (called **polymorphisms**) in some specific DNA sequences called **mini satellites.** It is used to analyse unique patterns in DNA fragments called Variable Number of Tandem Repeats (VNTRs) in order to genetically distinguish between organisms.

Principle : Genetic polymorphism is defined as the inherited genetic differences among individuals in over 1% of normal population. The RFLP technique exploits these differences in DNA sequences to recognize and study both intraspecies and interspecies variation.

Restriction endonucleases are enzymes that cut lengthy DNA into short pieces. Each restriction endonuclease targets different nucleotide sequences in a DNA strand and therefore cuts at different sites.

The distance between the cleavage sites of a certain restriction endonuclease differs between individuals. Hence, the length of the DNA fragments produced by a restriction endonuclease will differ across both individual organisms and species.

Steps of RFLP

The procedure of RFLP involves isolation of DNA samples, their restriction digestion and separation of fragments on gel followed by visualization. The details of these steps are as follows :

1. **DNA Extraction :** To begin with, DNA is extracted from blood, saliva or other samples and purified through salting out or other appropriate procedure.
2. **DNA Fragmentation :** The purified DNA is digested using restriction endonucleases. The recognition sites of these enzymes are generally 4 to 6 base pairs in length. The shorter the sequence recognized, the greater the number of fragments generated from digestion. For example, if there is a short sequence of GAGC that occurs repeatedly in a

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sample of DNA, then the restriction endonuclease that recognizes the GAGC sequence will cut the DNA at every repetition of the GAGC pattern. If one sample repeats the GAGC sequence 4 times whilst another sample repeats it 2 times, the length of the fragments generated by the enzyme for the two samples will be different.

3. **Gel Electrophoresis :** The restriction fragments produced during DNA fragmentation are analyzed using gel electrophoresis. The fragments are negatively charged and can be easily separated by electrophoresis, which separates molecules based on their size and charge. The fragmented DNA samples are placed in the chamber containing the electrophoretic gel and two electrodes.

   When an electric field is applied, the fragments migrate towards the positive electrode. Smaller fragments move faster through the gel leaving the larger ones behind and thus the DNA samples are separated into distinct bands on the gel.

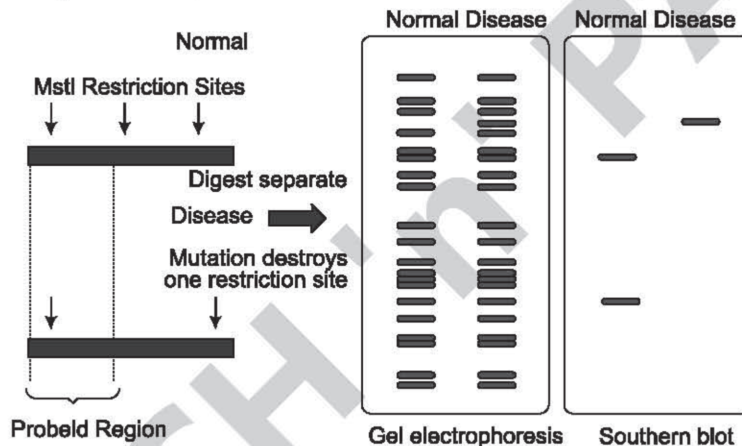

Fig. Restriction Fragment Length Polymorphism (RFLP)

4. **Visualization of Bands :** The gel is treated with luminescent dyes in order to make the DNA bands visible.

Disadvantages of RFLP

Since its invention, RFLP has been a widely used genome analysis technique employed in forensic science, medicine, and genetic studies. However, it has become almost obsolete with the advent of relatively simple and less expensive DNA profiling technologies such as the polymerase chain reaction (PCR).

The RFLP procedure requires numerous steps and takes weeks to yield results, while techniques such as PCR can amplify target DNA sequences in a mere few hours.

Additionally, RFLP requires a large DNA sample, the isolation of which can be a laborious and time-consuming process. In contrast, PCR can amplify minute amounts of DNA in a matter of hours.

Due to numerous reasons such as these, the PCR technique has largely replaced RFLP in most applications requiring DNA sequencing such as paternity testing or forensic sample analysis.

Furthermore, the identification of single-nucleotide polymorphisms in the Human Genome Project has almost replaced the need for RFLP in disease status analysis.

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Q.5. Explain in detail the method of gene amplification through PCR. Also discuss the applications of PCR in Biological science.

Ans.

Requirements for PCR

The essential requirements of PCR reaction comprise of a target double-stranded DNA, two primers that hybridize to adjoining sequences on each opposite strands of the target, all the four deoxyribonucleoside triphosphates (dNTPs), and a DNA polymerase, along with buffer, enzyme co-factors, and water. Because the reaction mixture is periodically heated to the high temperatures, PCR relies on using a heat-stable DNA polymerase. Many heat-stable enzymes derived from the thermophilic bacteria (bacteria that survive in hot environments) are now commercially available. The enzyme Taq polymerase from the thermophilic bacterium. *Thermophilus aquaticus* is the first and most frequently used polymerase in PCR.

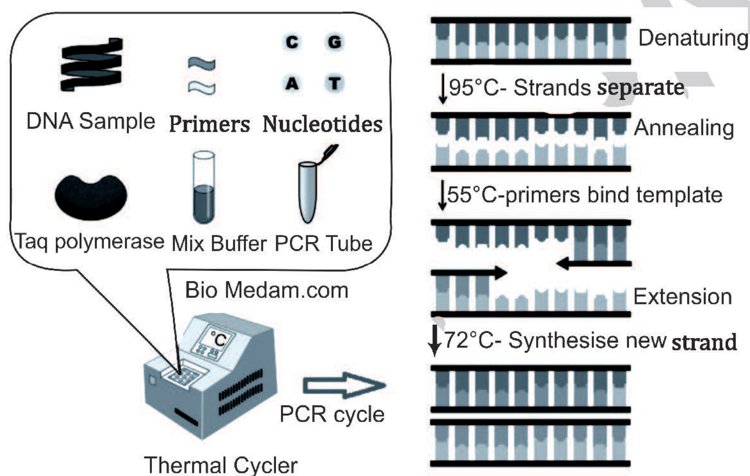

Fig. A. PCR components, B. PCR process (one cycle)

1. **Extraction and denaturation of target nucleic acid :** The first step in performing PCR is obtaining the nucleic acid which is extracted from the organism or a clinical sample containing the target organism using heat, chemical, or enzymatic methods. The extracted nucleic acid is mixed with all the PCR components (primers, nucleotides, covalent ions, buffer, and enzyme) and placed in a thermal cycler for amplification.
2. **Steps involved in amplification :** This is the most important phase of PCR and involves multiple (25-50) cycles of replication. Each cycle comprises of three sequential reactions - denaturation of target DNA, annealing of primer to target nucleic acid, and extension of primer on target duplex.
   (i) **Denaturation of Target DNA :** The target DNA is denatured by quickly heating the reaction mixture to 95°C (about 15-30 seconds) so that the single strands formed after denaturation might act as templates for the synthesis of DNA.
   (ii) **Annealing of primers :** The heated mixture of denatured DNA, primers and other PCR reagents is rapidly cooled to a predetermined temperature, allowing the two primers to bind to the sequences on the two strands flanking the target DNA. Primers are short, single-stranded nucleic acid sequences (usually 20 to 30

nucleotides long) meant to specifically hybridise (anneal) to a specific nucleic acid target, essentially acting as probes (usually around 55°C). Each strand is bound by a different primer. Because the primers are abundant in parental DNA, the two parental strands do not re-anneal with each other.

(iii) **Extension :** The extension of DNA on primers requires the temperature of the mixture to be raised to 72°C and held there for a predetermined amount of time to allow the DNA polymerase to elongate each primer in 3' direction by copying the single-stranded DNA templates. Taq polymerase is a commonly used enzyme for primer extension, which occurs at 72°C. This enzyme is used because of its heat stability which allows it to withstand the denaturing temperature of 94°C over multiple cycles. The ability to allow primer annealing and extension at high temperatures without harming the polymerase increases the stringency of the reaction, lowering the possibility of non-target nucleic acid amplification (*i.e.,* nonspecific amplification).

The three steps of PCR cycle mentioned above are allowed to repeat several times. The four DNA strands obtained after the completion of the first cycle bind primersand are extended in the second cycle. There is no need to add any additional reactants. The three steps are repeated for a third cycle, and so on for an indefinite number of cycles. By the third cycle, some PCR products only represent the DNA sequence between the two primer sites and do not extend beyond these sites. The number of double-stranded DNA synthesised and hence the amount of DNA formed increases with every new cycle. The initial amount of DNA amplifies to a million times after 20 cycles of PCR and after 30 cycles the amount increases a billion times.

3. **Analysis of product :** Amplification of DNA is followed by gel electrophoresis of the amplified product. It is subjected to size-based separation through agarose gel electrophoresis. The amplified DNA forms clear bands that can be visualized under ultraviolet (UV) light.

Applications of PCR

PCR has many uses in molecular diagnostics and biological research. Some of its applications are as follows :

1. PCR has the ability to amplify a single DNA molecule from a complex mixture which eliminates the need for DNA cloning. Variants of the technique can also amplify a particular RNA molecule from the complex mixture.
2. PCR has led to simplification of DNA sequencing.
3. PCR can be used for mutagenesis. By using appropriate primers, PCR can induce point mutations, deletions, and insertions of target DNA, greatly facilitating the analysis of gene expression and function.
4. PCR is extremely sensitive and capable of amplification of miniscule amounts of DNA. Thus, very small amounts of DNA/RNA from specific viruses and bacteria can be distinguished in tissues using appropriate primers, making PCR invaluable for medical diagnosis.

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5. PCR-based RAPD technique is fast replacing the use of RFLP in DNA finger printing procedure. Thus, it is becoming an indispensable tool for characterizing medically significant DNA samples.
6. PCR is a technique of high significance in forensic medicine due to its extreme sensitivity. It can be used to amplify DNA even from a microscopic droplet of blood or a single hair left at the crime scene.

Q.6. Explain the DNA fingerprinting and discuss their steps and applications.

Ans.

DNA Fingerprinting

"DNA fingerprinting is a technique that shows the genetic makeup of living things. It is a method of finding the difference between the satellite DNA regions in the genome."

DNA fingerprinting, also called DNA typing, DNA profiling, genetic fingerprinting, genotyping, or identity testing, in genetics, method of isolating and identifying variable elements within the base-pair sequence of DNA (deoxyribonucleic acid). The technique was developed in 1984 by British geneticist **Alec Jeffreys**, after he noticed that certain sequences of highly variable DNA (known as minisatellites), which do not contribute to the functions of genes, are repeated within genes. Jeffreys recognized that each individual has a unique pattern of **minisatellites** (the only exceptions being multiple individuals from a single zygote, such as identical twins).

Satellite DNA regions are stretches of repetitive DNA which do not code for any specific protein. These non-coding sequences form a major chunk of the DNA profile of humans. They depict a high level of polymorphism and are the basis of DNA fingerprinting. These genes show a high level of polymorphism in all kind of tissues as a result of which they prove to be very useful in forensic studies.

Any piece of DNA sample found at a crime scene can be analysed for the level of polymorphism in the non-coding repetitive sequences. After the DNA profile is traced, it becomes easier to find the criminal by performing the DNA fingerprinting for the suspects.

Apart from crime scenes, Fingerprinting applications also prove useful in finding the parents of an unclaimed baby by conducting a paternity test on a DNA sample from the baby.

DNA Fingerprinting Steps

Alec Jeffreys developed this technique in which he used satellite DNAs also called VNTRs (Variable Number of Tandem Repeats) as a probe because it showed the high level of polymorphism. Following are the steps involved in DNA fingerprinting :

Isolating the DNA

Digesting the DNA with the help of restriction endonuclease enzymes.

Separating the digested fragments as per the fragment size by the process of electrophoresis.

Blotting the separated fragments onto synthetic membranes like nylon.

Hybridising the fragments using labelled VNTR probes.

Analysing the hybrid fragments using autoradiography.

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DNA Fingerprinting Applications

The technique of fingerprinting is used for DNA analysis in forensic tests and paternity tests. Apart from these two fields, it is also used in determining the frequency of a particular gene in a population which gives rise to diversity. In case of the change in gene frequency or genetic drift, Fingerprinting can be used to trace the role of this change in evolution.

An early use of DNA fingerprinting was in legal disputes, notably to help solve crimes and to determine paternity. Since its development, DNA fingerprinting has led to the conviction of numerous criminals and to the freeing from prison of many individuals who were wrongly convicted. However, making scientific identification coincide exactly with legal proof is often problematic. Even a single suggestion of the possibility of error is sometimes enough to persuade a jury not to convict a suspect. Sample contamination, faulty preparation procedures, and mistakes in interpretation of results are major sources of error. In addition, RFLP requires large amounts of high-quality DNA, which limits its application in forensics. Forensic DNA samples frequently are degraded or are collected postmortem, which means that they are lower quality and subject to producing less-reliable results than samples that are obtained from a living individual. Some of the concerns with DNA fingerprinting, and specifically the use of RFLP, subsided with the development of PCR- and STR-based approaches.

Q.7. Explain various variants of ELISA and discuss their applications in medical diagnosties.

Ans.

Various Variants of ELISA Techniques

Depending on the nature of analytes to be detected, ELISA tests can be performed by following four methods which use different schemes of antigen-antibody binding and have their own merits and demerits: direct, indirect, sandwich, and competitive ELISA.

1. **Direct ELISA :** It is the simplest form of the ELISA in which the antigen or sample is immobilized directly on the microtiter plate which is the solid phase that passively soaks up the antigen on incubation (for one hour at 37°C or can be incubated at 4°C overnight). Any unbound antigen is washed away after incubation with the use of agents (BSA, ovalbumin, aprotinin, or other animal proteins) so that only the solid phase is left. Specific antibodies for the antigen designate carrying an enzyme (conjugate) are then added and the plate is incubated again. The unbound conjugate is

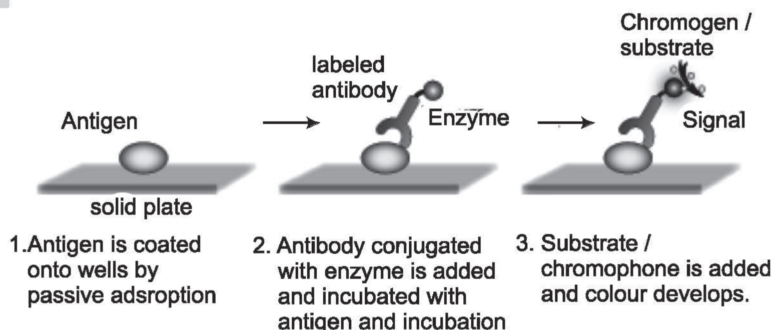

Fig. : Direct ELISA

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washed away after the enzyme-coupled antibodies bind with the antigen on the solid phase. A chromophore (color carrier) is additionally supplied which acts as a catalyst for the enzyme to produce a colored product. Finally, the reaction concludes after a brief period and the intensity of colour is measured through a spectrophotometer.

Direct ELISA is used to assess antigen-antibody specificity and for investigating blocking (inhibitory) reactions.

2. **Indirect ELISA :** It is similar to the direct ELISA with respect to the first step which involves immobilization of an antigen. However, unlike direct ELISA, it needs two antibodies—the main detection antibody that binds to the protein and a peripheral enzyme-linked antibody. First of all the main antibody is added, the plates are washed and then the peripheral enzyme-linked antibody (secondary antibody) is supplied and the plates are incubated. The steps are now similar to the steps of direct ELISA subject to the detection of a color change. Thus, indirect ELISA involves one additional step of washing. Through indirect ELISA, numerous antisera can be examined for binding to a given antigen by utilizing only a single anti-species conjugate. This method is mostly utilized for the diagnostic applications while exploring a large number of samples.

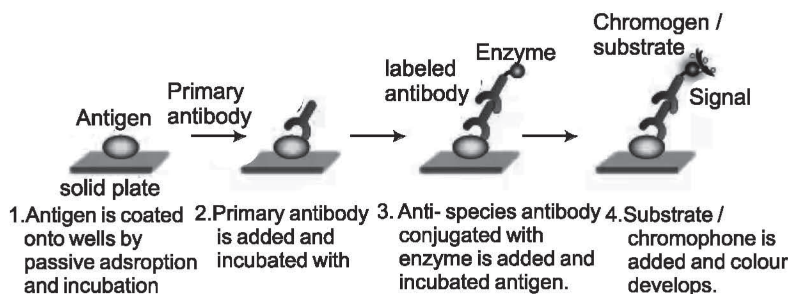

Fig. : Indirect ELISA

3. **Sandwich ELISA :** This is the most common type of ELISA and is extensively used in diagnostic procedures. The name of this method is based on the fact that antigen molecules are sandwiched between two antibody molecules. The antibody molecule that binds the microtiter plate is called capture antibody and the other one which carries the enzyme is called detection antibody. Both the antibodies bind to different epitopes on the antigen sandwiched between them. It is important to coat the well surface with a certain amount of bound antibody for acquiring the required antigen. After adding a particular primary capture) antibody to the plates and incubating it overnight, the plates are washed in the buffer at room temperature. After this the antigen is added and the plate is incubated to allow the binding of antigen with the primary antibody. A conjugated-detection antibody is then added which binds to a different epitope on the antigen. When the substrate is added it undergoes catalysis by

the enzyme linked to the detection antibody and a gives a coloured signal which is proportional to the amount of analyte present in the sample.

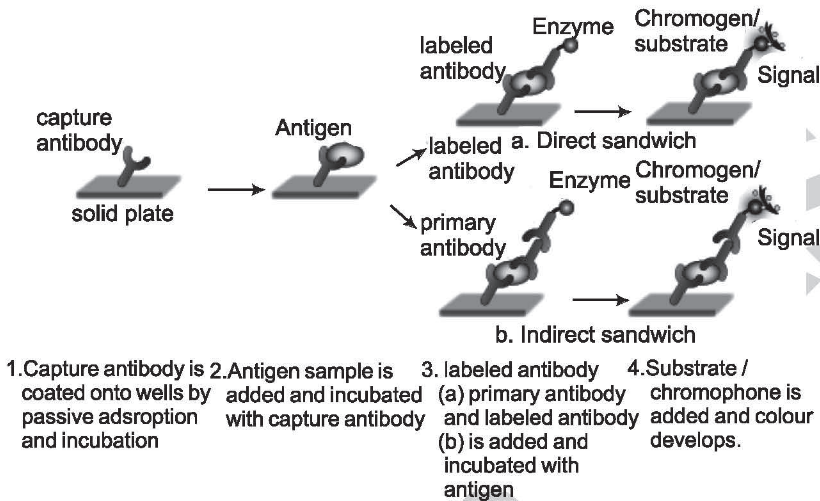

Fig. : Sandwich ELISA

4. **Competitive ELISA :** In this version of ELISA, there is competition for binding with the main antibody between the sample antigen and the antigen present on the wells of the microtiter plate. In the protocol of competitive ELISA, at first a primary or capture antibody (unlabeled) is incubated with sample antigen. Next, antigen-antibody complexes are added to 96-well plates which are coated with the same antigen. The plate is then washed to remove any unbound antibody. Here it should be noted that due to competition, if there is more antigen in the well, less antibody will be able to bind to the antigen in the well. Now a secondary antibody (detection antibody) conjugated with the enzyme is added which is specific for primary antibody. Addition of a substrate then elicits a colour reaction which can be measured. Thus, in this ELISA, a weaker signal indicates a higher concentration of sample antigen.

   Competitive ELISA is used for small molecules *i.e.,* when the protein of interest is too small to be sandwiched between two antibodies. This version of ELISA is helpful in measuring concentrations of small molecules such as hormones.

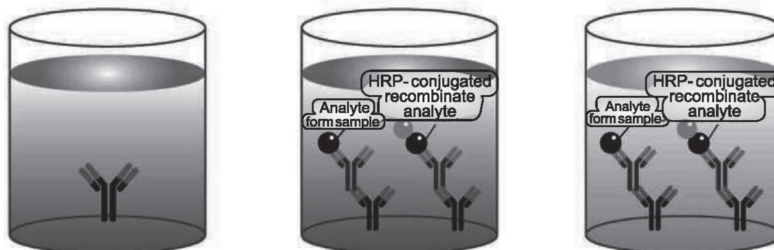

Fig. : Copetitive ELISA

Applications of ELISA

It is a very common and extremely reliable tool in medical diagnostics. It finds following important applications in biology and medicine :

1. It is used to determine the presence of antigens and antibodies in a sample.
2. It is commonly used in the food processing industry to detect the presence of food allergens.
3. It helps determine the serum antibody concentration in a virus test.
4. During a disease outbreak, rapid testing kits are used to confirm and quantify the presence of antibodies in blood samples which is critical in assessing the spread of the disease, such as during the recent COVID-19 outbreak.

Q.8. Explain the procedure of Western blotting. Also gives its applications and limitations.

Ans.

Procedure of Western Blotting

The technique of Western blotting consists of three major processes-PAGE, blotting, and detection using probes. The details of these steps are as below :

1. **Separation of Proteins :** At first the proteins are separated by gel electrophoresis (native PAGE or SDS-PAGE) which separates them according to their size.
2. **Transfer to Membrane (Blotting) :** The process of blotting is the most important part of the western blotting technique. It involves the act of transferring protein bands from the polyacrylamide gel to nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membranes. Blotting is done by keeping the nitrocellulose membrane just above the gel and putting a stack of papers along with weights above the membrane. In such an arrangement, proteins move upwards from polyacrylamide gels and get stuck onto the nitrocellulose membrane. The purpose of blotting is to immobilize proteins on nitrocellulose membrane where they can be detected by probes.
3. **Detection Using Probes :** After blotting is done, the protein bands on the nitrocellulose membrane can be detected using antibodies as probes. Protein bands on the nitrocellulose membrane are targeted either by a reporter-labeled primary antibody or by a reporter-labeled secondary antibody against the primary antibody. The secondary antibody binds to the primary antibody at one end and is also conjugated to an enzyme. The enzyme-conjugated primary or secondary antibody produces colour reaction on coming in contact with suitable substrate.

   In an alternative method the protein bands on the membrane can be radioactively labelled. By exposing such a membrane to an X-ray film in a process known as **autoradiography**, it is possible to detect the location of the protein of interest on the blot if it is bound by a radioactive antibody. However, enzyme-linked antibodies against the protein are safer than radioactively labelled antibodies and are used in the most widely practised detection methods. The formation of a colourful band at the location of the target antigen is caused by the addition of a chromogenic substrate after

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the enzyme-antibody conjugate has bound. This result is highly coloured and insoluble. If a chemiluminescent substance is combined with the appropriate boosting agents to create light at the antigen site, the location of the protein of interest may be identified with a much better sensitivity.

Applications of Western Blotting

Western blotting is a widely used method in molecular biology and has numerous applications, some of which are as follows :

1. Identification of a specific protein in a complex mixture of proteins is a very common use of Western blotting.
2. With Western blotting, it is also possible to estimate the size of the protein as well as the amount of protein present in the mixture.
3. It is most widely used as a confirmatory test for the diagnosis of HIV, where this procedure is used to determine whether the patient has antibodies that react with one or more viral proteins or not.
4. Western blotting has been used for demonstration of specific antibodies in the serum which is a part of the diagnosis of diseases like neurocysticercosis and tubercular meningitis.

Limitations of Western Blotting

Despite having several advantages, the technique of western blotting has certain limitations too. Some of these are as follows :

1. Since Western blotting is a highly sensitive process, any imbalance in the procedure can interrupt the outcomes of the whole process.
2. In some cases, no bands or erroneous bands may be observed due to deficient protein transfer.
3. With Western blotting, estimation or quantitative measurement is not always precise. Hence, the test can only be used as a semi-quantitative test.
4. Since Western blotting is a time-consuming and complex process, it should be carried out by properly trained personnel only.
5. Western blotting can only be done if the primary antibodies for the proteins are available.
6. Some antibodies may have off-target effects by interacting with multiple proteins in the sample.
7. The technique is quite expensive and requires the purchase of antibodies as well as expensive detection methods.
8. There is problem with both very small and very large proteins. Small proteins may be lost by the membrane, whereas larger proteins may be difficult to transfer to the membrane.

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MODEL PAPER

Molecular Biology, Bioinstrumentation & Biotechniques

B.Sc.-II (SEM-III) [M.M. : 75

Note : Attempt all the sections as per instructions.

Section-A : Very Short Answer Type Questions

Instruction : Attempt all **FIVE** questions. Each question carries **3 Marks**. Very Short Answer is required, not exceeding 75 words. **[3 × 5 = 15]**

1. What is gene in DNA?
2. What is central dogma?
3. What do you mean by gene expression?
4. What is the first step in eukaryotic gene expression?
5. Who created the centrifuge?

Section-B : Short Answer Type Questions

Instruction : Attempt all **TWO** questions out of the following 3 questions. Each question carries **7.5 Marks**. Short Answer is required not exceeding 200 words. **[7.5 × 2 = 15]**

6. What do you mean by gene silencing?
7. Write about the history of microscope.
8. Write a short note on the pH scale?

Section-C : Long Answer Type Questions

Instruction : Attempt all **THREE** questions out of the following 5 questions. Each question carries **15 Marks**. Answer is required in detail, between 500-800 words. **[15 × 3 = 45]**

9. What is collinearity principle of gene? Why is this not applicable in case of eukaryotes?
10. Write about the characteristics of genetic code.
11. Describe the composition and working of tryptophan in prokaryotes.
12. Explain the basic principles and applications of microscopes.
13. Explain the procedure of Western blotting. Also gives its applications and limitations.

●

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- यद्यपि इस पुस्तक को यथासम्भव शुद्ध एवं त्रुटिरहित प्रस्तुत करने का भरसक प्रयास किया गया है, तथापि इसमें कोई कमी अथवा त्रुटि अनिच्छाकृत ढंग से रह गई हो तो उससे कारित क्षति अथवा सन्ताप के लिए लेखक, प्रकाशक तथा मुद्रक का कोई दायित्व नहीं होगा। सभी विवादित मामलों का न्यायक्षेत्र मेरठ न्यायालय के अधीन होगा।
- इस पुस्तक में समाहित सम्पूर्ण पाठ्य-सामग्री (रेखा व छायाचित्रों सहित) के सर्वाधिकार प्रकाशक के अधीन हैं। अत: कोई भी व्यक्ति इस पुस्तक का नाम, टाइटिल-डिजाइन तथा पाठ्य-सामग्री आदि को आंशिक या पूर्ण रूप से तोड़-मरोड़कर प्रकाशित करने का प्रयास न करें, अन्यथा कानूनी तौर पर हर्जे-खर्चे व हानि के जिम्मेदार होंगे।
- इस पुस्तक में रह गई तथ्यात्मक त्रुटियों तथा अन्य किसी भी कमी के लिए विद्वत् पाठकगण से भूल-सुधार/सुझाव एवं टिप्पणियाँ सादर आमन्त्रित हैं। प्राप्त सुझावों अथवा त्रुटियों का समायोजन आगामी संस्करण में कर दिया जाएगा। किसी भी प्रकार के भूल-सुधार/सुझाव आप info@vidyauniversitypress.com पर भी ई-मेल कर सकते हैं।

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