DISTANCE LEARNING MATERIAL

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION

MIDNAPORE - 721 102

M. Sc. in Zoology

PART - I

Paper - II - Group : A & B

Module No. - 14, 15, 16, 17, 18, 19, 20, 22/A, 23 & 23/A

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M. Sc. in ZOOLOGY

Paper - II, Part-I, Group-A
Unit-I

Module No. 14

Mutation, Transposable element, Cancer, Hardy-Weinberg equilibrium

Chaper I - "Mutation"

A heritable change in base sequence can occur if one of the strands in a parental duplex DNA molecule undergoes a temporary change in sequence just at the time the chromosome is being replicated so that the daughter strand comes to contain one or more wrong bases. This is called tautomeric shift.

The purine and purimidines of DNA usually exist in particular tautomeric forms and that both the stability of an AT or a GC base pair and the accuracy of base pairing during replication are dependent on the existence of these stable chemical configurations.

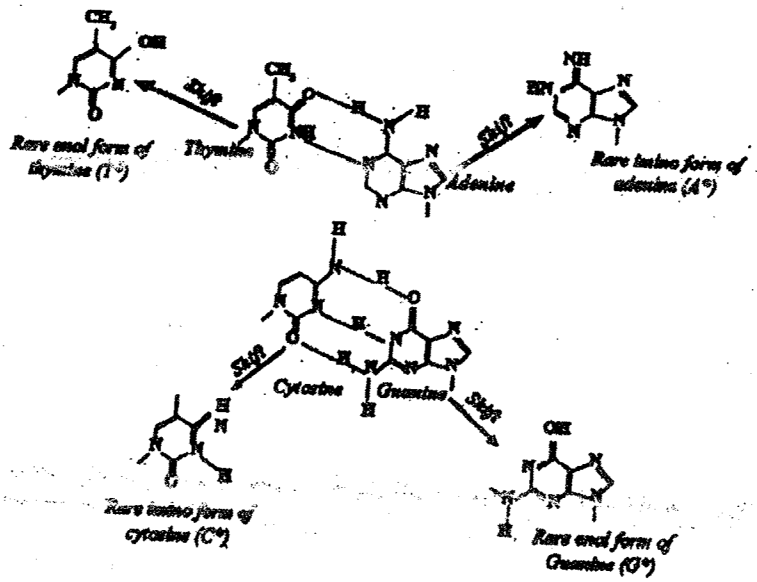


Fig. 1. The Tautomeric forms of pyrimidines and purines. In the center are shown the stable forms of thymine, adenine, cytosine and guanine as they participate in AT and CG pairing. The tautomeric from of each molecules is indicated at arrows.

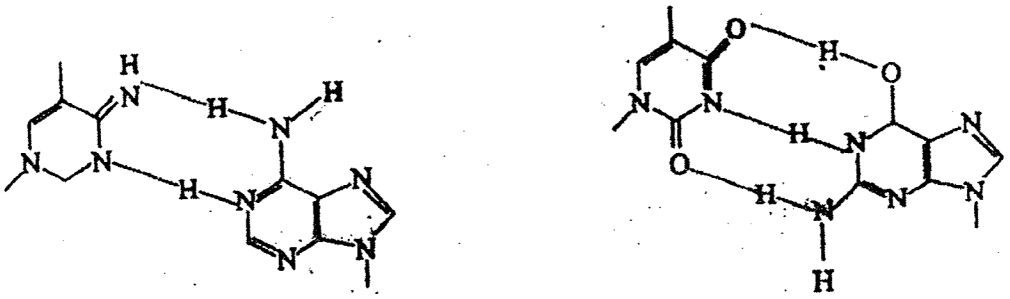


Fig. 2. Base pairing of C (tautomer of cytosine) with normal adenine and G* (tautomer of guanine) with normal thymine.*

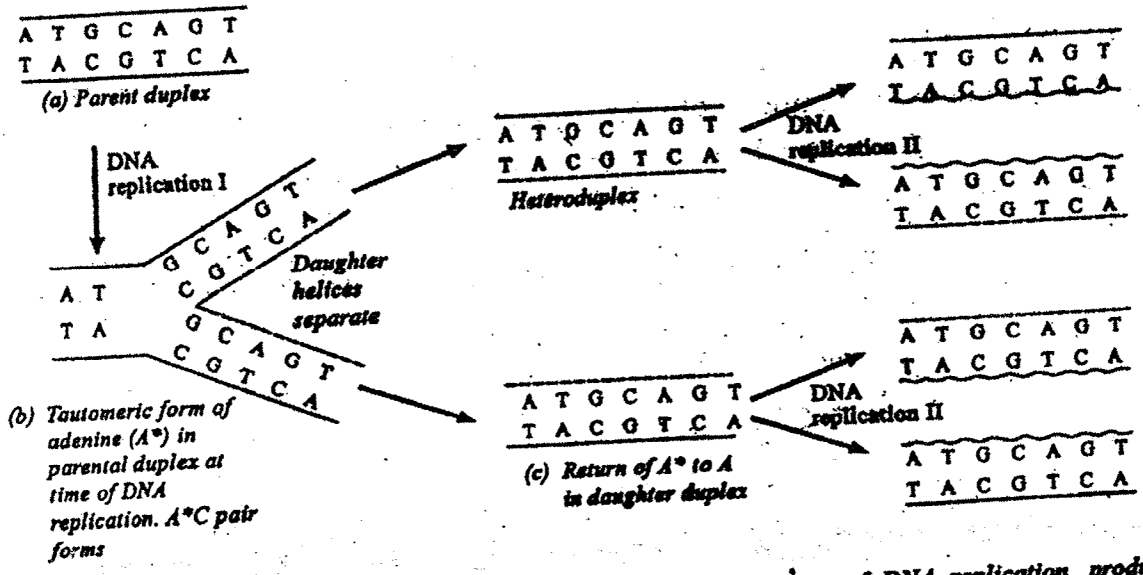


Fig. 3. Mutagenesis through tautomeric shift in an A molecule at the time of DNA replication, producing an AT → GC transition.

The stable tautomers of thymine, adenine, cytosine and guanine are shown in the center of Figure 1 and their normal hydrogen-bonded configurations are indicated. These bases can, on occassion, undergo tautomeric shifts in which certain hydrogen atoms migrate to new unstable positions. Fig. 1 illustrates their effect when they alter the stae of the atoms at the critical 1 and 6 positions on the purine & pyrimidine rings. In all cases the resulting molecules T*, C*, A* and G* have modified pairing properties. Specificially, such shifts endow adenine with base-pairing properties of guanine, guanine with those of adenine, cytosine with those of thymine and thymine with those of cytosine.

Figure 2 illustrate such mistaken pairing as it occurs between C* and adenine and between G* and thymine.

Waston & Crick predicted that on rare & short lived occasions such tautomeric shifts might happen at the moment a particular portion of a DNA strand is being replicated. A mistake in base pairing might then result & the daughter polynucleotide chain would then carry a wrong base. In the example shown in Figure 3. A is shown undergoing a temporary shift during replication in which it pairs with C instead of T. A heteroduplex molecule results and is inherited by a daughter cell. At the next round of replication the strand carrying the worng base will pair as it normally does in Figure 3, C is seen to pair normally with G – and the resulting duplex will thus carry a GC pair where the original molecule carried an AT pair. This replacement of AT by GC (or GC by AT) is known as a transition. More generally a transition can be defined as the insertion of one purine where the other is called for or the insertion of one pyrimidine where the other is called for.

It is to be noted that the pairing errors arise through the transition of the pairing relationship of one purine base (e.g. adenine) into the pairing form of another purine base (e.g. gaunine) or of one pyrimidine (e.g. thymine) into that of another pyrimidine (e.g. cytosine). Because of this they have been called trasitional errors Freese have also proposed the possibility of transversion in which a purine such as adenine for example, may be changed into a pyrimidine such as cytosine (Fig. 4). Thus for both transitions & transversions, a base substitution is produced which leads to a new nucleotide sequence.

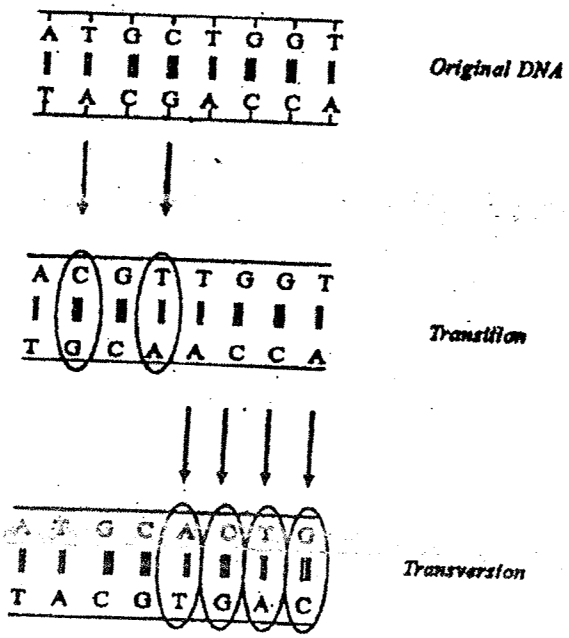


Fig. 4. Types of possible nucleotide substitution

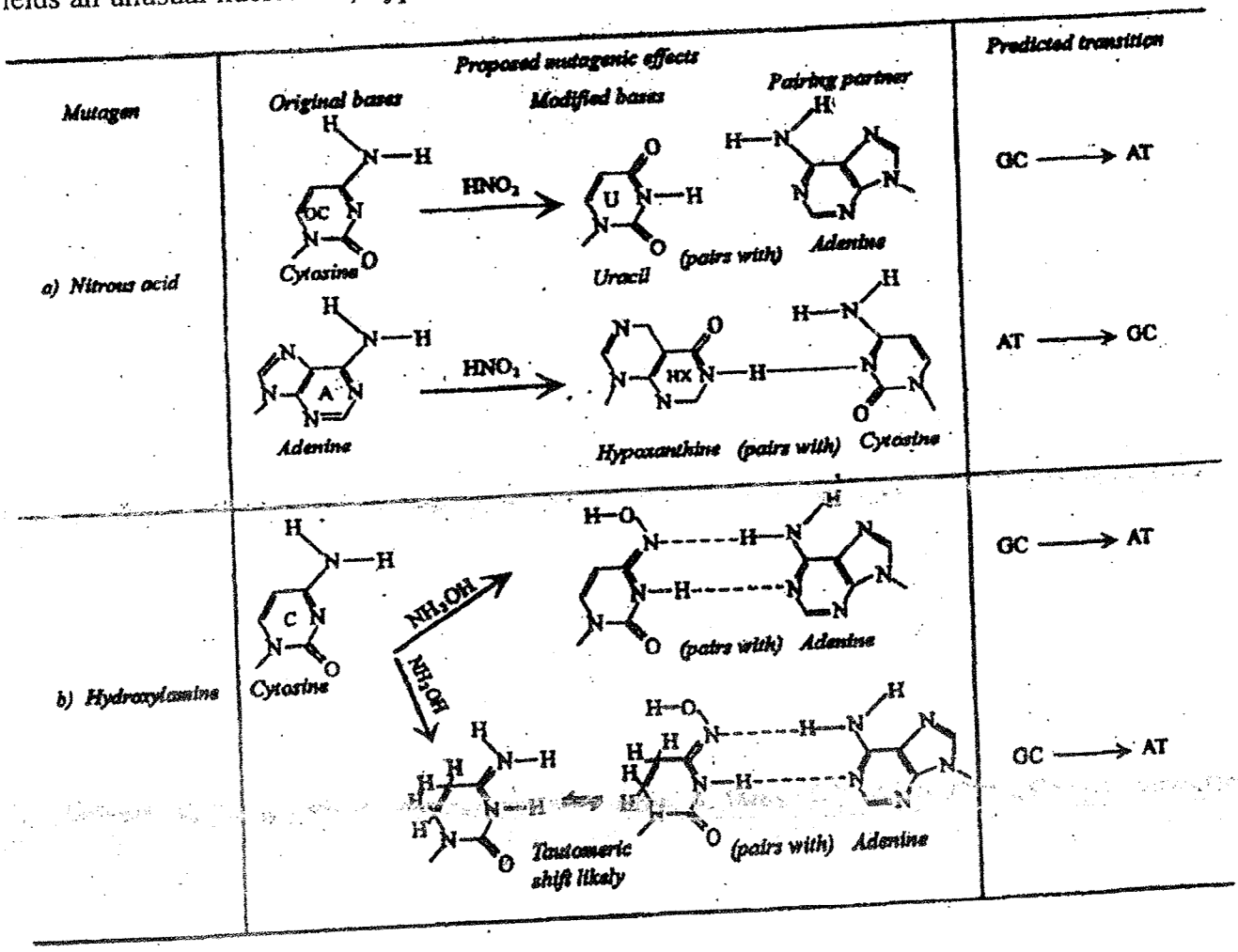
Permanent Alteration in Nucleotide Structure :

In contrast to the transitory tautomeric shifts, a no. of mutations are induced by permanently altering a base in a parental DNA molecule. The alteration need not occur at the time the DNA is replicating but to qualify as a mutation it must have as its end result a mistake made during replication.

Such permanent alterations in nucleotide structure can be effected by a variety of chemicals. The three most widely used mutagens of this sort are nitrous acid (HNO_2), hydroxylamine (NH_2OH) and a class of chemical that acts to alkylate the bases.

Nitrous acid :

HNO_2 deaminates nucleotides, removing amino ($-NH_2$) groups & substituting instead a keto group (=o). As shown in Figure 5 deamination of cytosine yields uracil & deamination of adenine yields an unusual nucleotide, hypoxanthine (HX). Uracil is seen to pair with adenine & thus the



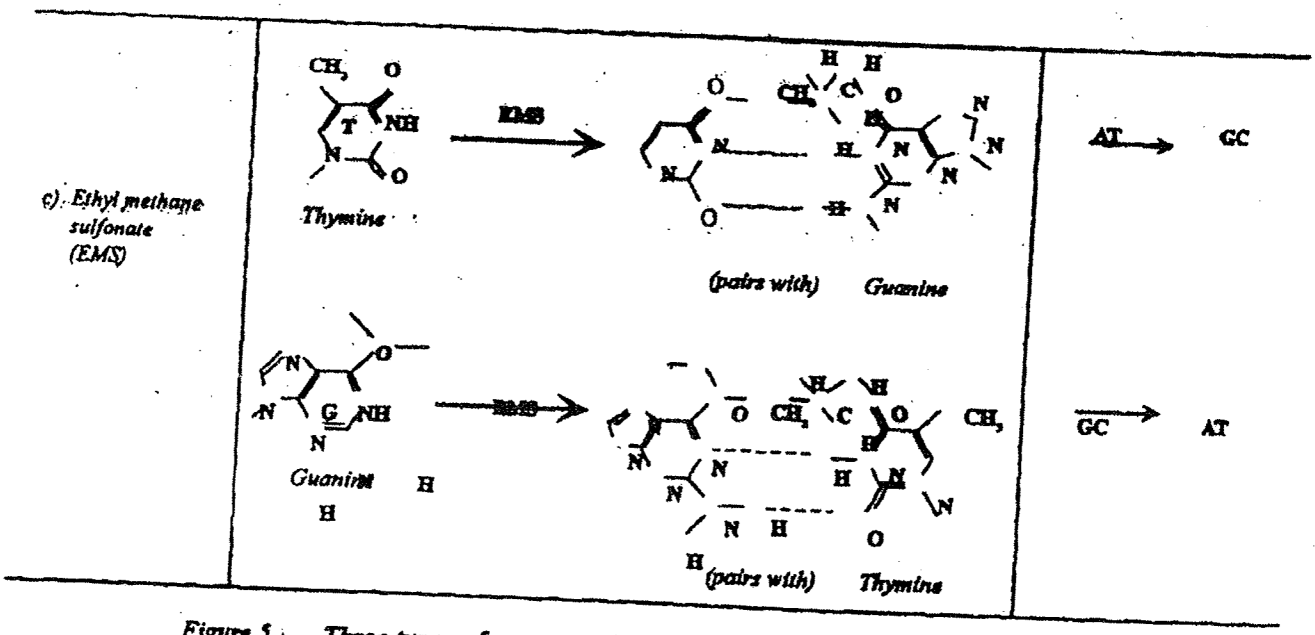


Figure 5. ***Three types of mutagens that modify the chemical structure of bases***

deamination of cytosine can produce a GC → AT transition. Hx resembles guanine at the critical 1 and 6 position on the ring & can pair with cytosine as shown in Figure 5. Again a transition results AT → GC. Figure 5 showing three types of mutagens that modify the chemical structure of bases.

Hydroxylamine :

Hydroxylamine reacts only with pyrimidines & probably only its reactions with cytosine is mutagenic. Hydroxylamine attacks the amino group in position 6 of cytosine converting it to an oxime (=N–OH), the resulting nucleotide preperantially pairs with adenine.

Alkylating Agents :

A variety of highly reactive chemicals introduces an alkyl group (CH_3^-, CH_3, CH_2^- etc.) into the nucleotides. These chemicals include mustard gas & the epoxides, dimethyl and diethyl sulfonate, methyl and ethyl methanesulfonate (MMS & EMS) and nitrosoguanadine (NG). EMS and NG appear to alkylate the keto groups at 6 position of thymine & guanine forcing them to undergo tautomerization and thus bringing about transitions.

Bast Analogs :

In the preceding section we considered mutagens whose mode of action is, at least primarily, the permanent alteration of the chemical structure of nucleotides in the parental DNA or RNA. They are thus capable of producing mutations even when the nucleic acid is not undergoing replication.

Another class of chemical mutagen does not affect the parental DNA or RNA at all & acts only at the time nucleic acids are replicating, known as base analogs. These mutagens have chemical structure analogous to naturally occuring bases but carry critical modifications.

Two of the most commonly used base analogs are 5-bromouracil (5-BU) and its nucleoside 5-bromodeyxyuridine (5-BUDR). 5-BU is an analog of thymine and carries a bromine atom in place of methyl group on carbon 5 (Fig. 6). The presence of bromine atom in this molecule changes its charge

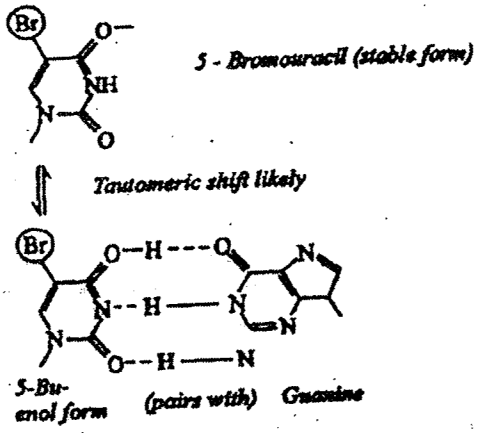
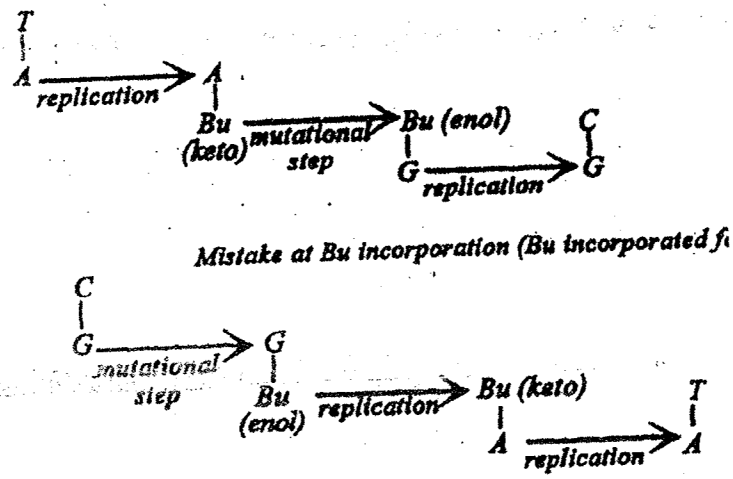
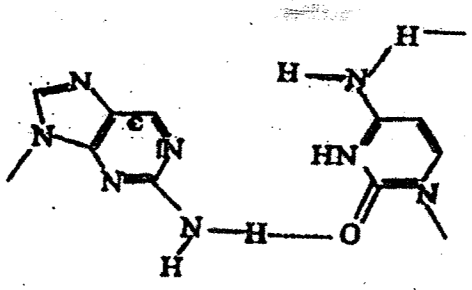


Fig. 6.

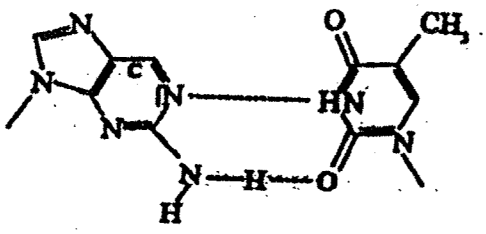
distribution & thus increased the likelihood that it will undergo a tautomeric shift. The tautomeric form 5-BU* possesses the hydrogen-bonding properties of cytosine rather than thymine. Therefore it a 5-BU* nucleoside triphosphate is mistaken for cytosine at the time of nucleic acid replication, it may be brought in by the polymerase to part with guanine. Figure showing mistake at BU replication (BU incorporated for T, but pairs with G)





2-Aminopurine (2-AP) (pairs with) Cytosine

2-aminopurine and its proposed base pair formation with cytosine and with thymine.

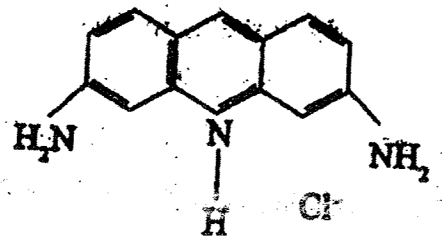


2-Aminopurine (2-AP) Thymine

Fig. 7 : Another widely used analog is 2-aminopurine can pair with C/T.

Acridines :

A third class of chemical mutagen consists of aromatic molecules known as acridines. The most widely used acridine, proflavin shown in Fig. 6. Acridine produce mutations that are not revertible by analogs or hydroxylamine. These mutations apparently involve deletion or addition of one to at least 20 bases in a nucleic acid.



Proflavin (Chloride) : 2, 8 - diamino acridine hydrochloride

Fig. The structure of proflavin, an acridine mutagen.

Acridine dyes such as proflavin and acridine orange are other mutagens that seem to produce direct effects on the DNA molecule. According to Lerman, acridine dyes act by inserting themselves between two neighboring purine bases in a single DNA strand. Thus acridine mutations would not be expected transitions as do base analogues, nitrous acid, hydroxylamine & alkylating agents.

Ref. Book :

Genetics by strickberger.

Sample Question :

1. A DNA mol. which contains the tetranucleotide sequence

   C – G
   G – C
   A – T
   T – A

   is subjected to the following mutagenic agents :

   a) 5-bromouracil

   b) 2-aminopurine

   c) Nitrous acid

   d) Hydroxylamine

   e) Ethyl ethane sulfonate.

   For each of these mutagenic agents, show the sequence of changes that mutational alteration in at best one nucleotide pair in replicates of this DNA chain.

2. Identify the following point mutations represented in DNA and in RNA as (1) transition (2) transversion or (3) reading frameshifts

   a) G → C

   b) G → C

   c) UAU ACC UAU → UAU AAC CUA

   d) UUG CUA AUA → UUG CUG AUA

Chapter II - Transposable elements in Bacteria :

Within the framework of the genome, however, are mechanisms that allow the movement of certain sequences. We obtained evidence of this type of rearrangement, involving *transposable elements* or *transposons*. Many such elements have been found in the DNA of prokaryotes. Bacterial transposition involves duplication of the element one copy remains at the original 'donor' site, while the other copy appears at a new 'recipient' or 'target' site.

The transposons are called *insertion sequences*. Each type is given the prefix 1S, followed by a number that identifies the type. The original classes were numbered 1s !-4. The 1S elements are normal constituents of bacterial chromosomes & plasmids. For example, one standard strain of E. coli has eight copies of 1s 1 and five copies of 1S 2. To describe an insertion into a particular site, a double colon is used, lambda :: 1S 1 describes an 1S 1 element inserted into phage lambda.

The 1S elements identify the simplest class of transposons, since their genetic functions are concerned only with the ability to transpose. Although each 1S element is different in sequence, all share a common form of organization. Each element possesses short inverted terminal repeats. Most commonly these are between 15 and 25 bp long. Usually the two copies of the repeat are closely related rather than idèntical. Figure 1 shows that the same sequence is encourntered proceeding toward the element from the flanking DNA on either side of it.

Fig. 1 showing transposons have inverted terminal repeats and generate direct repeats of flanking DNA at the raget site.

		Target site			
		MMMM ATGCA MMMM			
		MMMM TACGT MMMM			
MMMM	ATGCA	123456789 987654321		ATGCA	MMMM
MMMM	TACGT	123456789 987654321		TACGT	MMMM

Here Target is 5 bp sequence. The ends of the transposon consist 9 bp. When an 1S element transposes, a sequence of host DNA at the site insertion is duplicated. The 1S DNA is always flanked by very short *direct repeats*. In this context, 'direct' indicates that two copies of a sequence are repeated in

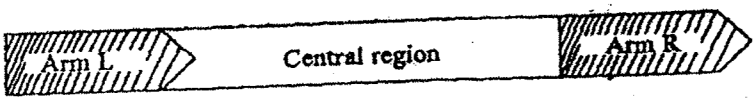
the same orientation, not that the repeats are adjacent. In Figure 1, the target site consists of the sequence $\frac{ATGCA}{TACGT}$. After transposition, one copy of this sequence is present on either side of the transposon. The most common length for the direct repeats are 5 bp and 9 bp.

As can be seen from Fig. 1, a transposon therefore displays a characteristic structure in which its ends are identified by the inverted terminal repeats. While the adjacent ends of the flanking host DNA are identified by the short direct repeats. When observed in a sequence of DNA, this type of organization is taken to be diagnostic of a transposon.

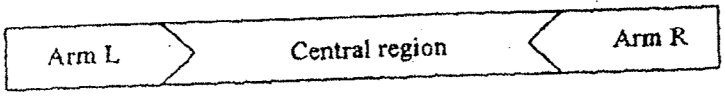
Composite transposons have IS modules :

Some transposons cary drug resistance markers as well as the functions concerend with transposition. These transpons are named Tn followed by a number. One class of larger transposons comprises *composite elements* that consist of a central region carrying the drug marker (S) flanked on either side by arms whose sequences are related. These arms sometimes have been called lengthy terminal repeats.

The arms may be in either the same or an inverted orientation. Thus a composite transposon with arms that are direct repeats has the structure.



In the arms are inverted repeats, the structure is



The arrows indicate the orientation of the arms, which are identified as L and R according to an

orientation of the genetic map of the transposon from left to right. The structure of a composite transposon is illustrated in more detail in Fig. 2.

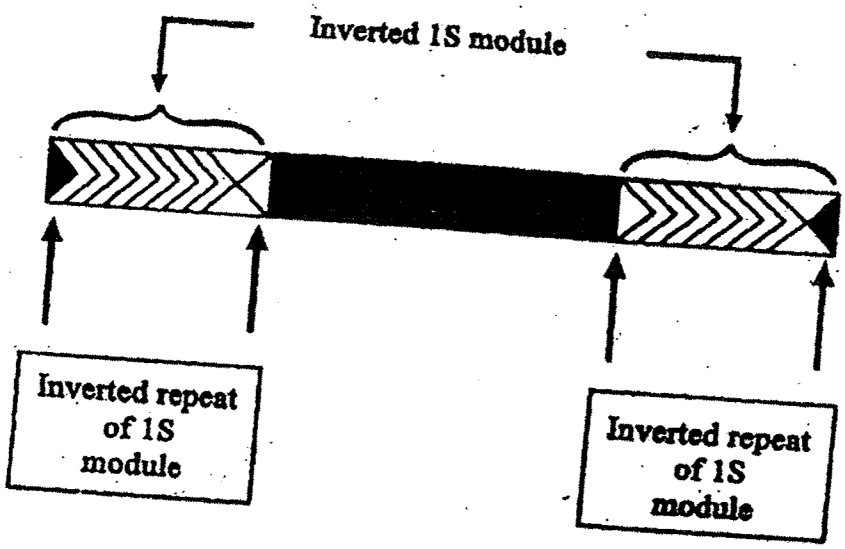


Fig. 2 shows a composite transposon has a central region carrying markers unconnected with transposition (such as drug resistance) flanked by IS module. The module have short inverted terminal repeats. If the modules themselves are in inverted orientation (as drawn), the short inverted terminal repeats at the ends of the transposon are identicals.

The arms may consist of identical IS elements. In the ease of Tn 9, a direct repeat of an IS 1 element is present at each end of the transposon. The properties of some composite transposons are summerized in the following table :-

Element	Length (bp)	Genetic markers	Terminal module	Module Orientation	Module function
Tn 10	9300	tat^R	IS 10R	Inverted	fully functional
Tn 5	5700	kan^R	IS 10 L IS 50R	Inverted	Reduced function Functional
Tn 903	3100	kan^R	IS 50 L IS 903	Inverted	Functional
Tn9	2500	cam^R	IS 1	Direct	Presumed functional

Two 1S element in fact are able to transposon any sequence residing between them, as well as themselves. If Tn 10 residing on a circular replicon, its two modules can be considered to flank either the tet^r gene of the original Tn 10 or the sequence in the other part of the circle. Thus a transposition even can involve either the original Tn 10 transposon or the "inside out" transposon with the alternate central region. Note that both transposons have inverted modules, but these modules evidently can function in either orientation relative to the central region. (Fig. 3)

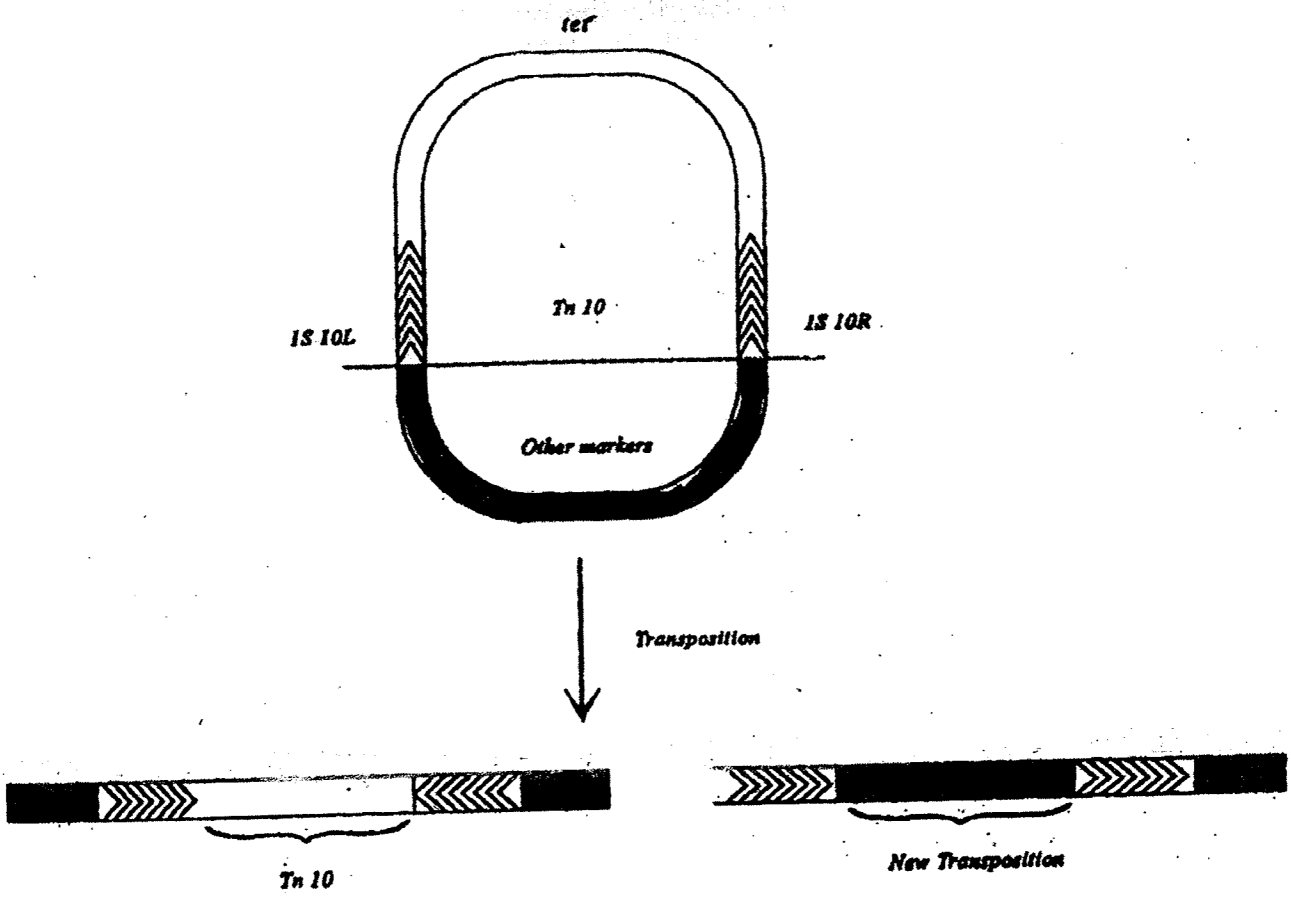


Fig. 3 shows that the IS 10 modules can cooperate to transpose any region of DNA that lies between them.

When Tn 10 is part of a small circular molecule, the IS 10 repeats can transpose either side of the circle. Transposition of tet^r corresponds to the movement of Tn 10. Transposition of the markers on the other side to creats a new "inside out" transposon.

Only one module of Tn 10 is functional :

The relationship between modules that are not longer identical may offer insights into the evolution of the composite transposon. The examples of Tn 10 and Tn 5 are the best characterized.

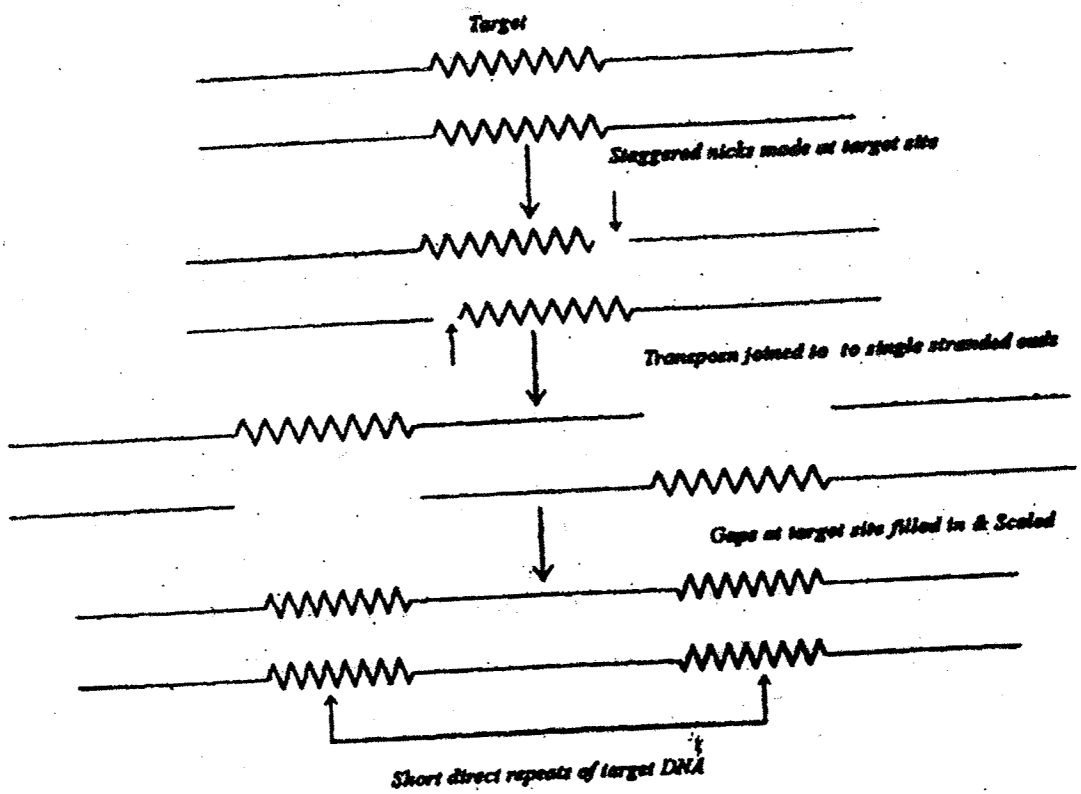
Only a few bases at each end of Tn 10 are needed to recognize the element as a substrate for transposition. The inverted terminal repeats are 22 bp long probably only the last 13 are needed for the transposition.

Tn 10 is an exceptional element that has a specific target sequence. The 9 bp direct repeats of flanking DNA generated by transposition display a consensus of a 6 bp sequence symmetrically within the target. Thus the repeats on either side of Tn 10 often take the form $\begin{matrix} NGCTNAGCN \\ NCGANTCGN \end{matrix}$ where N identifies any base pair. The stronger the hotspot, the more closely it conforms to the consensus. The element of 1s 10 R provides the active module of Tn 10. The 1S 10 L module is functionally defective and provides only 1-10% of the transposition activity of 1S 10 R.

Transposition involves replicative recombination :

'Transposition' is almost a misnomer in the sense that a transposon does not leave its former site in the course of transposition. Since the process involves duplication of the transposon, one copy remains at the original site, while the other is found at the new site. Thus transposition is accompanied by an increase in the no. of copies of the transposon.

Transposition involves two types of reaction, which in principle could occur in either order. One involves replication of the transposon without replication of adjacent chromosomal sequences. The other requires breakage of the target DNA sequence to generate a site into which the transposon is inserted. At no stage in this process is a free molecule of transposon DNA generated. The general nature of the insertion reaction is illustrated in Figure 4.



The above Fig. 4 shows the direct repeats of target DNA flanking a transposon are generated by the introduction of staggered cuts whose protruding ends ae linked to the transposon.

It consist of making staggered breaks in the target DNA joining the transposon to the protruding single - stranded ends & filling in the gaps. The generation & filling of the staggered ends explain the occurance of the direct repeats of the target DNA at the site of insertion. The stagger between the cuts on the two strands determines the length of the direct repeats, thus the target repeats characteristic of each transposon reflects the geometry of the enzyme involved in cutting target DNA.

The most revealing of the transposon mediated reaction is replicon fusion to form a cointegrate structure. A replicon containing a transposon may become fused with a replicon lacking the element. The resulting contrigrate has two copies of the transposon, one at each junction between the original replicosn, oriented as direct repeats.

A model for a transposition pathway in which a cointegrate is an obligatory intermediate is illuatrated in Fig. 5. Four single stranded cleavages initiate the process. The donor molecule is cleaved

at either end of the transposon by a site specific enzyme that recognizes the termini. The target molecule is cleaved at sites staggered (most commonly) by 5 or 9 bases.

The donor target strands are ligated at the nicks. Each end of the transposon sequence is joined to one of the protruding single strands generated at the target site. The linkage generates a chi-shaped structured held together at the duplex transposon. The formation of this structure is responsible for the movement of the transposon, the enzyme activity responsible for it is called the *transposase.*

A site specific recombination between the two copies of the transposon can regenerate the original donor replicon, releasing a target replicon that has gained a transposon flanked by short direct repeats of the hos target sequence. This reaction is called *resolution* & the enzyme responsible is called *resolvase.*

Ref. Book : Genes IV – Benjamin Lewin.

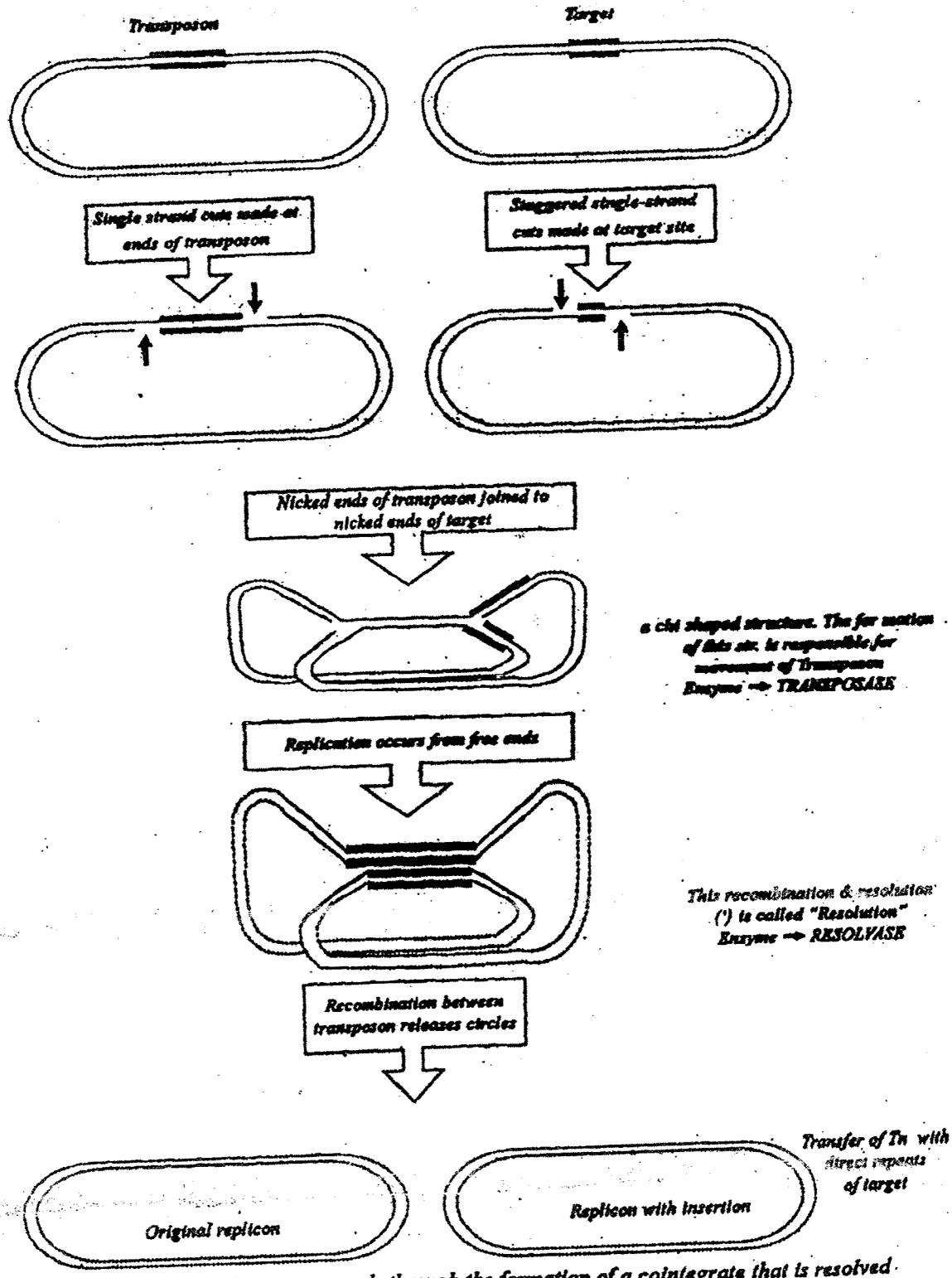


Figure 5. One pathway for transposition proceeds through the formation of a cointegrate that is resolved

Chapter III - Cancer :

Cancer may have many causes and may occur via multiple or individual events. A variety of agents increase the frequency with which cells are converted to the transformed condition, they are said to be *careinogenic*. Sometimes these carinogens ae divided into those that 'initiate' & those that 'promote' tumor formation, implying the existence of different stages in cancer development. We can sumarize the changes that occur when a cell becomes tumerogenic in 3 groups –

Immortalization describes the property of indefinite growth. *Transformation* describes the failure to observe the normal constraints of growth, for example trasformed cells become independent of factors usually needed for cell growth. Transformation subsumes immortalization, since a transformed cell must have been immortalized. *Metastasis* describe another feature in which the cancer cell gains the ability to invade normal tissue, so that it can more away from the tissue of origin & establish a new colony elsewhere in the body. Metastasis marks the distinction between a tumor that is clinically benign or malignant.

Transformation may occur spontaneously, may be caused bsy certain chemical agents & most notably, may result from infection with tumor viruses. There are many classes of tumor viruses, including both DNA and RNA viruses & they occur widely in the avian & animal kingdoms.

The transforming activity of a tumor viruse may reside in a particular gene or genes carried in the viral genome. Genes that confer the ability to convert cells to a tumerogenic state are called *oncogenes*. Table summarizes the general properties of four major classes of transforming viruses.

Viral Class	Type of virus	Oncogenes	Genome size
Papova	DNA duplex	T antigens	5-6 kb
Adeno	DNA duplex	E 1A & E 1B	~37kb
Retrovirus	RNA single str.	Individual	6-9 kb

Retrovirus :

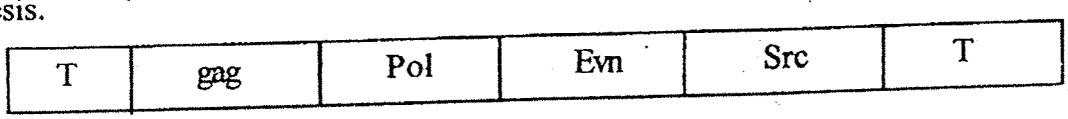
In 1911, Peyton Rous found the tumor causing agent in hen which is known as Rous Sarcoma virus (RSV). These viruses contain (+) RNA in their virions & propagate through a double-helical DNA intermediate. Hence they are known as retroviruses. In fact, retroviruses are the only RNA

viruses that can produce cancer. In 1964, Howard Temin observed that infection by RNA tumor viruses is blocked by inhibitors of DNA synthesis. This findings suggested that DNA synthesis is required for the growth of RNA tumor viruses. Hence transcription of DNA seemed to be essential for the muultiplication of RNA tumor viruses. These unexpected results led Temin to propose that a DNA provirus is an intermediate in the replication & oncogenic action of RNA tumor viruses. So genetic information.

RNA tumor virus → DNA provirus → RNA tumor virus

can flow from RNA to DNA. In 1970 Temin & Baltimore discovered an RNA directed DNA polymerase which is called reverse transcriptase, in the virions of some RNA tumor viruses.

Infecting virions bind to specific receptor on the surface of the host & enter the cell. The viral (+) RNA is uncoated in the cytosol. Reverse transcriptase then synthesizes a (–) DNA strand. This enzyme proceeds to digest the genomic RNA strand in the RNA-DNA hybrid. The (–) DNA then directs reverse transcriptase to synthesize a (+) strand. Thus reverse transcriptase carries out a sequence of three reactions. RNA-directed DNA synthesis, hydrolysis of RNA & DNA directed DNA synthesis.



Genetic map os avian sarcoma virus :

The 10 kb genome of avian sareoma virus contains four genes (See Fig. 1). Three of them – gag, pol and env are essential for productive infection. The gag gene specifies a important in the attachment of the virus to the surface of the host. The fourth gene src (for sarcoma) is not needed for viral multiplication but is required for transformation.

The transforming ability of several retrovirus has been localized in individual oncogenes. The oncogenic activity resides in a single gene. The new sequences present in a tumor retrovirus can be delineated by comparing the sequence of the virus with that of the parental (nontumorigenic) virus. Invariably there is a new region that is closely related to sequences in the cellular genome. The cellular sequences themselves are not oncogenic – if they were, the organism could scarcely have survived – but we may take them to be proto-oncogenes, cellular genes whose capture by a retrovirus & subsequent. modification may create an oncogene.

The viral oncogene & their cellular counterparl are described by using prefixes v for viral & c for cellular. Thus the oncogene carried by Rous sarcoma virus is called v-src & the proto-oncogene related to it in cellular genomes is called c-src.

Often the v-onc sequence is expressed as part of a fusion protein also containing viral functions. The most common structure is a gag-v-onc fusion when the v-onc coding sequence is closely related to the sequence of a gene or part of a gene in the cellular host genome, we may be able to define the boundaries where the recombination event occured to insert the cellular sequence in the virus.

Ref. Book :

GENES IV by Benjamin Lewin

Biochemistry by Stryer.

Chapter IV – Hardy – Weinberg Equilibrium :-

Process of evolutionary change :

Evolutionary change effects all aspects of living things – Their morphology, physiology, behaviour and ecology. Underlying these changes there are genetic changes, which in interaction with the eivironment determine what the organism are. At the genetic level evolution consist of changes in the genetic constitution population.

Evolution can occur if there is hereditary variability. The ultimate power of all hereditary variations is multiple and such variabilities are sorted out by independent assortment and recombination. Apparently it might appear that individuals exhibiting a dominant genotype should be more common than individuals with a recesine phenotype. But it must be remembered that a 3:1 ratio occur in the offspring of two individuals heterozygotus for the same two alleles. Entirely different ratios would occur in other mating combinations & the frequencies of these combinations would depend on the genotype frequencies in a population. So the question comes how we can determine the frequencies of a genotype in a population? Mendelism does not tell us anything about genotype frequencies. Unlike the expected 3 :1 frequencies of a dominant allele brachydactyly occur at a very few frequencies in the population. The first demonstration that gene frequencies are not dependent upon dominance or recessiveness, but may remain essentially unchanged from one generation to the next under certain condition was independently shown by the English mathematician G.H. Hardy an the German Physican W. Weinberg in 1908.

Hardy – Weinberg law :

The main lement in Hardy-Weinberg law is that in the absence of the evolutionary process (mutation, migration, drift & selection) gene frequencies remain constant from generation to generation.

Gene frequencies & genotype frequencies :

In a species we are characterized by many traits, most of us are tall, two eyed, fine fingered etc. But there are people who are short, some are albino, some has six fingers. But genes causing fine fingers are more frequent than those causing six fingers or more.

A preliminary step in analyzing the genetics of a population is to measure gene or allele frequencies. The allele frequencies of all gene loci would define a species precisely and a knowledge of how these frequencies of all gene loci would define a species precisely and a knowledge of how these frequencies change is tentamount to the knowledge of how evolution can occur.

Allele frequency :

Theoretically it is very simple to measure allele frequency. One simply looks at the population, assuming that each person is just one cell and counts how many alleles of each kind there are for any particular locus. Let us take the example of the locus controlling MN blood groups. Peoples are either MM, MN, NN, of the genotypes L^ML^M, L^ML^N at the L locus respectively. All these genotype identified directly.

Suppose in a sample of 100 people, 49 are of M blood group, 32 of MN table and 9 of N (Table 1).

Blood Group	Genotype	People	L^M alleis	L^W allels
M	L^ML^M	49	98	–
MN	L^ML^N	42	42	42
N	L^NL^N	9	–	18
			140	60
			Total allels = 200	

It can be seen from Table-1 that there are in total 200 E^M and L^N or 200 L alleles of them 140/200=0.7 (or 70 r) are L^M and 60/200 = 0.3 (or 30 r) that are L^N.

200 ..... 140 r

100 ..... $\frac{140\times100}{200} = 70r$

homozygote $+\frac{1}{2}$ heteroxygote

If there are only two alleles at a particular locus, their frequencies are generally called 'p' to 'q'. Let us call 'p' as the frequency of L^M and q that L^N ('p' is usually given to the dominant allele). In our example p + q = 0.7 to 0.3 = 1.0. One important point to notice here is that the ratios associated with families just don't show up in the population. We should not except at 1 : 2 : 1 of M : MN : N ratio because that is expected from only a MN x MN marriage. We had all possible matings in our population, which are :

1) MM x MN = MM : MN
   1 : 1
2) MM x MN = all MM
3) MM x MN = all MN
4) NN x NN = all NN
5) NN x MN = MN : NN
   1 1

& 6) MN x MN = MM : MN : NN
   1 2 1

Equilibrium :

We will now have to investigate whether there is any pontaneous possibility of these allele frequencies of change, because evolution involves the alternation of allel frequencies. For this let us take our population & allow it form another generation. Assuming a random mating the frequency of given genotype will simply be a produce of the frequencies of the two corresponding alleles.

		Eggs	
		L^M bearing (0.7) = p	L^N bearings (0.3) = q
Sperms	L^M bearing (0.7) = P	L^ML^M zygote (0.49) = p^2	L^ML^N zygote (0.21) = pq
	L^N bearing (0.3) = Q	L^ML^N zygote (0.21) = pq	L^NL^N zygote (0.09) = q^2

The total genotypes of the next generation will be therefore 0.49 blood group M, 0.21 + 0.21 = 0.42 group MN and 0.09 group N. In other words there will be no change in either gene or genotype frequencies from one generation to the next, this is an *equilibrium* situation. It is called a *Hardy-Weinberg Equilibrium.*

Hardy-Weinberg equilibrium (HWE) is defined by a set of genotype frequencies of p^2 AA, 2pq. Aa and q^2 aa on which A and a reperesent alleles at any locus and p and q are the frequencies of A and a. If the ration AA : Aa : aa is $p^2 : 2pq : e^2$ then the population is in HWE and the allele and genotype frequencies do not change from one generation to the other.

To show that this not just a trick of numbers we can use some allele frequencies, but non HWE genotype frequencies and see the consequence from one generation to the next.

Take for e.g. a population of 45M, 50MN and 3N blood group> Hee p=.45 + $^1/_2$.50 = .45 + .25=.70 and q=3. When this allele frequencies will be used in random mating we will again attain a $.49L^ML^M$, $.42L^ML^N$ and $.09L^{MN}$. So this time we do have a change, our 45 : 50 : 5 ratio becomes a 49 : 42 : 9 ratio on one generation. The original population is obviously not in equilibrium.

If population is not in equilibrium then it takes, only one generation of random mating to establish a HWE.

	AA	Aa	aa
1.	0.3	0.0	0.7
2.	0.2	0.2	0.6
3.	0.1	0.9	0.5

Algebric demontration of equilibrium :

Allele frequencies don't changes :

The frequencies of A allel is

p = homogygote + $^1/_2$ heterozygote

= $p^2 + pq$

$= P(p + q)$

$= p \times 1$

$= p$

or in other word no change in p has occured.

Genotype frequencies don't change :

If we start p^2 of AA, 2pq or Aa or Aa and q^2 of aa, we get six possible types of mating as follows:

	P^2AA	2Pq(Aa)	q^2AA
$P^2(AA)$	p^4 1	$2p^3q$ 2	p^2q^2 3
2Pq (Aa)	$2p^3q$ 4	$4p^2q^2$ 5	$2pq^3$ 6
q^2 (aa)	p^2q^2 7	$2pq^3$ 8	q^4 9

PROGENY

	Mating	**Freq.**	**AA**	**Aa**	**aa**
1)	AA x aa	p^4 (1)	p^4	–	–
2)	AA x Aa	$4p^3q$ (2+4)	$2p^3q$	$2p^3q$	–
3)	Aa x aa	$2p^2q^2$ (3+7)	–	$2p^2q^2$	–
4)	Aa x Aa	$4p^3Q$ (5)	$1p^2q^2$	$2p^3q$	$1p^2q^2$
5)	AA x aa	$4pq^3$ (6+8)	–	$2p^3q$	$2p^3q$
6)	aa x aa	p^4 (q)	–	–	p^3q

$p^4 + 2p^3q + 1p^2q^2 : 2pq^3 + 4p^2q^2 + 4pq^3 : 1p^2p^2 + 2pq^3 + q^4$

$= p^2 (p^2 (2pq + q^2) : 2pq (p^2 + 2pq + q^2) : q^2 (p^2 + 2pq + q^2)$

$= p^2 (p+q)^2 \quad : 2pq \quad : q^2$

$= p^2 \times 1 = p^2$

Ref. Book :-

Genetics – Strikeberger (3rd edition).

Questions :

1. The gamma-globulin portion of human blood serum is two types Gm^{2+} & Gm^{a-}. Gm^{a+} is dominant over Gm^{a-}. Assuming that HWE is established, what are the frequencies of heterozygotes (Gma^{+}/ Gm^{a-}) in the following population?

Region	No. of Tested	No. of Phenotypes	
		Gm^{a+}	Gm^{a-}
1	293	161	132
2	253	141	112
3	233	142	91
4	160	108	52

2. The incidence of recessive albinism is .0004 in a human population. What is the frequency of the recessive allele? (Assume random mating).

3. i) When transposition occurs, two base sequences are duplicated what are they?

   ii) Are all drug resistant genes located in transposons?

4. i) In what way, a retroviral provirus adjacent to the protooncoogena leads to the conversion of a normal cellular protooncogene to a cellular oncogene?

   ii) Mention the distinguishing caracteristics of v-src & e-src.

5. i) Draw a chi-shaped structure and a cointegrate structure which is responsible for the movement of the transposon.

   ii) What do you mean by resolution?

6. Consider the population whose genotypes are shown in Table.

Population	AA	Aa	aa
1.	1.0	0.0	0.0
2.	0.0	1.0	0.0
3.	0.50	0.25	0.25
4.	0.25	0.25	0.50
5.	0.33	0.33	0.33
6.	0.04	0.32	0.64

which of the populations are in Handy-Weinberg Equilibrium?

---- 0 -----

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M. Sc. in ZOOLOGY

Paper - II, Part-I, Group-A
Unit-1 (Cytogenetics)

Module No. 15

Contents :

1. Nature of the genetic code.
2. Mitotic apparatus.
3. Anaphase movement is diphasic.

Genetic Code :

Since, there are 20 different kinds of amino acids in proteins and only four kinds of nucleotides in DNA, the relationship between the gene & its most elementary functional product, i.e. between DNA and proteins, can be interpreted through a code of one nucleotide = one amino acid. A codon size of three nucleotides for one amino acid or triplet code seems more likely, since it prduces 4^3=64 possible codons (Fig. 1). It is to be noted that since only 20 amino acids need to be coded, 44 codons in a triplet code seem to be superfluous. To account for the excess of codons beyond the necessary 20, we can suppose that more than one codon can code for a particular amino acid. For example, if each kind of amino acid were coded for three different possible codons, 60 possible codons would be accounted for. A code in which there is more than one codon for the same amino acid is called degenerate. It is also possible that some or all of the codons in excess of 20 do not code for any amino acid and are therefore nonsense codons.

Singlet code

A
G
C
T

Doublet code

AA	AG	AC	AT
GA	GG	GC	GT
CA	CG	CC	CT
TA	TG	TC	TT

Triplet Code

AAA	AAG	AAC	AAT
AGA	AGG	AGC	AGT
ACA	ACG	ACC	ACT
ATA	ATG	ATC	ATT
GAA	GAG	GAC	GAT
GGA	GGG	GGC	GGT
GCA	GCG	GCC	GCT
GTA	GTG	GTC	GTT
CAA	CAG	CC	CAT
CGA	CGG	CGC	CGT
CCA	CCG	CCC	CCT
CTA	CTG	CTC	CTT
TAA	TAG	TAC	TAT
TGA	TGG	TGC	TGT
TCA	TCG	TCC	TCT
TTA	TTG	TTC	TTT

Figure - 1

Possible coding combinations using different-sized DNA nucleotide sequences. On the level of mRNA translation into protein, the mRNA codon sequences would be complementary, of course, to those shown here, e.g., ATC (DNA)=(UAG (mRNA). (After Nirenberg, 1963).

Whatever the codon size, however, the form of the code may be overlapping or nonoverlapping. If for example, the codons were triplets, this would mean that a sequence of six nucleotides could code either two amino acids, if it were nonoverlapping or more amino acids, if it were overlapping (Fig. 2). All evidence to date indicates that single nucleotide mutations affect only a single amino acid in a protein chain, meaning that the code is nonoverlapping.

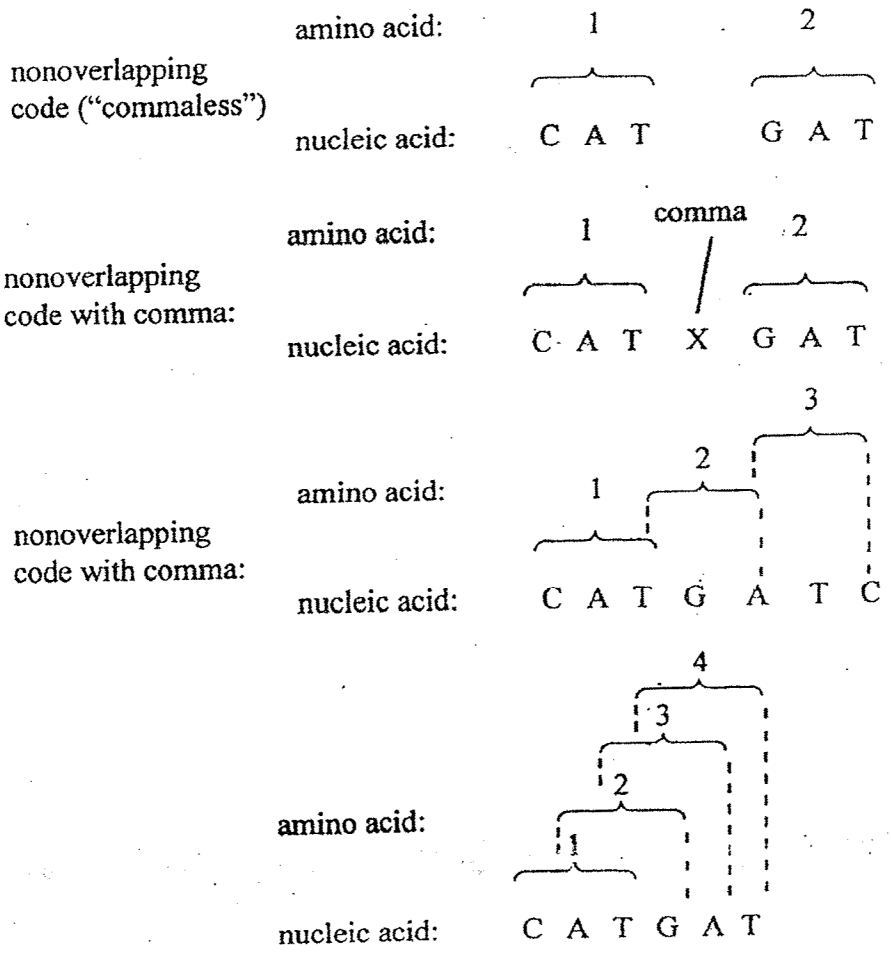


Figure - 2

Nonoverlapping and overlapping triplet codes.

For a nonoverlapping code two further possibilities exist (1) The code has "commas", consisting of one or more nucleotides placed between the codons; such commas might serve the purpose of particular starting point on the mRNA chain in a 'reading frame' consisting of codons placed side by side. Thus a reading frame in a triplet code might begin with the nucleotide sequence CATGAT....., in which CAT and GAT are subsequently transcribed and translated into two particular amino acids. Removal of the cytidine nucleotide (C) from the beginning of this DNA sequence, e.g., ATGAT....., would produce triplets ATG

that can translate into different amino acids than the originals ("missence" nutations) or yield no amino acids at all ("nonsence" mutations). These and other coding terms are discussed in Table 1.

Table - 1

Definitions of some common terms used in describing the genetic code

Term	Meaning	Term	Meaning
code letter	nucleotide, e.g., A, U, G, C (in mRNA) or A, T, G, C (in DNA)	*ambiguous code*	when one codon can code for more than one amino acid, e.g., GGA=glycine, glutamic acid.
code word, or codon	sequence of nucleotides specifying an amino acid, e.g., UUU=phenyl-alanine	*commaless code*	When there are no intermidiary nucleo-tides (spacers) between words, e.g., UUUCCC = two amino acids in triplet nonoverlapping code
anticodon	sequence of nucleotides on tRNA that complements the codon, e.g., AAA = anticodon for phenyl-alanine	*reading frame*	the particular nucleotide sequence that starts at a specific point and is then partitioned into codons until the final code word of that sequence is reached.
genetic code or coding dictionary	a table of all the code words or codons that specify amino acids		
word size or codon length	the number of letters in a code word e.g., three letters in a triplet code (these are the same as *coding ratio* in a nonoverlapping code)	*frame-shift mutation*	a change in the reading frame because of the insertion or deletion of nucleotides in numbers other than multiples of the codon length. This modifies the previous partitioning of codons in the reading frame, and causes a new sequence of codons to be read
nonoverlapping code	when only as many amino acids are coded as there are code words in end-to-end sequence, e.g. (triplet code), UUUCCC=phenylatanine (UUU)+ proline (CCC).		
overlapping code	when more amino acids are coded for than there are code words present in end-to-end sequence, e.g., UUUCCC = phenylalanine (UUU) + phenylalanine (UUC) + serine (UCC) + (proline (CCC)	*sense word*	a codon that specifies an amino acid normally present at that position in a protein
		missense mutation	a change in nucleotide sequence, either by deletion, insertion, or substitution, resulting in the appearance of a codon that produces a different amino acid in a particular protein, e.g., UUU (phenylalanine) mutates to UGU (cysteine)
nondegenerate code	when there is only one codon for each amino acid, e.g., 20 different amino acids have a total of 20 codons		
degenerate code	when there is more than one codon for a particular amino acid, e.g. UUU, UUC=phenylalanine, or 20 different amino acids have a total of more than 20 codons	*nonsense mutation*	a codon that does not produce an amino acid, e.g., UAG (also called a "stop" mutation or "termination" codon)
synonymous codons	different codons that specify the same amino acid in a degenerate code, e.g., UUU = UUC = phenyla-lanine	*universality*	utilization of the same genetic code in all organisms, e.g., UUU = phenylalanine in bacteria, mouse, man and tobacco

EVIDENCE ON THE TRIPLET CODON, THE DEGENERACY OF THE CODE :

(Crick, *et al* 1961)

Some mutations induced by acridine dyes represent deletions or insertions. Crick and his group tested a number of such mutations located in the B cistocon of the rII locus of T4. One of the mutations was arbitrarily designated as +, and if this mutant effect was suppressed by a different mutation the latter was then designated as --. FCO, for example, was called + and produced an rII mutant effect. FCI, another rII mutation, suppressed the action of FCO, producing wild type when they were both present in the cis configuration and was therefore designated as –. FC 58, on the other hand, did not produce wild type when present in cis configuration with FCO, but did produce wild type when present in cis configuration with FCI. Thus FC 58 could be designated as + ("a suppressor of a suppressor").

As shown is Table 2 for a sample group of mutations, these cis tests had illuminating results: when two + mutations or two – mutations were combined, the mutant effect was not modified; wild type only appeared in the + – combinations or in combinations of three '+' s or three '–'s. This mean that three changes of the same kind or a multiple of three were necessary to permit wild type to be formed. This relationship is visualized in Fig. 3, by considering that the rII effect of a single + mutation (e.g. insertion) can be corrected by either a – mutation (e.g., deletion) or by the presence of two further + mutations if the code is in triplets. Similarly a – mutant effect is restored to wild type by a + mutation or two further – mutations. It is to be noted that the restoration of wild type by these various combinations depends upon the commaless nature of this triplet code. That is, the wild type message can be restored by changes further along the nucleotide chain because all the intervening nucleotides are involved in the reading frame (i.e. none are used as commas).

Table - 2

Phenotypes produced by various *cis* combinations among a sample group of acridine-induced mutations at the *rII* locus of T4

Designations	
+	–
FC 0	*FC* 1
FC 40	*FC* 21
FC 58	*FC* 23

Wild-type combinations	rII combinations
FC 0, *FC* 1, (+ –)	*FC* 0, *FC* 40, (+ +)
FC 0, *FC* 21, (+ –)	*FC* 0, *FC* 58, (+ +)
FC 40, *FC* 1, (+ –)	*FC* 1, *FC* 21, (– –)
FC 58, *FC* 1, (+ –)	*FC* 1, *FC* 23, (– –)
FC 0, *FC* 40, *FC* 58 (+ + +)	
FC 1, *FC* 21, *FC* 23, (– – –)	

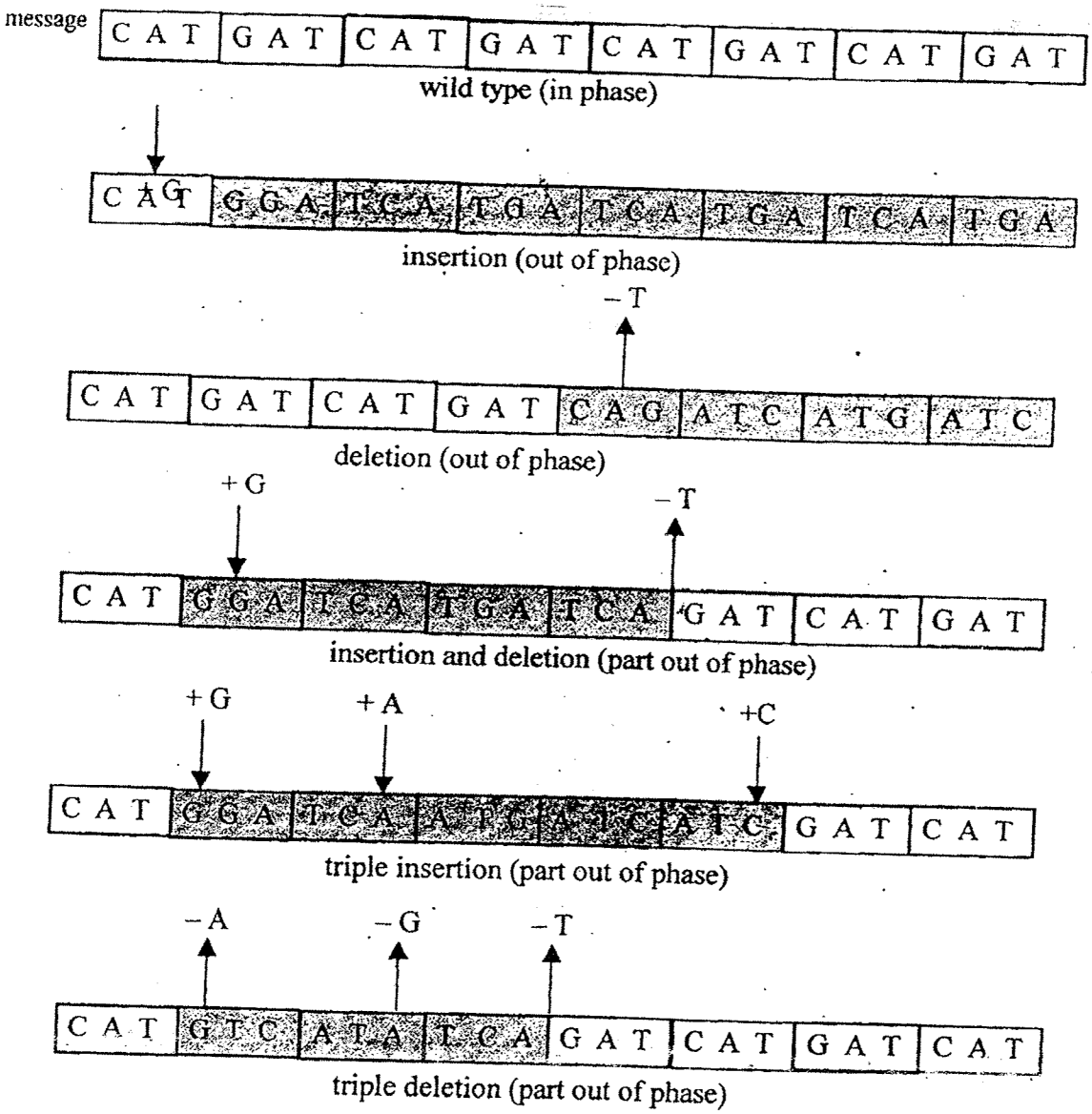


Figure - 3

Effect of nucleotide insertions, deletions, and their combinations upon a nucleotide chain "read" or triplet codons in a commaless sequence ("reading frame" from left to right. Since such insertions or deletions affect a number of codons beyond the one carrying the mutation, these changes are considered as reading-frame or frame-shift mutations.

CHARACTERIZATION OF THE CODON:

In 1961 Nirenberg and Matthei reported that a particular RNA sequence produced a particular amino acid sequence. They used a cell free system extracted from E. coli containing ribosomes, radioactively labelled amino acid, transfer RNA and the necessary enzymes and energy sources (ATP) for protein synthesis.

In the absence of mRNA no observable protein synthesis took place. They then added RNA from tobacco mosaic virus (TMV) and showed an increase in amino acid incorporation into proteins. In other words, mRNA was necessary for protein formation & an E. coli system could use TMV-RNA to synthesize TMV-like proteins. However, their most remarkable discovery occured when they added the synthetic polyribonucleotide, poly-U, to this system and obtained a polypeptide whose amino acids were all phenylalanine in spite of the presence of other amino acids in the original mixture. The results mean that phenylalanine, or more precisely the anticodon of a phenylalanine-carying tRNA, was coded by a sequence of uracil bases (UUU codon of mRNA=AAA anticodon of tRNA phe). It was immediately evident that other synthetic RNA messengers introduced into this system could be used to discern the code for a variety of amino acids. Thus poly-A was found to be the code for lysine and poly-C for proline. Poly G was also tried but could not attach to ribosomes, since it remained in solution as multistranded helixes caused by hydrogen bonding between its guanine bases. Aside from specifying the coding sequence of thse three amino acids, such finding also indicated that the code was commaless, without special intervening nucleotides between codons. The argument for this as follows:

i) If the code has specific commas, the incorporation of phenylalanine into poly-U means that the commas are all uracil.

ii) However, the incorporation of lysine into poly A and of proline into poly-C, in turn, means that the commas are made of adenine and of cytosine explanations which are extremely inconsistent.

Within a short period of time, synthetic ribonucleotides containing mixed bases like poly-UC also tried which contained only eight different codons: UUU, UUC, UCU, UCC, CUU, CUC, CCU, CCC. In this example poly-UC caused the polypeptide incorporation of leucine and serine in addition to the expected incorporation of phenylalanine (UUU) and proline (CCC). Modifying the proportion of bases in poly-UC so that it contains more uracil than cytosine causes more serine to be incorporated into the polypeptide, indicating that serine may either be UUC, CUU or UCU. Futher determination with other nucleotide polymer enabled other amino acids to be characterized by their general codes e.g., poly UG caused the polypeptide incorporation of cysteine, glycine, tryptophan and valine. Poly-UA led to incorporation of asparagine, isoleucine, leucine, lysine and tyrosine. As in serine, however, the precise nucleotide sequence within the codons of most amino acids could not be determined.

Evidence for the triplet nature of the code is obtained from the fact that the binding of specific amino acids to ribosomes occurs preferentially in the presence of trinucleotide mRNA rather than in the presence of dinucleotide mRNA. Thus, tRNA phe bearing ^{14}C radioactive phenylalanine becomes bound to ribosomes when UUU or UUC trinucleotides are used. Radioactive leucine becomes ribosomal-bound by the trinucleotides UUA, UUG, CUC and CUG. In the khorana technique longer ribonucleotide sequences are formed by first synthesizing specific DNA sequences and then using DNA directed RNA polymerase to make complementary RNA molecules. These precisely sequenced RNA messengers are then used in vitro translational systems from which the amino acid sequences in the resultant polypeptide are analyzed. Such studies not only reaffirmed the triplet code & provided information on disputed codons but also demonstrated that

the code was commaless and nonoverlapping.

To date, all the 64 possible ucleotides have been tested & found to incorporate among them all 20 amino acids. As shown in table 3, there are numerous instances in which a single amino acid is coded by more than one codon; i.e. some of the different codons are synonymous and the code is degenerate. One cause for this degeneracy arises from the fact that the tRNA molecules to which a particular amino acid attaches may consist of more than one variety, each variety with its own anticodon. For example, some types of tRNA bearing a specific anticodon to pair with two or more synonymous codons of a particular amino acid (e.g. yeast $tRNA^{ala}$ bearing anticodon CGI can pair with alanine codons GCU, GCC and GCA).

Table - 3

Nucleotide sequence of RNA codons based on binding of amino-acid-charged tRNA molecules of ribosomes and on incorporation of amino acids into polypeptides*

UUU, UUC *phe*	UCU, UCC, UCA, UCG *ser*	UAU, UAC *tyr*	UGU, UGC *cys*
UUA, UUG *leu*		UAA nonsense or chain	UGA nonsense
		UAG termination	UGG *trp*
CUU, CUC, CUA, CUG *leu*	CCU, CCC, CCA, CCG *pro*	CAU, CAC *his*	CGU, CGC, CGA, CGG *arg*
		CAA, CAG *gln*	
AUU, AUC, AUA *ile*	ACU, ACC, ACA, ACG *thr*	AAU, AAC *asn*	AGU, AGC *ser*
AUG ** *met*		AAA, AAG *lys*	AGA, AGG *arg*
GUU, GUC, GUA, GUG *val*	GCU, GCC, GCA, GCG *ala*	GAU, GAC *asp*	GGU, GGC, GGA, GGG *gly*
		GAA, GAG *glu*	

From data of Nirenberg and co-workers, Khorana and co-wrkers, and others.

* These codons are oriented on the mRNA molecule so that the nucleotide on the left is toward the 5′ end, and the nucleotide on the right is toward the 3′ end.

** AUG is the most common initiator codon.

DEGENERACY AND WOBBLE :

All the amino acids except methionine & tryptophan are specified by more than one codon (Table 3). Three amino acids leucine, serine, arginine are each specified by six different codons. Isolencine has three codons.

The other amino acids each have either two or four codons. The occurence of more than one codon per amino acid is called degeneracy. The degeneracy in the genetic code is highly ordered. Usually the multiple codons specifying an amino acid differ by only one base, the third or 3′ base of the codon.

The degeneracy is primarily of two types

1) Partial degeneracy occurs when the third base may be either one of the two pyrimidines (U and C) or alternatively, either one of the two purines (A and G).
2) In the case of complete degeneracy, any of the four bases may be present at the third position in the codon and the codon will still specify the same amino acid. For example, valine is specified by GUU, GUC, GUA and GUG.

Because of the degeneracy of the genetic code, there must either be several different tRNAs that recognize the different codons specifying a given amino acid or the anticodon of a given tRNA must be able to base-pair with several different codons. Actually both of these occur. Several tRNAs exist for certain amino acids and some tRNAs recognize more than one codon. The hydrogen bonding between the bases in the anticodon of tRNA and the codon of mRNA appears to follow strict base pairing rules (i.e. be tight) only for the first two bases of the codon. The base-pairing involving the third base of the codon is apparently less stringent, allowing what crick has called wobble at this site.

On the basis of molecular distances, Crick proposed that wobble would allow several types, but not all types, of base-pairing at the third codon base in the codon-anticodon interaction. His proposal has since been strongly supported by experimental data. Table shows the base-pairing predicted by the wobble hypothesis.

Table showing base-pairing between the 5′ base of the anticodon of tRNA and 3′ base of codons of mRNAs. According to the Wobble Hypothesis

Base in Anticodon	Base in codon
G	U or C
C	G
A	U
U	A or G
I	A, U or C

It necessitates that there be at least two tRNAs for each amino acid whose codons exhibit complete degeneracy at the third position. This has indeed been found to be true. The wobble hypothesis predicted the occurence of three tRNA for the six-serine codons. Three serine tRNAs have been characterized:

1. $tRNA_{Ser1}$ (anticodon AGG) binds to codons UCU and UCC
2. $tRNA_{Ser2}$ (anticodon AGU) binds to codons UCA and UCG.
3. $tRNA_{Ser3}$ (anticodon UCG) binds to codons AGU and AGC.

Finally, several tRNAs contain the base inosine. Crick's wobble hypothesis predicted that inosine could pair

(at the wobble position) with adenine, uracil or cytosine (in the codon). In fact, purified alanyl-tRNA containing inosine (I) at the 5′ position of the anticodon binds to ribosomes activated with GCU, GCC or GCA trinucleotides. The same results has been obtained with other purified tRNAs with inosine at the 5′ position of the anticodon. The wobble hypothesis thus fits several observations, whether it is entirely accurate remains unknown.

INITIATION AND TERMINATION CODONS :

The genetic code also provides for punctuation of genetic information at the level of translation. three codons, UAA, UAG and UGA, specify polypeptide chain termination. These codons are recognized by protein release factors, rather than by tRNAs. One of these proteins, designated RF1, is apparently specific for UAA and UAG. the other. RF2, causes termination at UAA and UGA codons. Two codons, AUG and GUG, are recognized by the initiator tRNA, $tRNAi^{Met}$, but apparently only when they follow an appropriate nucleotide sequence in the leader segment of an mRNA molecule. At internal positions, AUG is recognized by $tRNA^{Met}$ and GUG is recognized by a valine tRNA. In the case of initiation codons AUG and GUG and $tRNAi^{Met}$, the Wobble base appears to be the first or 5′ base of the codon. Since wobble at the first base in unique to initiation, it may be related to base-pairing at the P site rather than at the A site on the ribosome.

B. Chemistry of Mitotic Apparatus (MA)

I. Isolation - 'Selective solubilization procedure (Mazia & Dan'52)

Dividing cells stabilized by exposure to 30%-40% ethanol at -10^0C

↓

Cytoplasm dispersed (not MA) by means of digitonin (Mazia'55) or ATP (Mazia'57)

↓

Native MA free from other cell constituents.

MA have S–S bonds which must be protected by an excess of an S–S compound. The substance chosen is dithiodiglycol ($OHCH_2CH_2SS.CH_2CH_2OH$). When sea-urchin eggs placed in a 1M sucrose soln. containing 0.15M DTDG at pH 6.2–6.3, a structurally intact MA was set free.

II. Chemical description :

1. **Protein :** a major component of MA. It may account 10% or more of all the prteins in cell. 90% or more protein content in sea-urchin egg. The amino acid analysis by Roslansky'57 shows similarity with actin of mammalian muscle & much similar with protein of flagella of chlamydomonas (Johes and Lewin 1960).
   The mol. wt. of the protein 315000 + 20000 (Zimmerman 1960). So it is a major structural building block.
2. **Nucleic acid :** RNA present. RNase treatment of Cyclops egg shows 5% reduction of RNA. Sea-urchin egg contains 8% RNA.
3. **Polysaccharide :** The association of polysaccharide with the mitotic apparatus has been confirmed from its positive reaction with PAS. But is cannot be isolated.
4. **Lipids :** Mostly it is possible lipoprotein system of MA express itself as membrane, vesicle & tubular filament.
5. **Enzyms :** MA isolated by DTDG method, specific active ATPase bind with the fraction of MA soluble in .5 m KCl which includes the fibrous component & to exclude contaminating cytoplasmic particles.
   This finding of ATRase conform that biological system carry out movement possess this enzyme activity.
6. **Zinc :** According to Fugii'54, 55 dithizone staring substance deposited in the spindle when MA forms & leaves it at anaphase.
7. **Thiol group :** Glutathione decreased in quantity upto metaphase & then reappeared as division proceed.
8. **Other intermolecular bonds :** Cations, involve in the formation of mitotic gel. *Hydrogen* bond.

ANAPHASE MOVEMENT IS DIPHASIC

Majia & Daniel (1952) have first isolated mitotic apparatus (MA) by using *sea urchin* as mateiral. MA comprises the spindle & the asters which surround the centrioles. The spindle is made of the *chromosomal fibers* (joining the chr. to the poles), the *continuous fibers* (extending pole to pole), the *interzonal fibers* (observed between the daughter chromosomes nuclei). All these fibers, including those of the aster, are composed of microtubules.

Basically 2 groups of mitotic spindle microtubules are found - the *chromosomal* (or *kinetochore*) *microtubules* and the *polar* (or *interpolar*) *microtubules.*

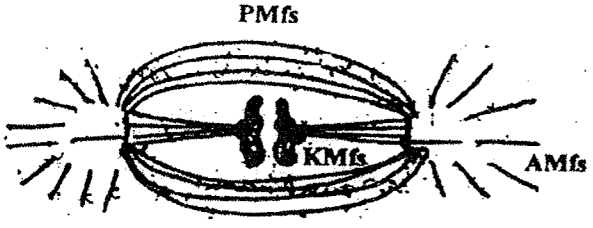


Diagram of the distribution of microtubules in the metaphase spindle.
KMfs – are kinetochore microtubules
AMfs – are Astral microtubules
PMfs – are Interpolar microtubules

[Note: In higher eukaryotes the centromeres of mitotic chr. s generally contain a specialized str. known as *a kinetochore*. The kinetochore is a *trilaminar disk* whose inner & outer layer consist of electron dense material probably composed of tightly packed chromatin in association with nonhistone chromosomal protein. - & middle layer of lower density]

Since the chromosome undergo condensation during prophase & prometaphase, each can be seen to be made of 2 members, termed *chromatid.* The 2 chromatids of each chromosome are connected to each other at their centromers. Anaphase begins with the sudden splitting apart of the kinetochores of the 2 sister chromatids of each chromosome & the chromatids begin their poleward migration. During metaphase an equilibrium of forces is established. But with the separation of the sister chromatids at anaphase, this equilibrium is broken.

ANAPHASE MOVEMENT :

1. Movements during anaphase are accompanied with the change in length of microtubules.
2. The movement of chromosomes from the equator to the poles is invariably accompanied by a *shortening of chromosomal microtubules* & the separation of poles is generally accompanied by a *lengthening of the polar microtubules.*
3. These 2 types of microtubular modifications, are involving the loss of subunits & the other involving the addition of subunits, occurs at the same time in different regions of the same dividing cell.
4. So, the anaphase events may be described in terms of 2 movements:-
   a) Inoné proposed that as the loss of subunits (Disassembly) occurs, the chromosomal fibers shorten. This dissolution of chromosomal fiber at one end provide much *pulling force* of 10^{-8} dyes (equivalent to 20-30 molecules of ATP) which is sufficient to pull the chromosomes forward.

b) Due to addition of subunits (assembly) to polar microtubules, 'interzonal' region obviously does increase in length, which acts as a 'pushing body' that pushes the poles farther apart.
So, it is justify to say that anaphase movement si *biphagic*.

OTHER VIEWS REGARDING MOVEMENTS :

1) **Interacting microtubules as a force generating system :** *Richard McIntosh* formulates a hypothesis to explain mitotic movement on the basis of sliding microtubules & movable cross bridges. In this model, the poleward movement of the chromosome would be accomplished by the sliding of the chromosomal & polar microtubules over one another. This idea of interaction of microtubules has received a boost in recent year with the finding that a particular protein *dynein*, appears to be a involved in pole - pole separation.
2) **Actinomyosin & microtrabeculae as force generating system:** It was discovered that the contractile protein *acting & myosin* might be present within the region of the mitotic spindle. It was proposed that the interaction between these two contractile proteins might serve to generate the force that drives the chromosomes to the poles, while the microtubules would represent passive structures through which the force would be transmitted.

Regulation of the movement :

1. Ca is a potent inhibitor of microtubule polymerization in vitro & elevated levels of Ca within the cell lead to the depolymerization of cytoplasmic microtubules. Examination of a variety of deviding cells indicates that large nos. of smooth surfaced vesicles are present in and around the mitotic spindle. The local release of Ca^{2+} in the cytosol would bring about the disassembly of microtubule at the spindle poles during chr. movement, while a local uptake of Ca^{2+} from the cytosol would be expected to promote the assembly of microtubules in the equatorial region during spindle elongation.
2. In the year 1987 **GRAVEL et al** established that 2 factors which influence the movement of chromosome along microtubules. ***Calmodulin & STOP*** play important role in this process. Both of them are under genetic control. **STOP protein is attached to microtubule calmodulin is associated** with stop protein. Ca are mediated through calmodulin. **Due to interaction between calmodulin &** stop protein chromosome may slide along microtubules.

Velocity :

The speed of chromosome movement in anaphase are somewhat variable, the range being from about 0.2μ/ min to about 5μ/min at normal temp.

THE MITOTIC APARATUS :

The mitotic apparatus has been defined (Mazia and Dan 1952) as "the ensemble of structures

constituting the 'inromatic' and 'achromatic'. It includes spindle, aster, centrioles.

A. Physical property-

1. **MA as a gel :**
   (a) MA occupies a *large part of the cell volume.* Minimum 10% (as in sea-urchin egg) & maximum 50% of the cell volume.
   (b) Visually, MA displays itself as *an unbound region* within cell. EM study reveals that there is no boundary layer.
   (c) By isolation it is found that MA is *a coherent body* whose molecular units are associated in a distinctive way. Thus MA may be characterised as a *gel.* Recent work suggests that mitotic gel may be composed of *a compact mass of vesicular & tubular elements.*

BIREFRINGENCE PROPERTY - (from optical study)

Sea-urchin eggs when blooked by mercaptoethanoz, MA is converted to a spherical clear space but isolation shows it as am or phous mass of gel. This loss of orientation is described as a dissolution or disappearance of mitotic apparatus or modification of *birefringence property.* It is a gelled region composed of fibers which are highly elastic. It is a discrete body & its movement in centribufal field depends on its relative density, at high forced it is distorted which supports it orientation & attachment of chromosome.

Problems :

1. Define the following terms as they apply to the genetic code:
   (a) Reading frame, (b) Overlapping code (c) Non-overlapping code (d) Initiation codon (e) Termination codon, (f) Sense codon, (g) nonsense codon.
2. What is the significance of the fact that many synonymous codons differ only in the third nucleotide position.
3. What three different methods were used to help break the genetic code? What did each reveal & what were the advantages and disadvantages of each?
4. How is the reading frame of a nucleotide sequence set?
5. Prove that anephase movement is biphasic.
6. Mention the chemistry of Mitotic apparatus.

Suggested reading :

Genetics _ A conceptual Approach by Benjamin A. Pierce.
Principles of Genetics - Snustad. Simmons (Third edition).

CELL - CYCLE

Cells reproduce by doubling their contents and then dividing into two. This cell division cycle is the fundamental means by which all living organisms propagate. The time from the end of one mitosis to the start of the next is called **interphase**. The period between two mitotic divisions is called the somatic cell cycle. Two daughter cells are formed from a mother cell after one **cell cycle**. These nearly formed daughter cells can themselves grow and divide giving rise to a new cell population formed by the growth and division of a single parental cell and its progeny. Thus through repeated cycles of cell growth and division results in the development of a single fertilized egg (zygote) into the more than 10^{13} cells that constitute the human body.

The divisions of the cell cycle :

In general, every cell has two major periods in its life cycle; interphase (non-division) and division (which produces two daughter cells). This cycle is repeated at each cell generation, but the length of the cycle varies considerably in different types of cells.

The interphase is composed of the G_1, S and G_2 where the divisional phase is popularly known as Mitotic phase or M phase. (Fig - 1). The time from the end of one mitosis to the start of the next is called **interphase**. The period of actual division, corresponding to visible mitosis is called **M Phase.**

Functional activities of the different cell cycle phase :

G_1 — Cell those are released from mitosis enter into **G_2 phase**: During this phase RNAs and proteins are synthesized, but there is no DNA replication.

S-Phase — The essential function of the nucleus is to store and make available to the cell the information present in its DNA molecule(s). This molecule duplicates during a special period of interphase called the S **Phase (or Synthesis Phase)** in preparation for cell division. The initiation of DNA replication marks the transition from **G_1 Phase** to the period of **S Phase**. S Phase is defined as lasting until all of the DNA has been replicated. During S Phase, the total content of DNA increases from the diploid value of 2n to the fully replicated value of 4n. S Phase is so called as the synthetic period when DNA replication takes place. G_1 and G_2, stand for the two 'gaps' in the cell cycle when no DNA synthesis takes place.

G2 — The period from the end of S Phase until mitosis is called **G_2 Phase** : during this period the cells get two complete diploid sets of chromosomes,

M - Phase: In this phase the segregation of one diploid set of chromosomes to each daughter cell takes place. Individual chromosomes become visible only during this phase, when nuclear envelope dissolves, and the cell is reorganized on a **spindle**.

Duration of various cell cycle phases :

The duration of the phases of cell cycle varies consideraley in different kinds of cells. For a typical rapidly proliferating human cell with a total cycle time 24 hours, the G_1-Phase might last about 11 hours, S-Phase about 8 hours, the G_2 about 4 hours, and M about 1 hour. Other types of cells, can divide much more rapidly. Budding yeasts, can progress through all four phases the cell cycle on only about 90 minutes. A single bacterium can make a colony consisting of millions of progeny cells during overnight incubation on a plate of nutrient agar medium. In case of early embyonic cell cycles rapidly divide the egg cytoplasm into smaller cells. There is no G_1 or G_2 phase, and DNA replication occurs very rapidly in these early embryonic cell cycles, which therefore. consist of very short S-phase alternating with M-phase.

G_0-Phase : Some cells in adult animals ceases division altogether (e.g. nerve cells) and many other divide only occasionally (e.g. skin fibroblast, kidney, liver, lung, cells). In case of these cells G, phase enters into a quiescent stage of cell cycle called G_0, where the cells remain metabolically active but no longer proliferative unless they are induced.

The cell-cycle control system:

The cell-cycle control system is a cyclically operating biochemical device constructed from a set of interacting proteins that induce and co-ordinate the essential **downstream processes** that duplicate and divide the cell's contents. In a standard cell-cycle the control system is regulated by brakes that can stop the cycle at specific **check points**. The brakes allow the cell cycle control system to be regulated by signals from the environment. The progression of cells through the cell division cycle is not only regulated by extra cellular signals from the environment but also by the internal signals are responsible for the co-ordination of the stages.

In case of many types of cells a major cell-cycle regulatory point occurs date in G_1 and controls progression from G_1 to S. One such regulatory point was known as **START**, discovered for the budding yeast cell cycle. Once

cells have passed **START**, they are committed to entering S phase and undergiong one cell-division cycle. **START** not only monitor extracellular signals but also act as a point at which cell growth is co-ordinated with DNA replication and cell division, (Fig - 2).

In case of most animal, proliferation of cells is also regulated in the G_1 phase. A decision point in late G1, called the **restriction point** functions analogously to **START** in the yeasts. However animal cell proliferation mostly depend upon the extracellular growth factors rather than by the availability of nutrients as required for the yeast cells, (Fig - 3).

Cell-cycle checkpoint:

In most cells, the co-ordination between different phases of the cell-cycle is dependent on a system of checkpoints and feedback controls that prevent entry into the next phase of the cell cycle until the events of the proceeding phase have been completed.

Several cell-cycle checkpoints function to ensure that incomplete or damaged chromosomes are not replicated and passed on to daughter cells. One of the most defined checkpoint is called G_2 checkpoint. This checkpoint senses unreplicated DNA, which generates a signal that leads to cell cycle arrest. Operation of the G_2 checkpoint therefore prevents the initiation of M phase before completion of S phase. as a result, cells remain in G_1 until the genome has been completely replicated. Sensing completion of replication of DNA the inhibition of G_2 progression is relieved following the cell to initiate mitosis and distribute the completely replicated chromosomes to daughter cells (Fig - 4). DNA damage not only arrests the cell-cycle in G_2, but also slows the progression of cells through S - phase and arrests cell-cycle progressin at a checkpoint in G_1. This arrest may allow repair of the damaged DNA before the cell enters S phase.

In mammalian cells, arrest at the G_1 checkpoint is mediated by the action of a portein known as p53. Loss of p53 function as a result of mutations prevent G_1 arrest in response to DNA damage; so the damaged DNA is replicated and passed on to the daughter cells instead of being repaired (Fig - 5).

The molecular mechanism that restricts DNA replication to once per cell cycle involves the action of a family of proteins (called MCM proteins) that bind to replication origins together with the origin replication complex (ORC) proteins. The MCM proteins act as licensing factors' that allow replication to initiate.

Phosphorylation (Catalyzed by kinases) and dephosphorylation (Catalyzed by phosphatases) are the critical events

that regulate the cell-cycle. They are used both to control the activities of the regulatory circuit itself and to control the activities of the substrates that execute the decisions of the regualtory circuit.

Cell cycle regulation :

The cell-cycle regulatory circuit consists of a series of kinases and phosphates that respond to external signal and checkpoints by phosphorylating or dephosphoryiative the next member of the circuit is to determine the activity of M phase kinase (or the S phase kinase) by controlling its state of phorphosylation.

Regulators of cell-cycle Progression :

The cell-cycle regulation system is based upon two key families of proteins. The first family is known as 'cyclin - dependent protein kinases (**Cdk**), which induce downstream process by phosphorylating selected proteins on serines and threonines. The second family is a specialized activating proteins, called cyclins. that bind to Cdk molecules and control) their ability to phosphorylate appropriate target proteins. (Fig - 6).

The cell cycles of higher enkaryctes are controlled not only by multiple cyclins, but also by multiple Cdc2 - related protein kinases. These Cdc-2 related kinases are known as Cdk's (Cyclin-dependent kinases). As the original member of this family Cdc2 is also known as Cdk1, other members are called Cdk2 through Cdk-8. These multiple members of the Cdk family associate with specific cyclins to drive progression through the different stages of the cell cycle. The activity of Cdk's during cell cycle progression is regulated by at least four molecular mechanisms (Fig - 8).

The cyclic assembly, activation and disassembly of cyclin-Cdk complexes are the pivotal events driving the cell-cycle.

Experiments done by two groups of researches independently in 1971 on Oocytes show that certain factor called maturation promoting factor (MPF) act as a regulator of the transition from G_2 to M. MPF is now understood to stand for **M phase promoting factor**. Chemically MPF is a dimer of Cdc2 and cyclin B (Fig - 7), Cyclin B is a regulator subunit required for catalytic activity of the Cdc2 protein kinase.

A number of studies have confirmed that MPF activity is controlled by the periodic accumulation and degredation of cyclin B during cell-cycle progression. The availability of MPF to induce mitosis implies that the M phase kinase, directly or indirectly, triggers these activities. Activation of M phase kinase triggers onset of M phase.

Inactivation is necessary to exit M phase. It is therefore suggested that M phase kinase activity are reversible. That is, phosphorylation of substrates is required for the reorganization of the cell into a mitotic spindle, and dephosphorylation of the same substrates is required to return to an interphase organization. (Fig - 9).

Two alternate roles have been attributed to the functioning of M phase kinase

(i) It may be 'master regulatior' that phosphorylates target proteins that in turn act to regulate other necessary functions — a classic cascade.

(ii) It may be a 'workhorse' that itself directely phosphorylates the crucial substrates need to execute the regulator events or cell reorganization involved in the cycle.

In addition to regulation of the Cdk's by posphorylation, their activities can also be controlled by the binding of inhibitory proteins (called Cdk inhibitors or CKIs) to Cdk/cyclin complexes. In mammalian cells, two families of Cdk inhibitors are responsibles for regulating different Cdk/cyclin complex.

Cell Cycle (Summary) :

The cell-cycle consists of transitions from one regulatory state to another. The change in regulatory state is separated by a lag period from the subsequent changes in the cell phenotype. The transitions take the form of activiting or inactivating a kinase(s), which modifies substrates that determine the physical state of the cell, Checkpoints can retard a transition until some intrinsic or extrinsic conditions are fulfilled. There are two key control points in the cell cycle. They are in G_1 and at the end of G_2. During G_1, a commitment is made to enter a replication cycle; the decision is identified by the restriction points in animal cells, and by START in yeast cells.

After this decision has been taken, cells are committed to begining on S phase, although there is a lag (gap) period before DNA replication initiates. The end of G_2 is marked by a decision that is executed immediately to enter mitosis.

A common feature in the cell cycles of yeasts and animals is the existence of an M-phase kinase, consisting of two subunits : Cdc2, with serine / threonine protein kinase catalytic activity : and a mitotic cyclin of either A or B class. The activity of the M-phase kinase is controlled by the phosphorylation state of the catalytic sybunit. The active form requires dephosphorylation on Tyr - 15 (in yeasts) or Thr - 14 / Tyr - 15 (in animal cells) and phosphorylation on Thr-161. The cyclins are also phosphorylated. In animal cells, the kinase is inactivated by

degradation of the cyclin component, which occurs abruptly during mitosis.

By phosphorylating appropriate substrates, the kinase provides MPF activity, which stimulates mitosis or Meiosis. Phosphorylation of H_1 could be concerned with the need to condense chromatin at mitosis. Phosphorylation of lamins (another factor) causes the dissolution of the nuclear lamina. Phosphatases are required to reverse the modifications introduced by M-phase kinase.

Transition from G_1 into S phase requires a kinase related to the M-phase kinase. In mammalian cells, a family of catalytic subunits is provided by the Cdk genes. There are ~10 Cdk (cyclin - dependent kinase) genes in animal genome.

Checkpoints control progression of the cell cycle. One checkpoint responds to the presence of unreplicated or damged DNA by blocking mitosis. Others control progress through mitosis.

Model questions :

1. What are the divisions of a cell cycle? State the functional activities of the different stage. Comment on the duration of various cell-cycle stages.
2. Expound your knowledge on the cell cycle control mechanisms.
3. Narrate the roles of regulators of cell-cycle progression.

Glossary :

Interphase, Cytokinesis, M-phase, GO, START, p53, Cdk's Cdc - 2, MPF.

References :

1. Geoffrey M. Cooper (2000). The Cell (a Molecular Approach). Second Edition. ASM Press. Washington, D. C.
2. Bruce Alberts et al. (1994). Molecular Biology of The Cell Third Edition. Garland Publishing Inc. New York & London.
3. Benjamin Lewin (2000). genes VII. Oxford University Press.

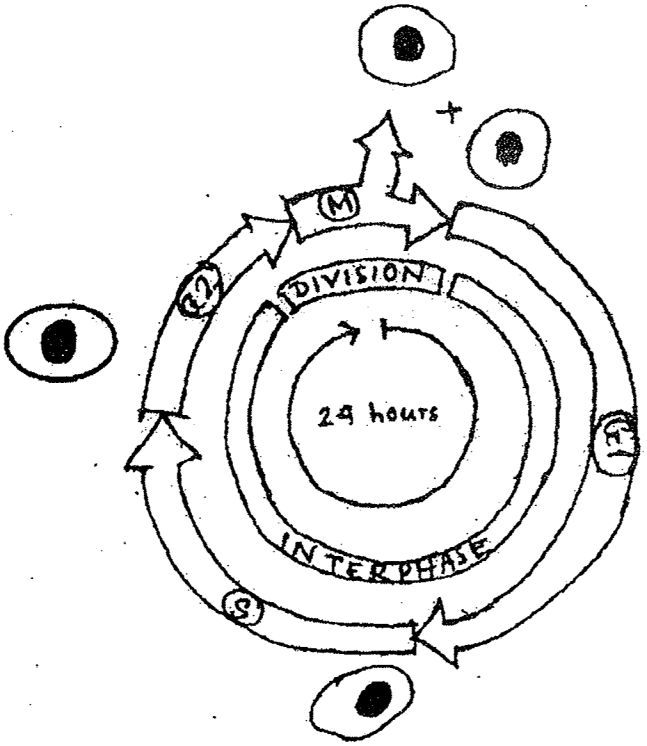


Fig. - 1 : The four successive phase of a typical eukaryotic cell cycle.
During interphase - Cell grows continuoulsy.
During M Phase - Cell divides

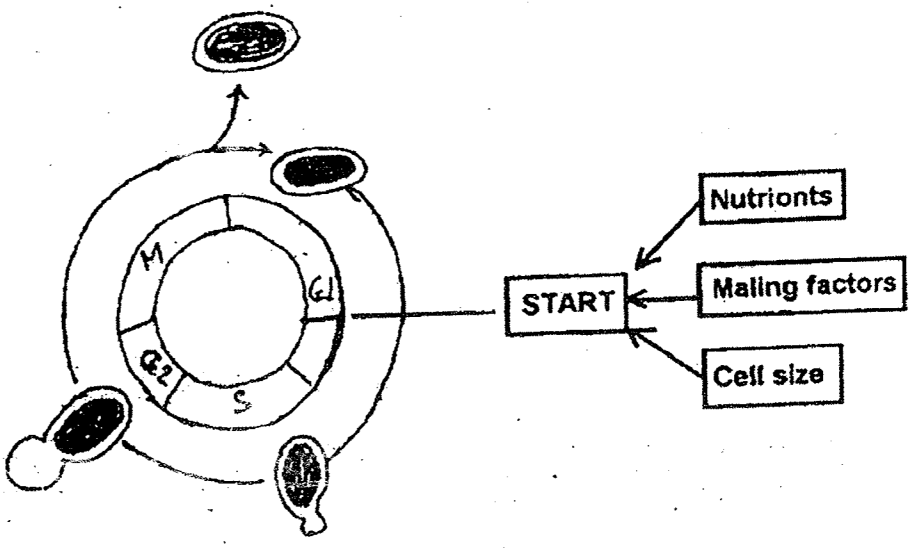


Fig. - 2 : Regulation of the cell-cycle of budding yeast.

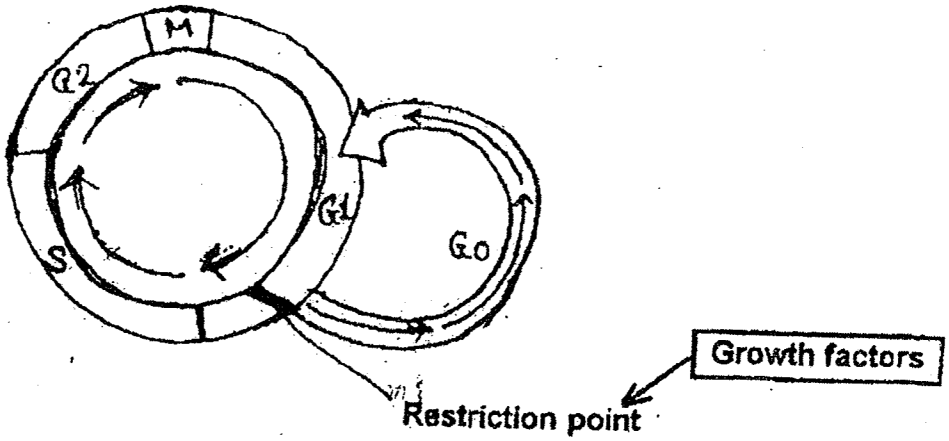


Fig. - 3 : Regulation of animal cell cycle by growth factors.

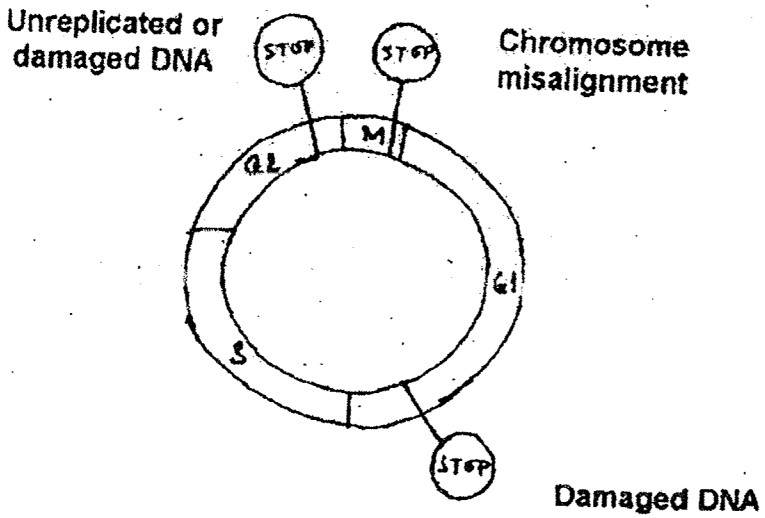


Fig. - 4 : Cell cycle checkpoints. (Several checkpoints function to ensure that complete genomes are transmitted to daughter cells).

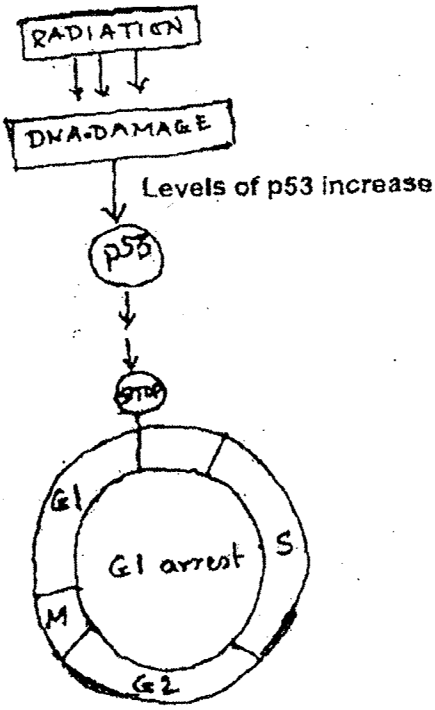


Fig. - 5 : Role of p53 in G1 arrest induced by DNA damage.

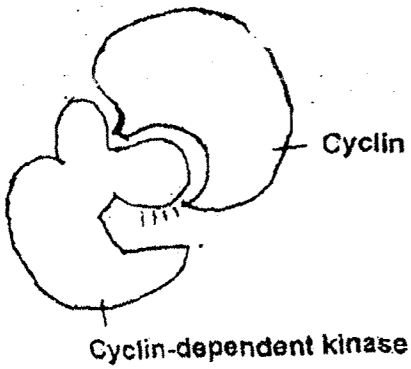


Fig. - 6 : Two key components of cell-cycle control system in mammals

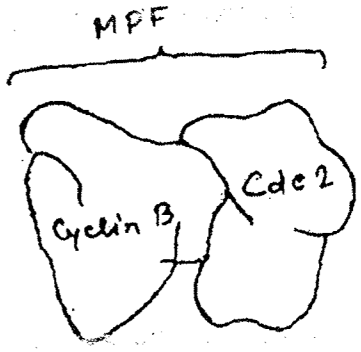


Fig. - 7 : Structure of MPF. (MPF is composed of cyclin B and the Cdc2 protein kinse).

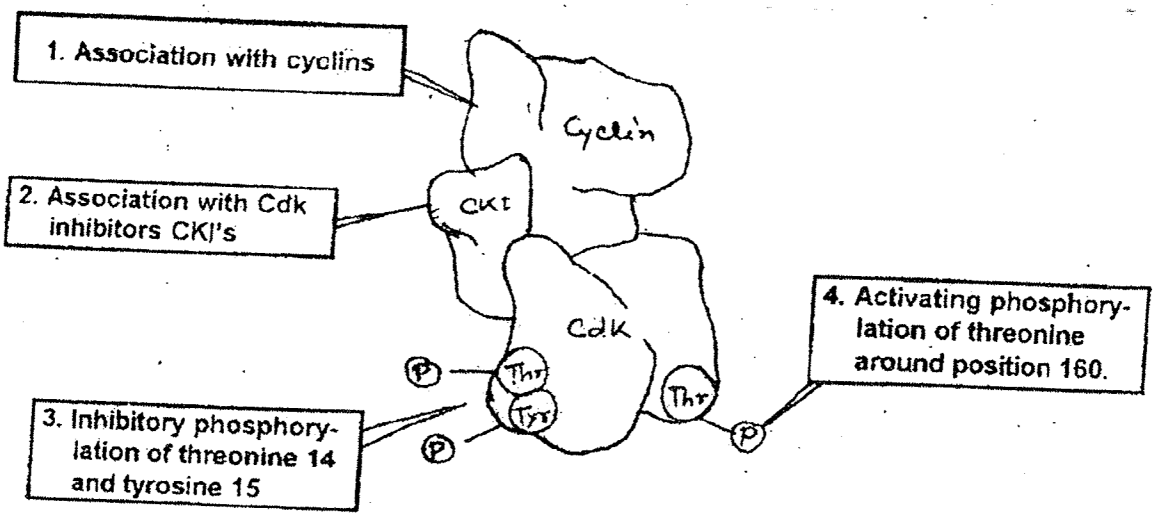


Fig. - 8 : Mechanisms of Cdk regulation

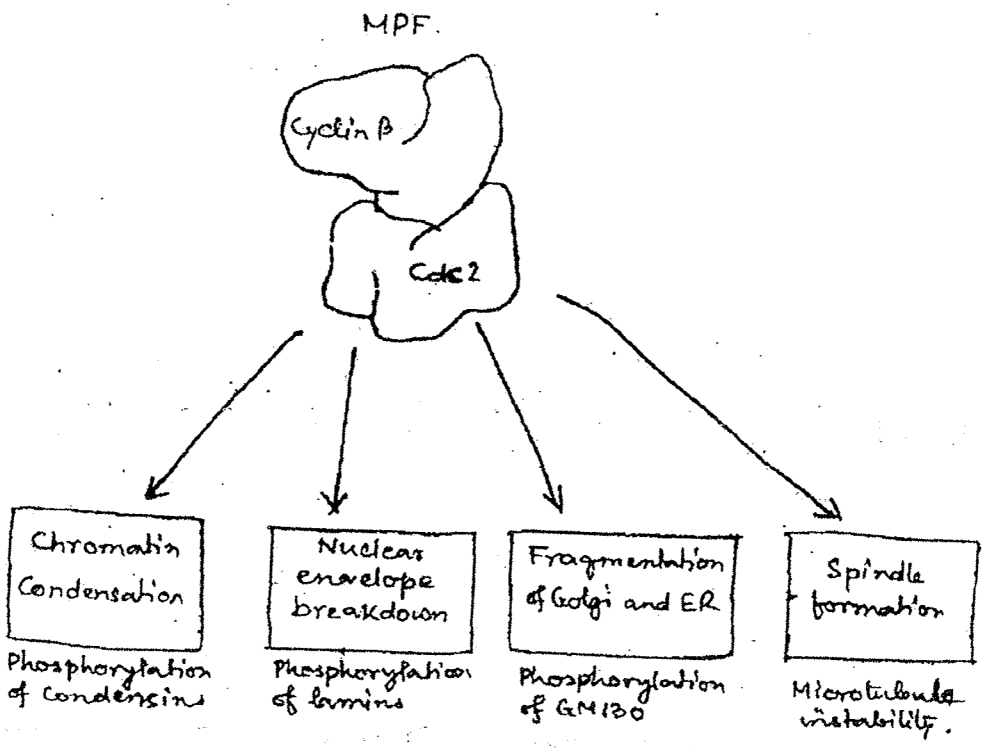


Fig. - 9 : Targets of MPF.
MPF induces multiple nuclear and cytoplasmic changes at the onset of M Phase, both by activating other protein kinases and by phosphorylating proteins such as condensins and the nuclear lamins.

THE CHROMOSOMES

During cell division, Chromatin becomes condensed to form some distinct structures called chromosomes, clearly visible. These are under the optical microscopes. Specific number of chromosomes are found in the cells of the organisms of a particular species. Therefore these elements are very much important for the analysis of genetic characters.

Shape of the Chromosomes :

Shape of and size of the chromosomes very with the state of condensation of the chromatin material. At the metaphase state of cell division chromosomes become condensed to such extent that they can be studied very well at this stage. A typical metaphase chromosome posses two arms connected a point called centromere. Each arm is with two chromatids. For better observation of the chromosomes under the light compound microscope squash preparation technique is usually followed. For this purpose chromosomes are stained with basic dyes (e.g. Orcein, Giemsa) and then squashed between the slide and coverslip by gentle pressure.

Chromosomes are classified into four types according to their shape, which is determined by the position of the centromere. These are telocentric, acrocentric, submetacentric and metacentric.

Telocentric chromosomes posses the centromere at one end of the chromatids. Acrocentric chromosome have centromere near the tip, thus one arm is much longer than the other. Submetacentric chromosomes have unequal arms because the centromere is located not at the middle, metacentric chromosomes have equal or almost equal arms, therefore during anaphase movement metacentric chromosomes take 'V' shape, submetacentric 'J' shaped and acrocentric 'I' shaped.

Nomenclature of chromosome :

A typical metaphase chromosome posses chromatid, chromonema, chromomeres, centromere and Telomere. Besides these Secondary constrictions, Nucleolar Organizers, Satellites are the features of some chromosomes (Fig. - V)

Chromatid :

At metaphase each chromosome consists of two symmetrical structures; the chromatids, each one of which contains a single DNA molecule. The chromatids are attached to each other only by the centromere and

become separated at the start of anaphase: when the sister chromatids migrate to opposite poles. Therefore, anaphase chromosomes have only one chromatid while metaphase chromosomes have two.

Chromonema(ta) :

During prophase (rarely in interphase) the chromosomal material becomes visible as very thin filaments, which are called chromonemata and which represents chromatids in early stages of condensation "chromatid" and "chromonema" therefore, are the two names of the same structure : a single linear DNA molecule with its associated proteins.

Chromomeres :

These are bead like accumulations of chromatin material that are occasionaly visible along interphase chromosomes. Chromomeres are especially visible in polytene chromosomes, where they are arranged side by side, constituting the chromosome bands. In metaphase stage the chromosomes are tighly coiled and the Chromomeres are no longer visible.

Centromere or kinetochore:

This is the area of the chromosome that becomes attached to the mitotic spindle. The centromere lies within a thinner segment of the chromosome, called **the primary constriction**. The regions flanking the centromere frequently contain highly repetitive DNA and may stain more intensely with basic dyes. This area is called heterochromatic. Centromeres contain specific DNA sequences with special protein bound to them, forming a disc shaped structure, called kinetochore. Microtubules become attached with this area. (Fig. - 2')

Telomere :

This is the tip of the chromosomes (Fig - 2'). Telomeres contain the ends of the long linear DNA molecule contained in each chromatid. The telomeres are composed of repeats of short, GC rich sequences. The G-rich strand of a telomere is added at the very 3'-ends of DNA strands, not by semiconservative replication, but by an enzyme called telomerase. The exact sequence of the repeat in a telomere is species - specific, in vertebrates including humans, it is TTGGGG/AACCCC.

Secondary constrictions :

This is an area where chromatin material is also less besides the primary contriction but not present in all

chromosomes. Some chromosomes do possess this area secondary constrictions are distinguished from the primary constriction by the absence of marked angular deviations of the chromosomal segments during anaphase.

Nucleolar organizers :

These areas are specific secondary constrictions that contain the genes coding for 18S and 28S ribosomal RNA and that induce the formation of nucleoli. The secondary constriction may arise because the rRNA genes are transcribed very actively interfering with chromosomal condensation.

Satellites :

Some chromosomes posses the element called satellites. These are rounded body of chromatin material separated from the rest of the chromosomes by a secondary constriction. The satellite and the constriction are constant shape and size for each particular chromosome.

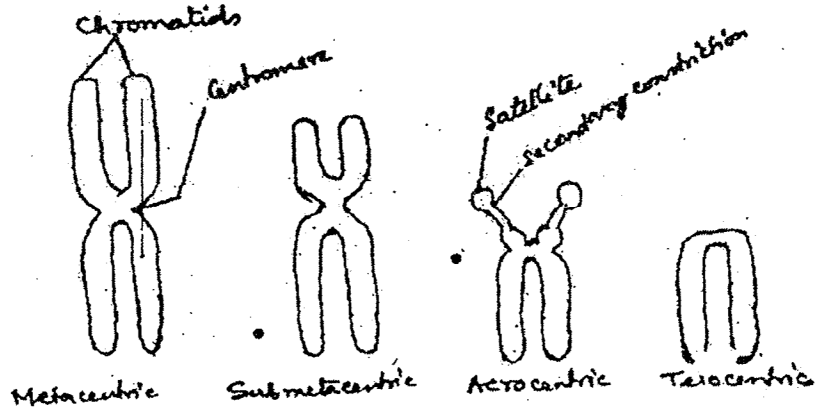


Fig. - 1' : Classification of chromosomes on the basis of centromeric position

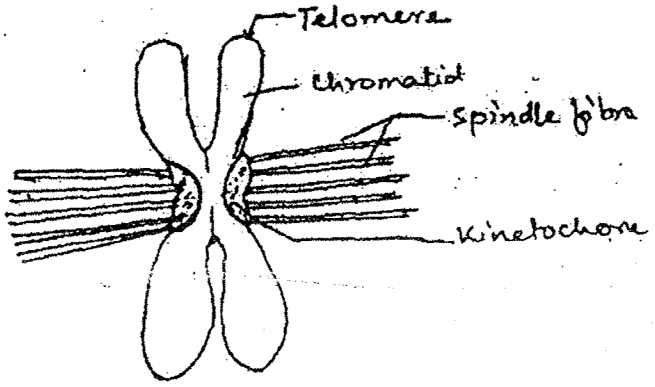


Fig. - 2' : Centromere and kinetochore of a metaphase chromosome.

CHROMOSOME & CHROMATIN

DNA is the principal genetic constituent of cells, carrying information in a coded form from cell to cell and from organism to organism. The complexes between eukaryotic DNA and proteins are called chromatin, which typically contains about twice as much protein as DNA. The major proteins of chromatin are the **histones**. These histones are small proteins containing a high proportion of basic amino acids (arginine and lysine) which facilitates binding to the negatively charged DNA molecules.

Histones :

Most eukaryotic cells contain five different kind of histones, H1, H2A, H2B, H3 and H4. These are very similar among different species of eukaryotes, (Table - 1). The histones are extremely abundant proteins in eukaryotic cells; together, their mass is approximately equal to that of the cells DNA. In addition, chromatin contains an approximately equal mass of a wide variety of nonhistone chromosomal proteins. Most of the histones are well conserved from one organism to another. The most extreme example is histone H4 of cow differs from H4 of pea in only two aminoacids out of a total of 102. Histone H3 is also extremely well conserved, histones H2A and H2B are moderately well conserved; but bistone H1 varies considerably among organisms. Histones are not found in eubacteria (e.g. *E. coli*), other proteins do the functions of histones. Archaebacteria, however, do contain histones.

Nucleosomes :

The basic structural unit of chromatin is called the nucleosome, first described by Roger Kornberg in 1974. Nucleosomes, as originally defined, contained about 200 bp of DNA, associated with a histone octamer that consists of two copies of each of H2A, H2B, H3 and H4. These are known as **core histones**. 200 bp long DNA is released by subjecting chromatin to a mild nuclease treatment. Further digestion with nuclease yields an intermediate, sometimes called a 'chromatosome', which retains about 165 bp of DNA and histones H1. Exhaustive digestion gives a nucleosome core particle with about 145 bp of DNA and no histone H1. (Fig. - 3 a', b', c').

The packing of DNA with histones yields a chromatin fibre approximately 10nm in diameter which is composed of chromatosomes separated by linker DNA segments averaging about 80 bp in length. In electron microscope, this 10 nm fibre has the beaded appearance that suggested the nucleosome model, Packaging of DNA into such a 10 nm chromatin fibre shortens its length approximately six fold.

The chromatin can then be further condensed by coiling into 30 nm fibre. Interaction between histone H1 molecules appear to play an important role in this stage of chromatin condensation.

The 30 nm Fibre :

When chromatin is examined in the electron microscope, two types of fibres are seen : the 10 nm fibre and 30 nm fibre. The 10 nm fibre is essentially a continuous string of nucleosomes, appearing as 'beads - on - string' form, when chromatin is visualized in conditions of greater ionic strength the 30 nm fibre is obtained. The 30 nm fibre probably arises from the folding of the nucleosome chain into a solenoid structure having about six nucleosomes per turn. In chromatin fibre it can be folded into such structures simply by increasing the salt concentration. At 1 mM the nucleosomes come closer together, and at concentrations over 60 mM a 30 nm thick fibre is found. The whole structure is stabilized by interactions between H1 molecules in neighbouring nucleosomes. The H1 molecules can interact with each other because they are located in the central 'hole' of the solenoid. Although other structural arrangements have been proposed for the 30 nm fibre (superbeads, invoked by Manfred Ren and Wolf stratling; Irregular condensed fibre shape was described by K.V. Holde and J. Zlatanova. Woodcock and colleagues have proposed a variable zig zag ribon structure for the 30 nm fibre) the solenoid model remains the most likely form of chromatin folding.

The role of Histone H1 in chromatin folding :

Earlier evidence suggested that H1 histone must play a critical role in forming the 30nm fibre because no such fibre occured in the absence of this histone. Other evidence suggested that H1 -H1 interaction is possible even in the absence of the 30nm fibre.

A compromise hypothesis suggests that the role of histone H 1 is to space the nucleosomes properly on chromation. This proper spacing then favours 30nm fibre formation.

The DNA of a 30nm solenoid has a packing that is about 40 folds. The DNA of a metaphase chromosome however, is packed between 5000 and 10,000 times i.e. the 25nm (30nm) fibre must be further folded more than 100 fold during mitosis.

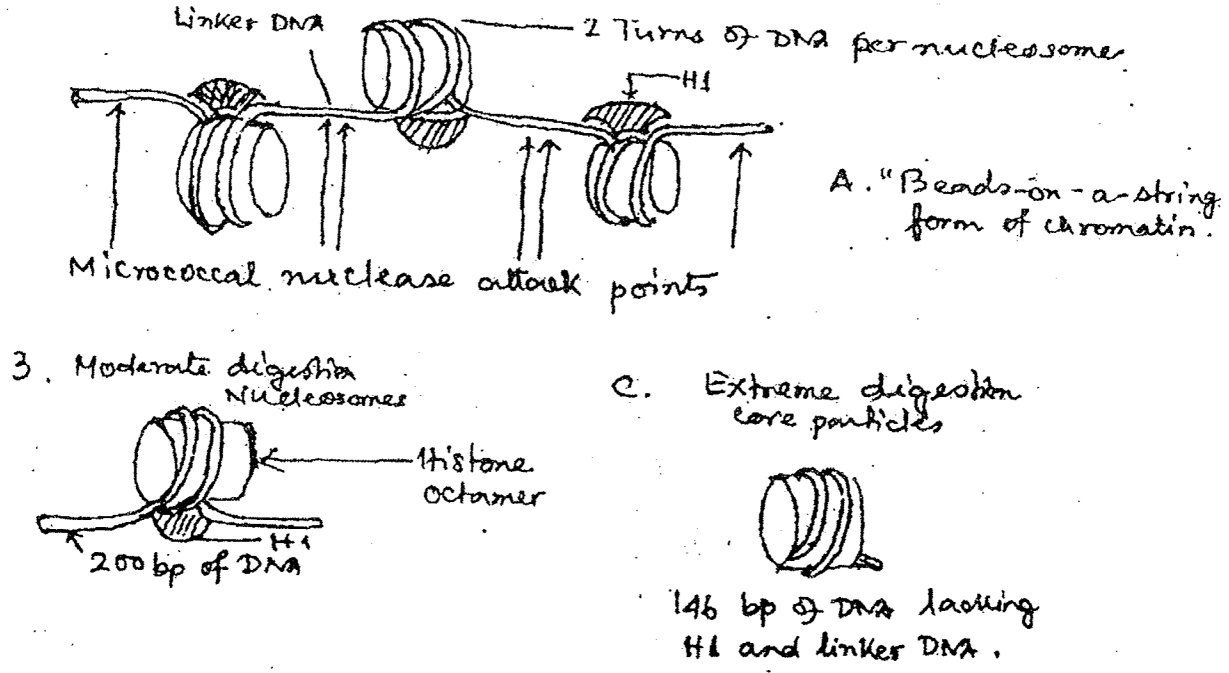


Fig. - 3a' : Effect of nucleases on chromatin.
A) intact chromatin B) 200 bp DNA with H1 in nucleosome
C) core particle with 146 bp DNA

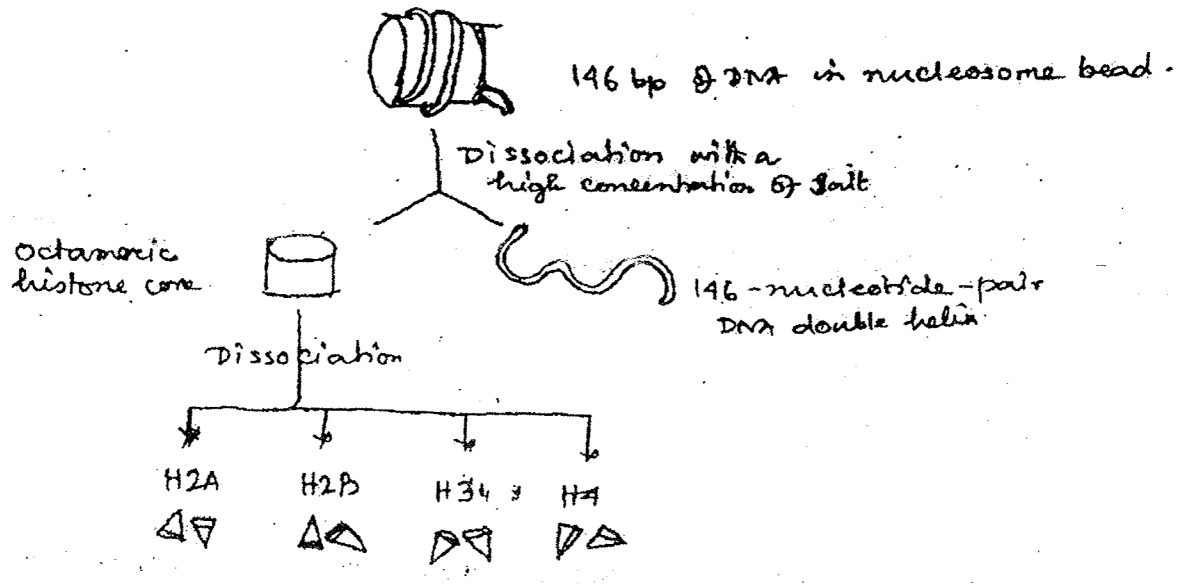


Fig. - 3b' : Histones are 146 nucleotide pair DNA double helix separated by high concentration of salt treatment.

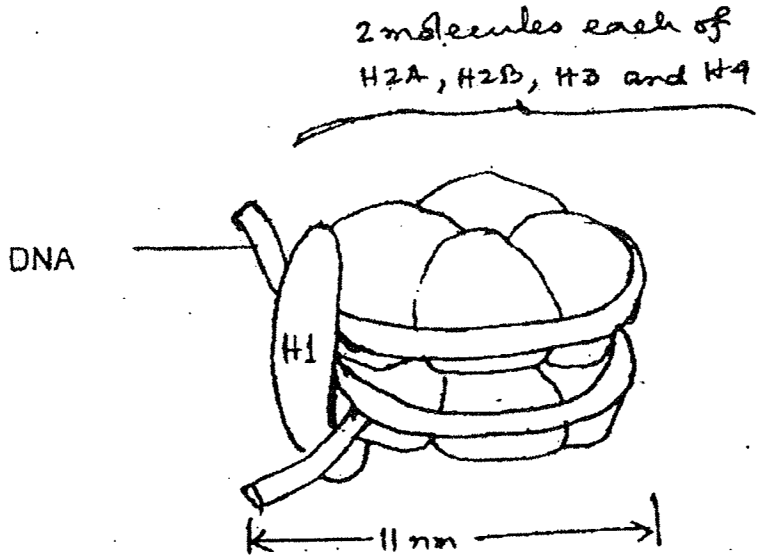


Fig. - 3c' : Structure of Chromatosome

Table – 1

*Characterization of the Mazor histone proteins of the nucleosome

Class	Histone	Molecular wt.	Number of amino acids	Percentage Lysine + Argenine
Very Lysine rich	H1	22,500	244	30.8
Lysine-rich	H2A	13,960	129	20.2
	H2B	13,774	125	24.2
Arginine rich	H3	15,373	135	22.9
	H4	11,236	102	24.5

*Data based on the studies of calf thymus for histones and H1 for rabbit

Higher-order chromatin folding: The Supersoleniod

It is now generally accepted that the chromatin fibre has a solenoid type ultrastructure, with the run of the coil made up of linear nucleosome units, 30-nm solenoid undergo supercoiling to form 400-nm tubuler structure called supersolenoid. Dissecting a supersolenoid a hierarchy of helices can be found. Starting with a single DNA double helix of 2-nm diameter we can contruct a metaphase chromosome hypothetically. The DNA duplex is wound around histone octamers with nearly two turns of DNA required per octamer. In the presence of histone H1, the speacer histone, the nucleosome beads may be somewhat drawn together. Thus forming 10 nm fibre. 10 nm string of nucleosome fibre upon soling form a 30 nm solenoid. The 30-nm solenoid is supercoiled in a very shallow helix of 30-nm pitch with a mean diametre of 400 nm. Each turn of the supersolenoid helix contains 150,000 base pairs of DNA which gives a packing ratio (length ÷ 30 nm pitch) for DNA of about 1400. This correspond well with the required packing ratio in metaphase chromosomes.

According to this model, there are therefore, three hierarchies of helices. One helix is in the nucleosome, a second in the solenoid and a third in the supersolenoid. Finally, the diameter of wet, unifixed human chromatids is estimated at about 1.4 μm. To account for this final dimension, the supersolenoid must be further contracted by a factor of about S. This additional contraction may be either helical or by a folding mechanism (Fig. - 4).

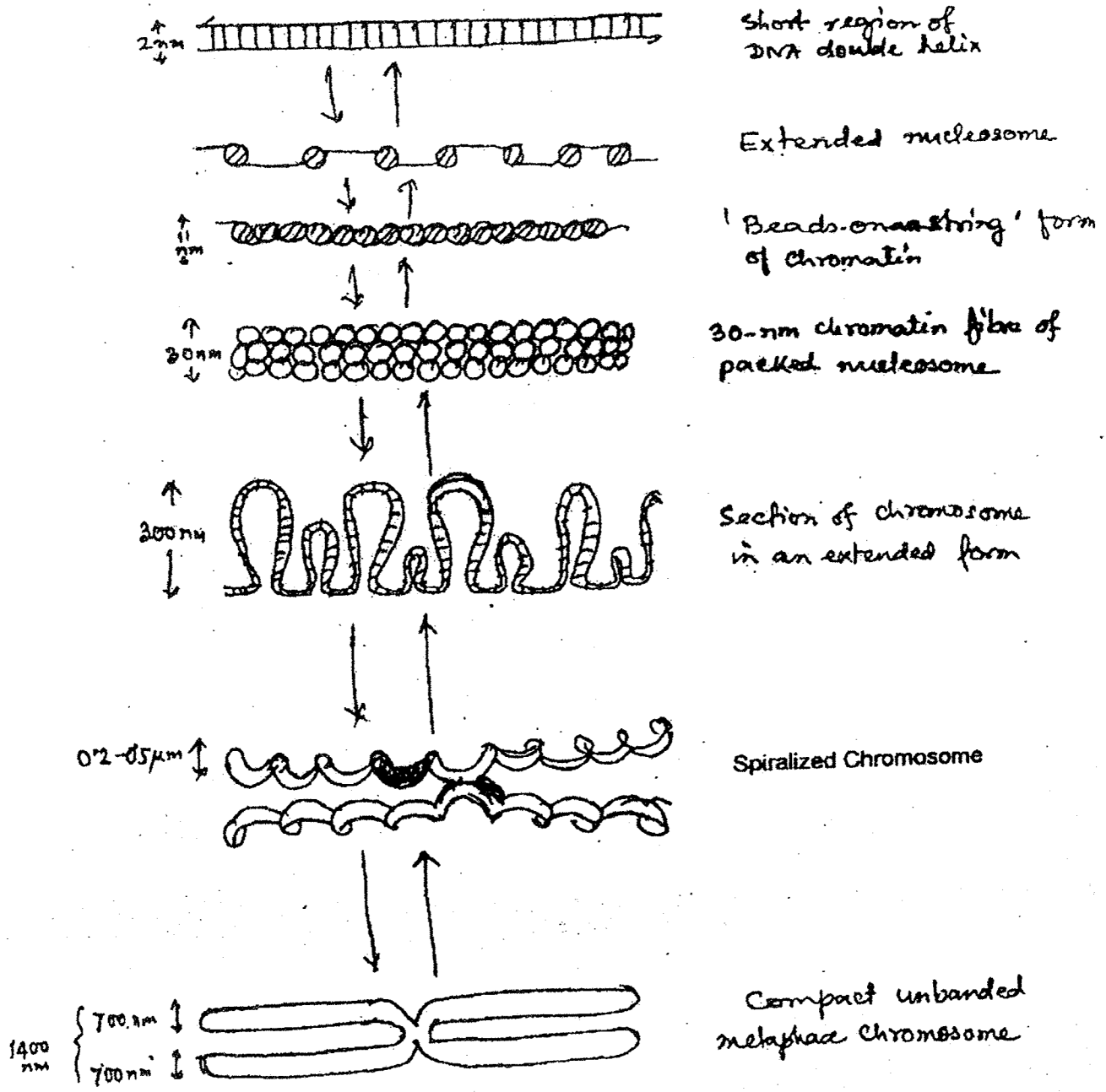


Fig. - 4' : Schematic diagrams showing the levels of organization of chromosomes from DNA to metaphase structure and reverse.

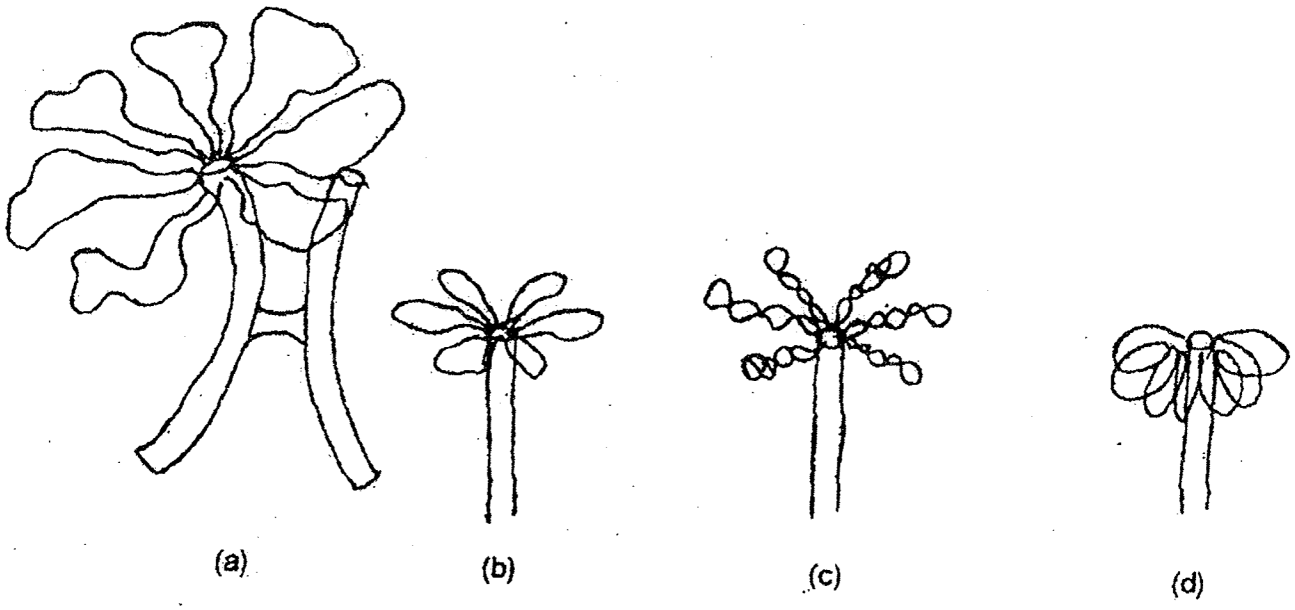


Fig.-5 : The Scaffold model of chromosome structure
(a) The DNA in the loops is condensed by interaction with histories and may take on a variety of conformations (b - d).

Chromosome Scaffolding:

Using chromosomes from which histones were removed by competing with the polyanions dextran sulfate and heparin, Laemmli and co-workers have uncovered a proteinaceous scaffolding structure in the core of the chromosome. A fundamental and reproducible observation is that DNA strands in histone depleted chromosomes cluster around and are anchored to a central protein scaffolding that still retains the skeletal structure of two chromatids. The DNA strand runs as a continuous loop from a point of exiting the scaffold to an adjacent point of entry. The majority of loops are about 15 to 30 μm long, representing 45,000 to 90.000 base pairs.

Laemli et al. also isolated intact scaffolds depleted of DNA and showed that the scaffold structure is of the same length as the original intact chromosome and has very much the same morphology as the chromosome from which it is isolated. The material of the scaffold is nonhistone protein. It is resistant to RNase but is disrupted by proteases, urea and the detergent sodium dodecyl sulfate (Fig.-5)

POLYTENE CHROMOSOMES

The interphase nuclei of some tissues of the larvae of Dipteran flies contain chromosomes that are greatly enlarged relative to their usual condition. They posses both incleased diameter and greater length. These chromosomes are called polytene chromosomes. Polytene chromosomes are found in certain cells of dipteran larvae. The most commonly studied source is salivary gland tissue of Drosophila and Chironomus.

Polytene is achieved by replication of the DNA several times without nuclear division (endomitosis) and the resulting daughter chromatids do not separate and remain aligned side by side, These giant chromosomes are generated during early lerval development as a result of cessation of mitotic divisions in these tissues without a concomitant halt of DNA replication. DNA continues to replicate as many as 10 times, yielding 210 - 1024 copies of DNA fibres, which remain together and exactly aligned.

Banding and Genes :

In polytene cells, the chromosomes are visible during interphase with light microscopy. These chromosomes provide insight into chromosome structure during interphase of the cell cycle.

One of the characteristics of interphase chromosome is banding. Each chromosome consists of a visible series of bands (chromomeres). The bands range in size from the largest with a breadth of ~ 0,5 μm to the smallest of — 0.05 4m. The bands contain most of the mass of DNA and stain intensely with appropriate reagents. The regions between bands stain more lightly and are called interbands, Band numbers and positions are predictable, with as many as 6900 band reported present on the four polytene chromosomes of Drosophila.

The centromeres of all four chromosomes of *Drosophila melanogaster* aggregate to form a chromocentre that consists largely of heterochrmatin (in the male it includes the entire Y chromosome). The length of the chromosome set is — 2000 μm. The DNA in extended form would stretch for ~ 40,000 I.1m, so the packing ratio is ~ 20.

The banding pattern is characteristic for each strain of Drosophila. The constant number and linear arrangement of the bands was first noted in the 1930s, when it was realized that they form a **cytological map** of the chromosomes. Rearrangements — such as deletions, inversions, or duplications - results in alterations of the order of bands. The linear array of Hands can be equated with the linear array of genes. So genetic rearrangements, as seen in a linkage map, can be correlated with structual rearragements of the cytological map. Ultimately, a

particular mutation can be located in a particular band. Since the total number of genes in D. melanogaster to exceed the number of bands. there are probably multiple genes in most or all 'bands.

The positions of particular genes on cytological map can be determined directly by the technique of in situ or **cytological hybridization**. Cioned DNAs can be mapped by in situ hybridization to polytene chromosomes, after with sufficient resolution to localize cloned genes to specific bands. Polytene chromosomes are very suitable for in situ hybridization because of their more than 1000 DNA molecules aligned side by side, thereby greatly facilitating the deletion of single copy genes.

Puffs (Balbiani rings) :

One of the most remarkable characteristics of polytiene chromosomes is that it is possible to visualize in them the genetic activity of specific chromosomal sites at local enlargements called puffs. A puff can be considered a band on which the DNA unfolds into open loops as a consequence of intense gene transcription. Sometimes in a large puff as typified by the Balbiani rings, the swelling is so extensive as to obscure the underlying array of bands.

The pattern of puffs is related to gene expression. During larval development puffs appear and regress in a definite, tissue-specific pattern. A characteristic pattern of puffs is found in each tissue at a given time. Puffs are induced by the hormone ecdyson that controls Drosophila development. Some puffs are induced directly by the hormone: others are induced indirectly by the product of the earlier puffs.

Since puffs are sites where RNA is being synthesized, therefore puffing has been viewed as a consequence of transcription.

Summary (Chromosomes, Nucleosomes and Polytene Chromosomes)

Chromosomes :

The morphology of chromosomes can be best studied during metaphase and anaphase. Depending on the position of the centromere (the primary constriction) chromosomes are of four types i) telocentric ii) acrocentric iii) submetacentric and iv) metacentric. Each metaphase chromosomes has two chromatids attached at a point called the centromere. Each chromatid consists of a single linear DNA molecule with its associated proteins. Mitotic chromosomes are made of chromatin fibres of 30 nm, Some regions of the chromosome remain condensed during interphase and are stained differentially by basic dyes. These heterochromatic regions are late replicating and genetically inert. Two types of heterochromatin can be recognized : constitutive and facutative heterochromatin

constitutive heterochromatin is permanently condensed in all types of cells and is related to highly repetitive DNAs (satellite DNAs). Facultative heterochromatin is condensed in certain cell types or at special stages of development. The genes contained in facultative heterochromatin are not expressed as shown in the Barrbodies of mammals.

Chromatin & nueleosomes :

The genome of the eukaryotic cell is sequestered in the nucleas as part of nucleoprotein complex referred to as **chromatin**. In most cells, the genome is present in units of chromatin, each unit a condensed form of chromatin called a **chromosome**.

Prokaryotic genomes are circular duplex DNA molecules that are organized and stabilized by attachments to proteins and RNA. The DNA extends out from the organizing centre in domains that are supercoiled.

All eullaryotic chromatin consists of nucleosomes. A nucleosome contains a characteristic length of DNA, usually - 200 bp, wrapped around an octamer containing two copies each of histones H2A, H2B, H3 and H4. A single H1 protein is associated with each nucleosome. Virtually all genomic DNA is organized into nucleosomes. Treatment with micrococcal nuclease can divide the nucleosomal DNA into two regions. The linker region is digested rapidly by nuclease but the core region of 146 bp is resistant to digestion. The path of DNA around the histone octamer creates 1.65 supercoils. The DNA 'enters' and 'leaves' the nucleosome in the same vicinity and could be 'sealed' by histone H1.

Nucleosomes are organized into a fibre of 30 nm diameter which has 6 nuleosomes per turn and a packing ratio of 40. Removal of H1 allows this fibre to unfold into a 10 nm fibre that consists of a linear string of nucleosome. The 30 nm fibre probably consists of the 10 nm fibre wound into a solenoid. The 30 nm fibre is the basic constituent of both euchromatin and heterochromatin; non histone proteins are responsible for further organization of fibre into chromatin or chromosome ultrastructure.

Polytene Chromosomes :

Chromosomes are generally decondensed during interphase, so their structure is difficult to discern. Notable exceptions are the lampbrush chromosomes of Amhibian oocyte and the polytene chromosomes of insects salivaly gland. Studies of these two types of interphase chromosomes suggest that each long DNA molecule in a chromosome is divided into a large number of discrete domains that are folded differently.

Polytene chromosomes from dipteran species are banded. When genes are expressed, bands or

chromomere, puff out by an uncoiling of the elementary chromatin fibre. Transcription takes place in puff regions.

Polyterie chromosomes provide the first evidence that eukaryotic gene activity is regulated at the level of RNA synthesis.

When dipteran chromosomes become polytenic, the DNA replicted by endomitosis, and the resulting daughter chromatids remain aligned side by side. These chromatids produce distinct dark bands and interbands during interphase.

Model Questions :

1. With suitable sketch describe the structure of a typical metaphase chromosome.
2. Elucidate the chemical component of eukaryotic chromatin.
3. Describe the model for the formation of chromosome from the nucleosome fibre.
4. Expand the polytene organization of any dipteran insect.

Glossary: Chromatin, histone, nucleosome, nucleosome core particle, chromatosome, euchromatin, heterochromatin, centromere, kinetochore, telomere, Balbianiring, chromocentre.

References :

1. Molecular Biology of the Cell. Third Edition. Bruce Albert et al. (1994). Garland Publishing, inc.

2. Cell and molecular Biology. Eighth Edition. E.D.P. De Robertis & E.M.F. De Robertis, Jr. (1999) B.I. Publications Pvt. Ltd.

3. The Cell – A molecular approach. Second Edition. Geoffery M. Cooper (2000). A.S.M. Press. Washington D.C.

4, Molecular Biology International Edition. Robert F. Weaver (1999). WBC Mc Graw-Hill.

5. Genes VII. Benjamin Lewin. (2000) Oxford University Press.

6. Cell Biology Neal O. Thorpe. John Wiley & Sons.

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION
MIDNAPORE - 721 102

M. Sc. in ZOOLOGY

Part-I
Paper - II, Group-A, Unit-II

Module No. 16

Topoisomerase I catalyzes the relaxation of supercoiled DNA :

The interconvension of topoisomerse of DNA is catalyzed by enzymes called topoisomerases that were discovered by James Wang & Martin Gellert. These enzymes after the linking number of DNA by catalyzing a three step process:

i) eleavage of one or both standards of DNA
ii) passage of a segment of DNA through this
iii) resealing of the DNA break.

The type I topoisomerase of E. Coli catalyzes t relaxation of negatively supercolied DNA. This enzym acts first as a nuclease & then as a ligase. However inte relaxed circles were formed in the absenc of ATP, NAI & other energy donors. In fact, a covalent enzyme DN complex has been formed. The 5′ phosphate moiety o DNA strand was found to be linked to a tyrosine hydrox gr. of the 100 kd enzyme (fig. 1). The 3′ - 0H at t other end of the cleaved chain nucleophilically at tac this activated intermediate to restore the continuity the circle. The only role of this topoisomerase is create a transient break that allows the passage of segment of DNA. The linking no. increased by +1 wi each catalytic cycle i.e. by one less turn of negeti supercoil.

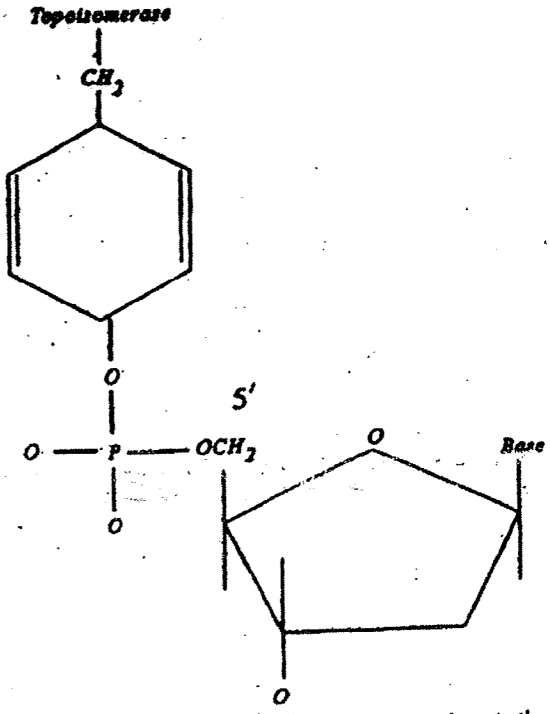


Figure 1 Showing enzyme-sunstrate intermediate in the action of topisomerase. The 5′-phosphate end of the cleaved DNA strand is covalently linked to the OH group of a specific tyrosine residue of the enzyme.

DNA gyrase catalyzes the ATP-driven introduction of negative Supercoil into DNA :

Supercoiling in E.coli is catalyzed by DNA gyrase, a topoisomerase consisting of two 105 kd. A chains and two 95 kd-B chains. DNA gyrase, an energy-transducing device; it converts the free energy of ATP into torsional energy of supercoiling. The reaction begins with the wrapping of about 200 bp DNA around the lozenge-shape enzyme. The binding of ATP then triggers the cleavage of both strainds of DNA : the four nucleotides from the site on the other strand. The 5′-phosphate terminus of each cleaved strand is linked to a specific tyrosine residue of an A subunit of the enzyme. The anchoring of the two ends of the cut DNA is essential for preventing their free rotation which would quickly lead to the loss of supercoiling.

The next step is the passage of a segment of DNA through the ~35 Å gap between the fixed ends. This passage is vectorial –DNA gyrase allows facile movement only in the direction leading to negative supercoiling. The cleaved DNA ends then come together to reestablish the continuity of both strands of the duplex. Finally, hydrolysis of bound ATP leads to the release of the transported DNA segment, enabling the enzyme to begin an other round of catalysis. DNA gyrase decreases the linking number of its substrate in steps of two, because duplex DNA passes through a break in both strands. About two negative supercoils are introduced per second. DNA gyrase can act repeatedly on the same DNA substrate without dissociating from it.

The degree of supercoiling of bacterial DNA is thus determined in the opposite actions of two enzymes. Negetive supercoils are introduced by DNA gyrase and are removed by topoisomerase I.

DNA polymerase I, the first template-directed enzyme :

The search for an enzyme that systhesizes DNA was initiated by ARthur Kornberg & his assotiates in 1955, DNA pol I is not the enzyme that replicates most of the DNA in E.coli. However it plays a critical role in replication & also in the repair of DNA.

Function of DNA Pol I

*** Catalyzing function :**

DNA pol I, a 103 kd monomer catalyzes the step by step addition of deoxyribonneleotide units to the 3' end of a DNA chain.

$$(DNA)_n residues + dNTP \rightleftarrows (DNA)_{n+1} + PPi$$

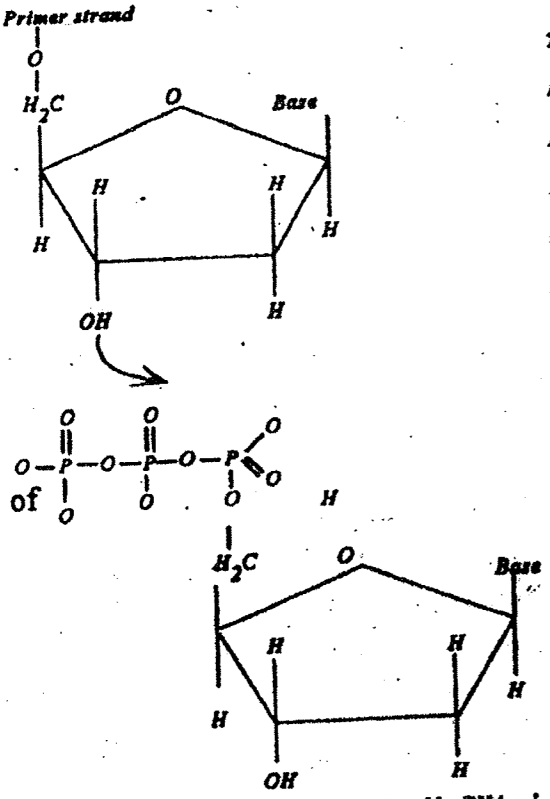
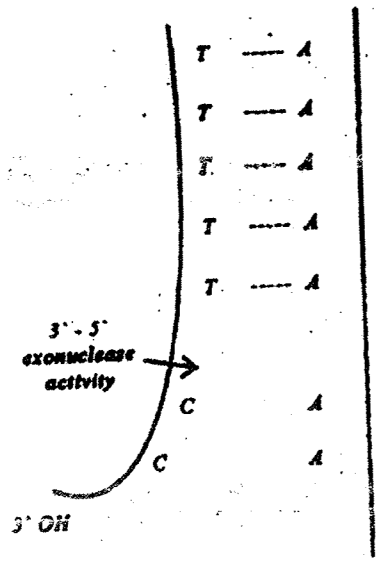


Figure 2 showing elongation reaction catalysed by DNA pol

T E M P L A T E S T R A N D

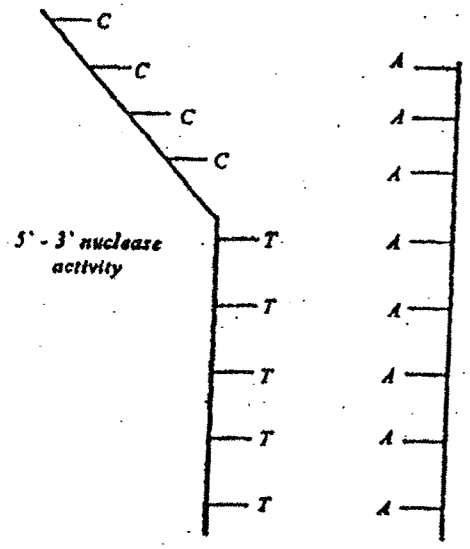
DNA pol I requires all four deoxyribonucleoside 5′ triphosphates – d ATP, d TTP, dCTP & dGTP and Mg++ to sysnthesize DNA. The enzyme adds deoxyribonneleotides to the free 3′-OH of the chain undergoing elongation which proceeds on the 5′→3′ direction. See fig. 2. A primer chain with a free 3′ – OH gr. is needed at start. A DNA template containing a single stranded region is also essential. The pol catalyzes the nueleophilic attack of the 3′-OH terminus of the primer on the innermost phosphorus atom a dNTP. A phosphoriester bond is formed and pyrophosphate is released. DNA pol is a moderately processive enzyme – it catalyzes multiple polymerization steps (~20).

A striking feature of the enzyme is that it takes instructions from its template.



*** Proof reading function :**

DNA pol I catalyzes the hydrolysis of nucleotides at the 3′-end of DNA chains. Thus DNA pol is a 3′ → 5′ exonuclease. To be removed, a nucleotide must have a free 3′–OH terminus. The 3′→ 5′ nuclease activity has an editing function in polymerization. The polymer shown in figure containts an unpaired C at the end of a sequence of T residues that form a double helix. On addition of DNA pol I & dTTP the unpaired C is first excised. dCMP is realeased and a 3′–OH gr. is exposed on the terminal T at the primer strand. Additional T are addied only after removal of the unpaired C. In general, DNA pol I residues mismatched residues at the primer terminus before proceeding into polymerization.

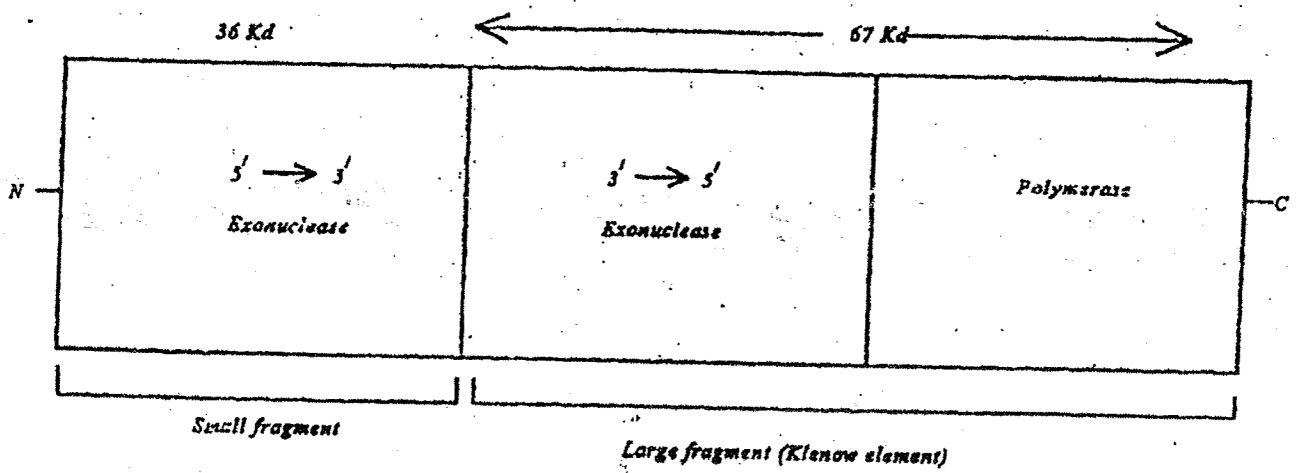


The $3' \rightarrow 5'$ exonuclease activity markedly exhances the accuracy of DNA replication.

*** Error Correcting Function :**

DNA pol I can also hydrolyze DNA starting from the $5'$ end of a chain. This $5' \rightarrow 3'$ nuclease activity is very different from the $3' \rightarrow 5'$ exonuclease action. First the cleaved bond must be in a double helical region. Second cleavage can occur atthe terminal phosphodiester bond or at a bond several residues away from the $5'$ terminus. Third $5' \rightarrow 3'$ exonuclease activity is enhanced by uncomitant DNA synthesis. Fourth, the active site for exonuclease action is cleanly separate from the active sites for polymerization and $3' \rightarrow 5'$ hydrolysis. Thus DNA pol I contains three different active sites on a single polypptide chain.

The $5' \rightarrow 3'$ exonuclease activity plays a key role in DNA replication by removing RNA primer. Moreover, the $5' \rightarrow 3'$ exonuclease complements the $3' \rightarrow 5'$ exonuclease activity by correcting errors of a different type.



DNA polymerase I has three enzymatic activities in a single polypeptide chain

Polymerase II and III :

In fact E. coli contains two other DNA polymerase named II and III which were found some

fifteen years after the discovery of DNA pol I. DNA pol II and II are like polymerase I in these respects –

1. These catalyze a template directed synthesis of DNA from deoxyribonucleoside 5′ triphosphate precursor.
2. A primer with a free 3′–OH group is required.
3. Synthesis is in the 5′ → 3′ direction.
4. They posses 3′ → 5′ exonuclease activity.

DNA replication starts at a Unique origin (ori C) & proceeds sequentally in opposite direction:

A unit of DNA capable of being replicated. A replicon contains a unique origin and a unique termination site. An entire replicon is replicated once in a cell cycle. The whole E. coli chromosome is a single replicon. The relative frequencies of a number of E. coli genes were determined by hybridization with complementary probes. The results of these experiments clearly showed that the relative gene frequencies depend on map position (see figure below) & revealed that :

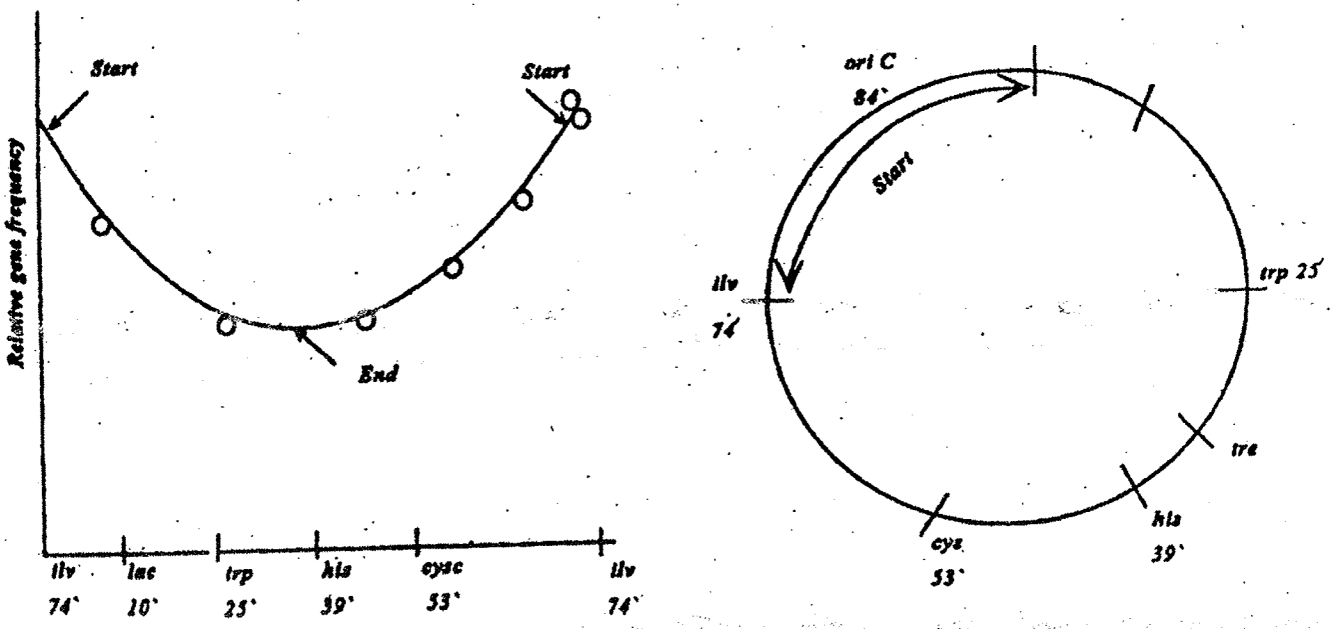
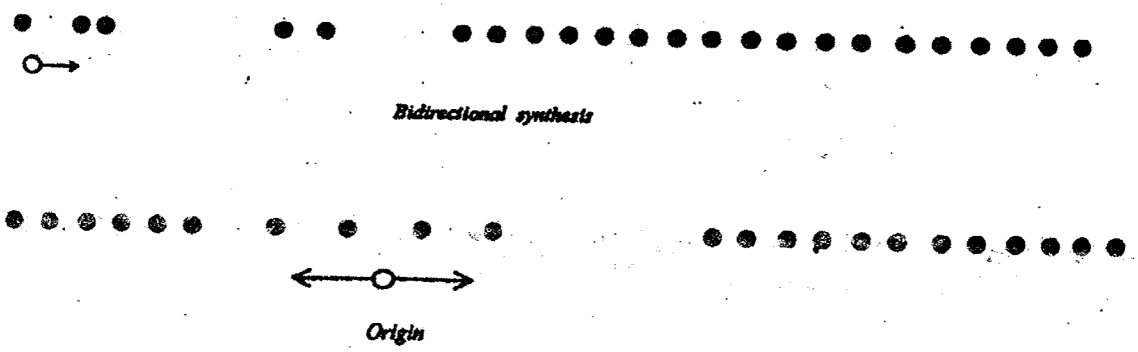


Fig. shows that amounts of different genes under conditions of raprid DNA systhesis in E. coli as a function of position on the genetic map.

1. Replications starts at a unique site – This origin locus has since been identified as ori C, located at 83 minutes on the standard 100 minute genetic map of E. coli.
2. Replication proceeds simultaneously opposite directions at about the same velocity. In other words, there are two replication forks one moves clockwise, the other counter clockwise.
3. The two replication forks meet at the (near 31′ on the map), the termination region, which is opposite the region of replication.

Further evidence for the bidirectionality of replication of E.coli DNA comes from autoradiography. Replication was initiated in a medium containing moderately radioactive tritiated thymine. After a few minutes of replication, the bacteria were transferred to a medium containing highly redioactive tritiated thymidine. Two levels of radioactivity were used to create two kinds of grain tracks in the autoradiographs : a low density track for DNA synthesized early & a high density track for DNA synthesized later. It replication were unidirectional tracks with a low grain density at one end a high grain density at the other end would be seen. On the other hand, it replication were bidirectional, the middle of a track would have a low density (see figure below).



The autoradiographic pattern provide a vivid answer. All of the grain tracks were denser on both ends than in the middle indicating that replication of the E. coli chromosome is bidirectiona;.

Replication begins with the unwinding of the ori C site :

The ori C locus is a sequence of 245 base pairs in the E. coli chromosome. The origin has some

unusual features. It contains a tandem array of three 13 nuecleotide sequences. Each begins with GATC, a sequence that appears at ori C. Methylation of adenine in GATC may be important in controlling when replication begins.

The binding of the dna A protein (named for its gene) to four sites of ori C initiates an intricate sequence of steps leading to the unwinding of the template DNA and the synthesis of a primer. The DNA must be negatively supercoiled to enable dna A to bind. A complex of the dna B and dna C proteins join. dna A to bend and open the double helix. dna B protein is a helicase : it catalyzes the ATP-driven unwinding of double helix DNA. The unwound protein of DNA is then stabilized by single strand binding protein (SSB), a tetramer of 19kd subunits which binds cooperatively to single stranded DNA. The unwinding of DNA at the origin would lead to the positive supercoiling of the circular DNA if the linking number remain constant and unwinding would soon stop. This is prevented by the compensatory action of DNA gyrase.

An RNA primer synthesized by Primase :

RNA synthesis is essential for the initiation of DNA synthesis. Kornberg found that nascent DNA is covalently linked to a short stretch of RNA.

In fact RNA primes the synthesis of DNA. A specific RNA polymerase called primase joins the prepriming complex in a multisubunit assembly called the primosome. Primase synthesizes a short stretches of RNA (~5 nucleotides) that is complementary to one of the template DNA strands. This primer RNA is removed at the end of replication by the $3' \rightarrow 5'$ exonuclease activity of DNA pol I.

DNA polymerase III holoenzyme, a highly processive & precise enzyme syntehsizes most of the DNA :

Composition of DNA pol II holoenzyme :

Subunit	Mass (kd)	
α	140	core
ε	27	
θ	10	
β	37	
τ	78	

DNA pol III holoenzyme, a multisubunit assembly is characterised by very high processivity, catalytic potency & fidelity. The holoenzyme catalyzes the formation of many thousands of phosphodiester bonds before releasing its template, compared with only twenty for DNA pol I. The much lower degree of processivity of DNA pol I is well suited to its role as a gap filler & repair enzyme. DNA pol III on the other hand, is

Subunit	Mass (kd)
γ	140
δ	32
δ	32

designed to grasp its template & not let go until it has been completely replicated. A second distinctive feature of this enzyme is its catalytic process. 1000 nucleotides added per second compared with only 10 per second by DNA pol I. This accelerated catalysis is not accomplished at the cost of accuracy. The holoenzyme consists of at least 8 kinds of polypeptptide chains and a mass of 800 kd. The role of some of the subunits of the holoenzyme have been elucideated. The catalytic activity resides in the α subunit, and the $3' \rightarrow 5'$ exonuclease activity in the α subunit. The α, ε and θ subunits form a core that is catalytically active but nor processive.

DNA replication proteins of E.coli :

Protein	Role	Size (kd)
dna B protein	Begins unwinding the double helix	300
primase	Synthesizes RNA primer	60
rep protein	Unwinds the double helix	65
SSB	Stabilizes single standard regions	74
DNA gyrase	Introduces negative supercoils	400
DNA pol III	Synthesizes DNA	800
DNA pol I	Erases primer and fills gap	103
DNA ligase	Joins the ends of DNA	74

Discontinuous synthesis and okazaki fragments :

At a replication fork, both strands of parental DNA serve as templates for the synthesis of new

DNA. The parental strands are antiparallel. The overall direction of DNA synthesis must be $5' \rightarrow 3'$ for one daughter strand & $3' \rightarrow 5'$ for the other.

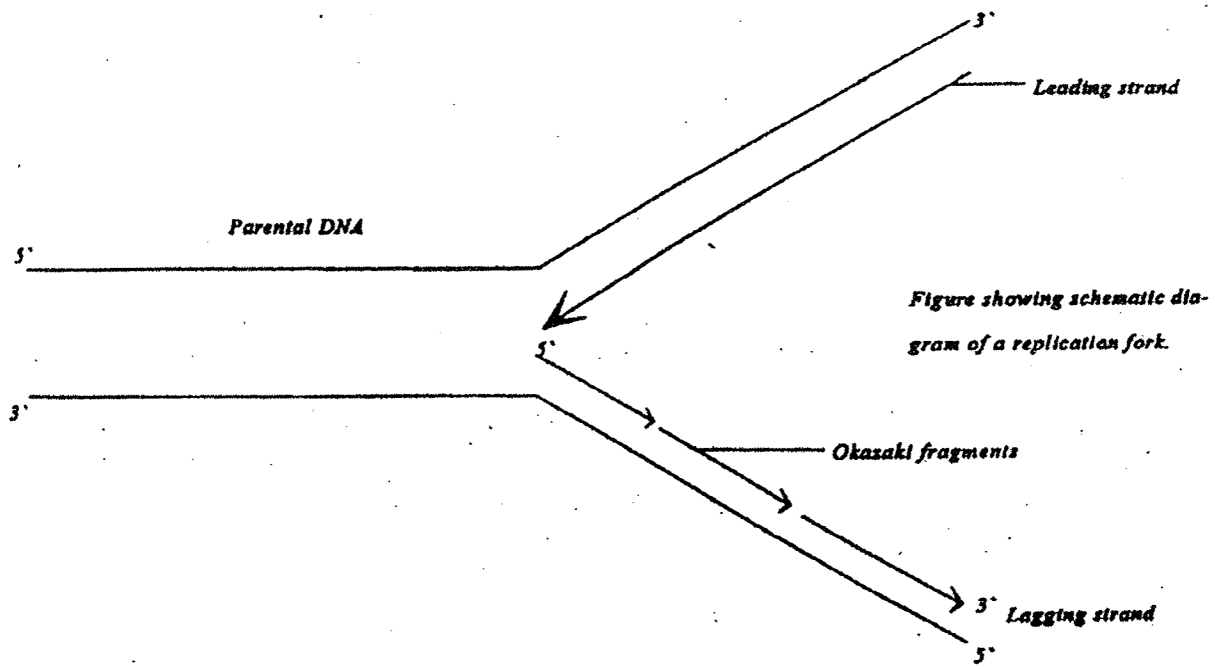


Figure showing schematic diagram of a replication fork.

The currently accepted model of DNA replication is not too unlike the original proposal of Waston and Crick, and conforms to the experimental results of Meselson & Stahl. The molecule unwinds in a localized area, thus forming replication "forks". The replication process is generally referred to as a semidiscontinuous process. Because the actual polymerizing enzyme can add nucleotides only in the $5' \rightarrow 3'$ direction. Synthesis on one strand (leading Str) is continuous in the $5' \rightarrow 3'$ direction toward the fork from the point of initiation. On the other strand (lagging strand), as the form opens, multiple sites of initiation are exposed. Synthesis then proceeds inshort segments in the $5' \rightarrow 3'$ direction away from the fork. Therefore, the DNA pol add new deoxyribonuclotides in the $5' \rightarrow 3'$ direction by adding at the $3'$ end of the nascent complementary strand, that is, both daughter strands are extended only in the $5' \rightarrow 3'$ direction: This means that the lagging strand and to a lesser extent the leading strand is synthesized in discontinuous segments. The major evidence for discontinuous synthesis was obtained by okazaki & his colleagues. Reiji okazaki found that a significant proportion of newly synthesized DNA exists as small fragments. These units of about a thousand nucleotides (called okazaki fragments) are presents briefly in the replication fork. A replication proceeds, these fragments becomes covalently joined by DNA ligase to form one of the daughter strand. The other

new strand is synthesized continously. The strand formed from okazaki fragments is termed lagging strand whereas the one syntehsized without interruption is the leading strand. Both teh okazaki fragments and the leading strand are synthesized in the $5' \rightarrow 3'$ direction. The discontinuous assembly of the lagging strand enables $5' \rightarrow 3'$ polymerization at the nucleotide level to give rise to overall growth in the $3' \rightarrow 5'$ direction.

REPETITIVE DNA AND SEQUENCE ORGANIZATION AND C-Value Paradox

Repetitive DNA

The chromosome of procaryotes DNA molecules with unique (nonrepeated) base pair sequences. That is, each gene (consider a gene to be a linear sequence of a few thousand bas pairs) is present only once in the genome. The gene for rRNa molecules are an exception. If the prokaryotic chromosomes are broken into many short fragments, each fragment will contain a different sequence of base pairs. The chromosomes of eucaryotes are much more complex in this respect. Certain base sequences are repeated many times in the haploid chromosome complement, sometimes as many as million times. DNA containing such repeated sequence, called repetitive DNA often represents a major component of the eukaryotic genome.

Satellite DNA :

The first evidence of repetitive DNA came from density gradient analysis of eukaryotic DNA. When the DNA of a prokaryotic DNA such as E. coli, is isolated, fragmented and centrifuged to equilibrium in a cesium chloride (Cscl) density gradient, the DNA usually forms a single band in the gradient. For E. coli this band will form at position where the Cscal density is equal to the density of DNA containing about 50 percent A-T and 50 percent G-C base pairs. DNA density increase with the increase of G-C base pairs content. The extra hydrogen bond in G-C base pairs is believed to result in a tighter association between the bases and thus a higher density than for A-T base pairs. On the other hand Cscl-density gradient analysis of DNA from eukaryotes usually reveals the presence of one large band of DNA (usually called mainband DNA) and one to several small bands. These small bands of DNA are called satellite bands, and the DNA in this bands in often referred to satellite DNA. Drosophila virilis DNA for example, contains thus the distinct satellite DNAs. (Fig. below) when

isolated and analyzed, each satellite DNA was found to contain a repeating sequence of seven base-pairs. One satellite repeat sequence is 5′-ACAAACT-3/ (one strand, other strand will have complementary sequence). A second satellite DNA has a 5′-ATAAACT-3′ thus differe from each other at only two positions. In three related species of crabs, a satellite DNA is present that contains 97 percent A-T base pairs. Some satellite DNAs in other eukaryotes have longer repetitive sequences.

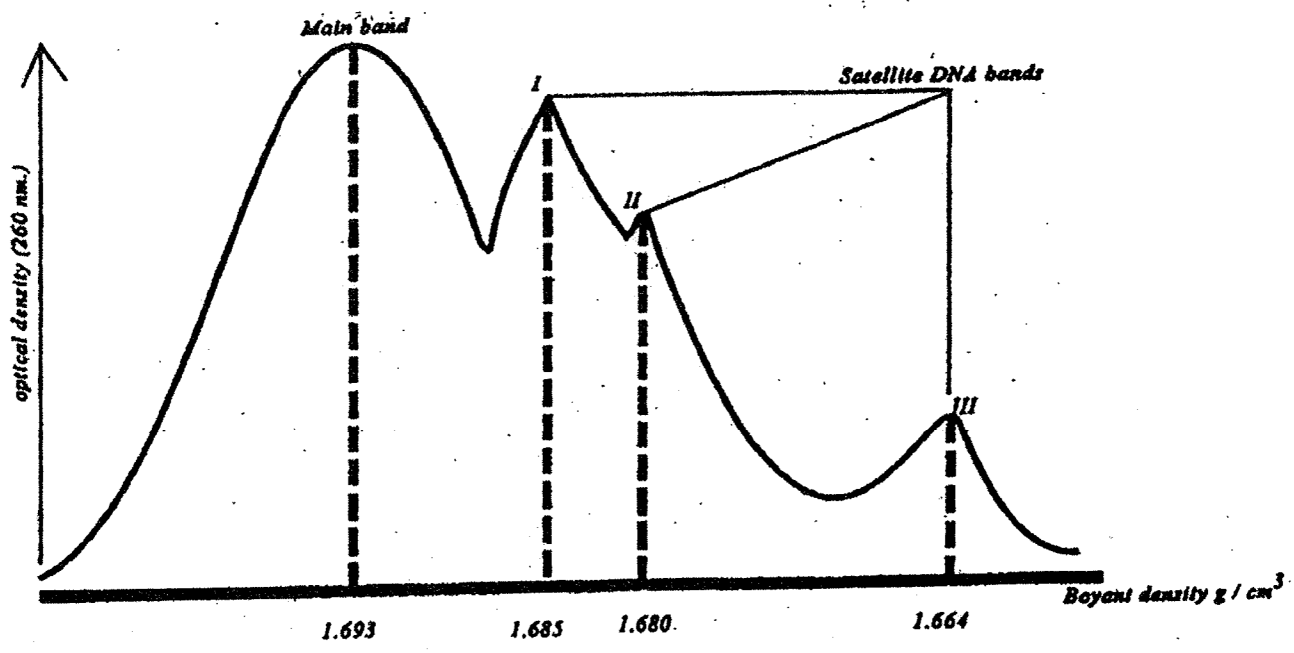


Fig :- The three satellite DNAs of Drosophilla virilis DNA from D .virilis embryos was centrifuged to equilibrium in 6M CsCl. The DNA in the bands were quantitated by measuring u-v (Ultra violet) light (260 nm).

The chromosomal location of several satellite DNAs have been determined by a technique called in situ hybridization. The complementary strands of DNA molecule are separated by heat or alkaline denaturation. Condition can then be reversed by lowering the temperature or lowering the pH, and the sepeated strand will renature to reform base-paired double helixes, a process called DNA Renaturation. DNA renaturation is a specific type of Nucleic acid hydridization, the formation of hydrogen bonded

double helices by single stranded molecules containing complementary base sequence. Hybridization will occur between the complementary single strands regardless of their source. If both participating strands are DNA, the process is called DNA hybridization. If one strands is DNA and the complementary strand is RNA the process is called DNA – RNA hybridization.

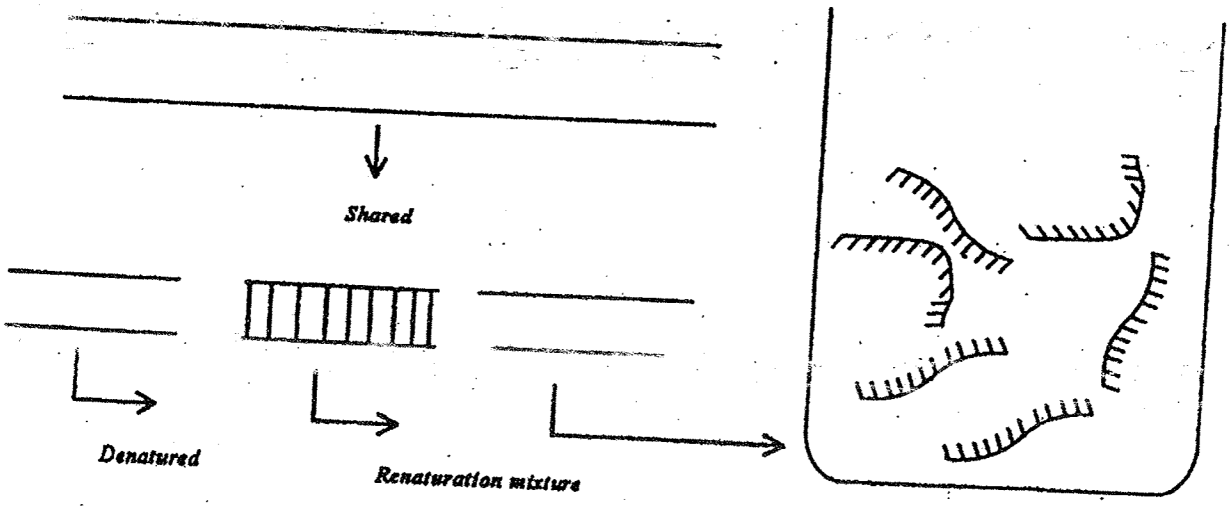
In situ hybridization in the case of the satellite DNAs usually involves annealing single strands of isolated radioactive satellite DNA, or complementary RNA sequences synthesized using satellite DNA as template, directly to denature DNA in chromosome squash preparation. After washing out the nonhybridized radioactive material, the location of satellite DNA sequences in chromosome determined by autoradiography. All satellite DNAs characterised to date are located in heterochromatic regions of chromosomes.

DNA renaturation kinetics :

A much more complete picture of the frequency and complexity of repetitive DNA sequences in eukaryotes has resulted from studies of DNA renaturation kinetics, consider a long DNA molecule with no repeated sequences (such as the DNA of prokaryote). If such a molecule is shared into fragments of a particular length, say 400 neucleotide-pairs is denatured and is then allowed to renature under appropriate conditions, the rate of renaturation will depend on

1. The concentration of DNA in solution and
2. The complexity of DNA, that is the number of different 400 base-pairs gragments.

Consider a particular fragments composed of two complementary strands, a and a′.



Reassociation of a and a′ will require a specific colision between these two single strands. Collisions between a or a′ and any other single strand will not lead to hybridization. For a given concentration, the larger DNA molecule, and thus the more nonidentical 400 base-pair fragments, the slower the reassociation reaction will be, because a smaller proportion of the random collisions will be between the complementary single strands such a and a′.

Note that every reassociation events, like a with a will require a collision between complementary single strands that present in the renaturation mixture in equal concentration. Since the reaction requires the interaction of two equally frequent molecules, the rate of renaturation should be a function of the square of the concentration of single strands (so caled second order or millimoleculár reaction kinetics) or

$$\frac{-dc}{dt} = KC^2$$

(or $\frac{-dc}{C^2} = Kdt$, rearranged for integration)

where C = the concentration of single stranded DNA in moles of nucleotide per it.

c = time in second.

and K = a second order rate constant in lit. per mole second.

Literally, the equation states that the change (decrease) in concentration of single stranded DNA (-dc) with time (dt) is equal to the proportionally constant (K) times the square of concentration of single stranded DNA.

Integration of the preceding equation from the initial condition (t = 0 seconds and C = Co, where Co equals the concentration of single stranded DNA at t = 0) yields

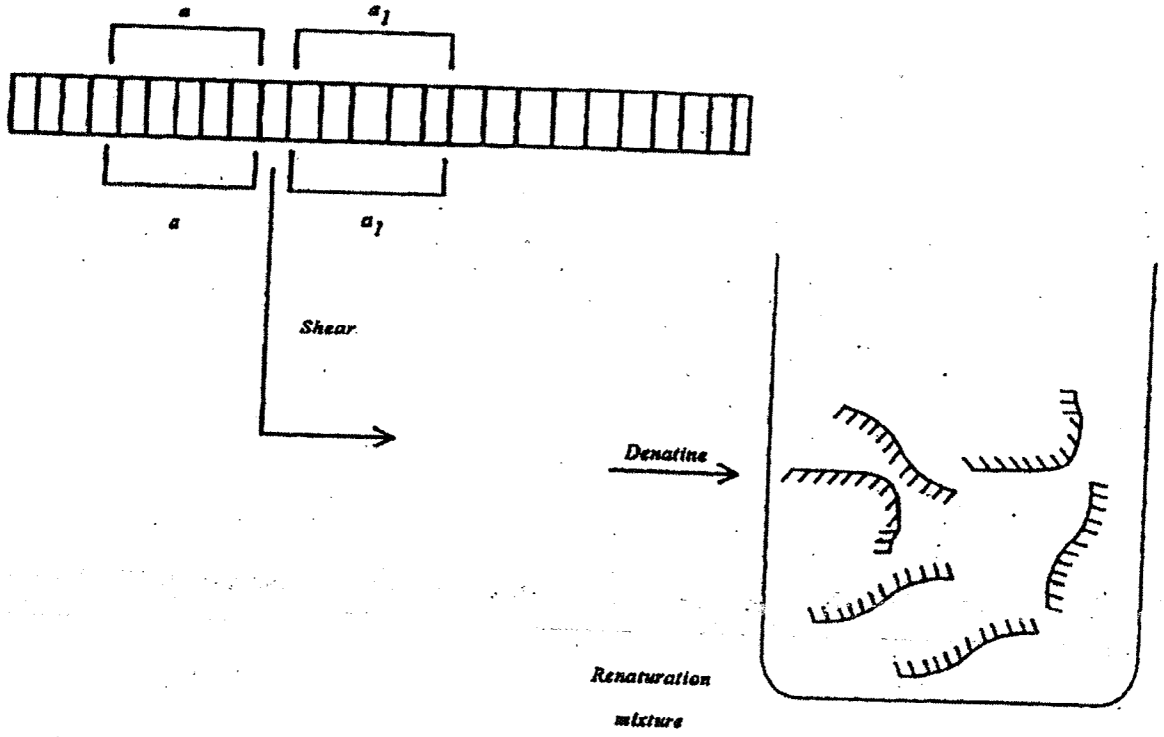
$$\frac{1}{C} = \frac{1}{Co} = Kt$$

or rearranged, $\frac{1}{Co} = \frac{1}{1 + KCot}$

This equation states that the fraction of input single stranded DNA remaining in a renaturation reaction mixture (C/Co) at any given time (t) is a function of the initial concentration (Co) times

elapsed time (t), or Cot. It is thus convinent to present data on hybridization kinetics in a plot of C/Co versus Cot. This is called Cot (pronounced caught) curves have provide a great deal of information about the types of repetitive DNA in eukaryotic genomes. Consider a DNA molecule containing a 400 base-pair sequence that is repeated.

Now a and a_1 an identical single strands a and a'_1 are identical complementary strands Reassociation of each repeated sequence will take only half as long as in the previous example, where each fragment contained a unique base sequence. Now reassociation will result from a collision of a with either a′ or a'_1 (likewise for a_1 and either a′ or a'_1). Thus the time required for reassociation of a particular DNA sequence is inversely proportional to the number of times that sequence is present in the genome. Clearly highly repetitive DNA sequence will ranature very rapidly.

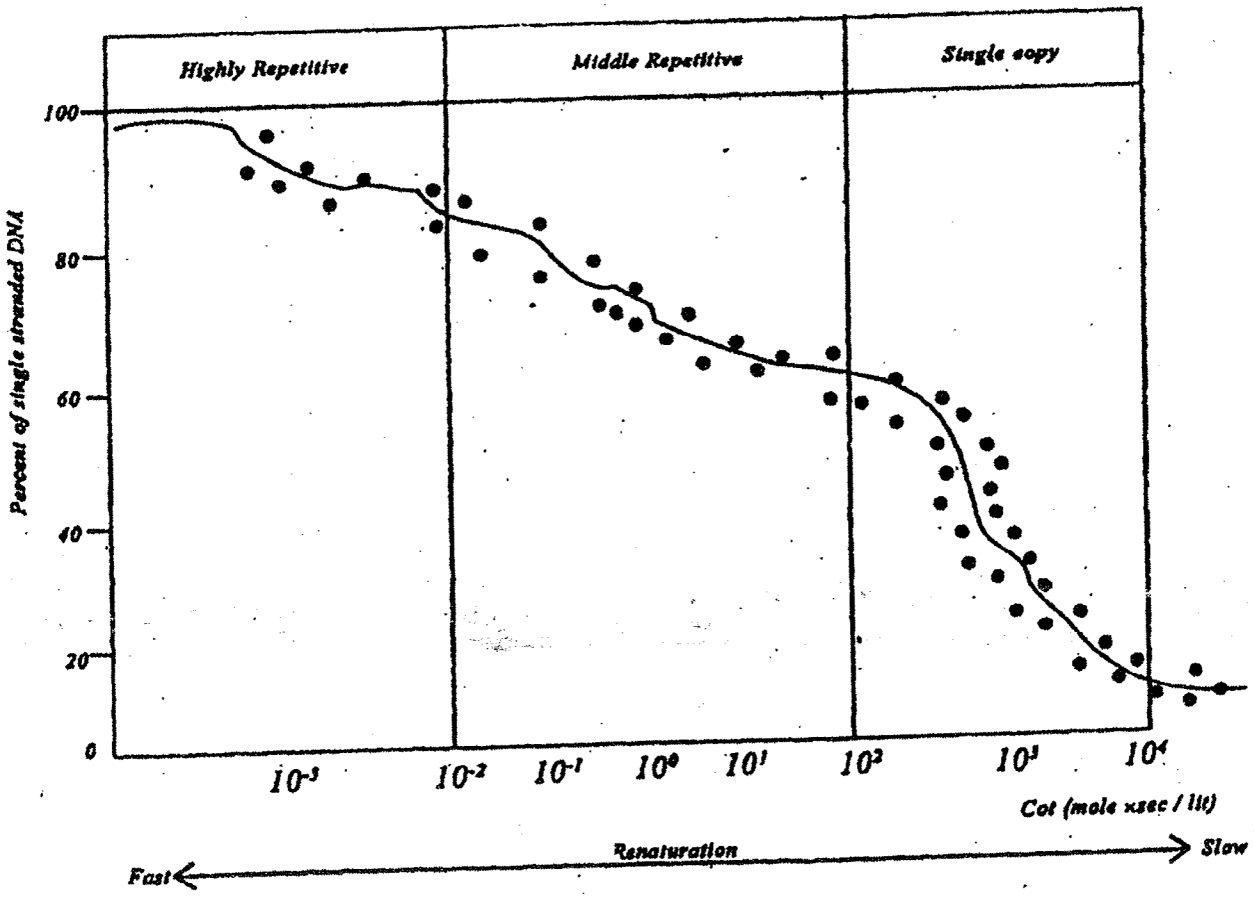


Renaturation kinetics indicate that eukaryotic genomes characteristically contain

1. a fraction (upto 90%) of unique of single copy of DNA sequence present in from one to few copies.
2. a fraction of middle repetitive DNA sequences present in 10^5 copies.
3. a fraction of highly repetitive DNA sequences present in greater than 10^5 copies.

Interspersion of Single Copy and Middle Repetitive DNA Sequences :

Where the sequences organization of eukaryotes genomes is studied - by combining the techniques of density gradient analysis, hybridization, kinetic analysis, bio-chemical analysis and electron microscopy - a pattern emerges. Much of the genome consists of middle reptitive sequences interpesred with single copy sequence. In toads, sea urchin, and human, the sequences are quite short. The middle repetitive sequences average 300 nucleotide-pairs in length; the single copy sequences are about 800 -1200 nucleotide pairs long. Drosophila melanogaster DNA also exhibits interspersion of middle repetive and single copy sequences, but the sequences are much longer (5600 and 13,000 nucleotide-pairs respectively.



Many geneticists have postulated that most middle repetitive sequences may be involved in the regulation gene expression. Their interpersion with single copy sequences, and thus their location

adjacent to structural genesis certainly have a regulatory role. Recently several intermediate repetitive sequences of Drosophila melanogaster have been shown to be capable of moving from one location in a chromosome to another location in the same chromosome or even to a different chromosome. These intermediate repetitive DNA sequences have been called nomadic sequences or transposable element, because of their ability to migrate from one position to another in the genome. The 'nomadic' middle repetitive DNA is well-characterized transposable of prokaryotes.

Function of highly Repetitive DNA sequence :

The function(s) of highly repetitive DNA - most if not all of which is located in genetically inactive heterochromatic regions of chromosomes - is also completely unknown postulated functions for high repetitive DNA include

1. **Structural or organizational roles in chromosome.**
2. **Involvement in chromosome pairing during meiosis.**
3. Involvement in cross-over of recombination.
4. Protection of important structural genes, like histone, r-RNA, or ribosomal protein genes.
5. A respiratory of unessenontial DNA sequences for use in the future evolution of the species and no junction at all just 'Junk' DNA that is carried along by processes of replication and segregation chromosome. The validity of any of these postulated roles remain to be established.

TRANSCRIPTION

Why gene expression is regulated ?

During its growth cycle a cell may not require functioning of all the genes it carries in its chromosome.

As the expression of the genes to othe levels of both RNAs and proteins involves the expenditure of a huge amount of energy, a cell expresses only the genes that are needed in a particular environment to avoid the wastage of such energy.

Even a cell may need to express a gene for functioning for a very short period of time during its growth cycle.

So, the expression of genes during the growth of a cell must be regulated.

Transcription is the first stage in gene expression. It is an autonomous activity of an enzyme which attaches to DNA at the start of a gene & then moves along, transcribing RNA. Transcription involves systhesis of an RNA chain representing one strand of a DNA duplex. It takes place by usual process of complementary base pairing.

PURPOSE OF TRANSCRIPTION :

The purpose of transcription within the cellular economy is not just to make RNA but to make appropriate types of RNAs in appropriate amounts at appropriate stages or moments of cell cycle.

(a) The enzyme must recognise the begnning of the RNA sequence to be transcribed within the random length of ds DNA sequence of the entire genome;

(b) The enzyme must insert the correct nucleotide residues into each positions as determined by he coding sequence of the template DNA following the principle of base complementarity;

(c) It must carry out RNA synthesis with total processivity; this means that RNA must be transcribed from the beginning to the end as a consequence of one polymerase binding and initiation event;

(d) The enzyme must recognize the termination signals in order to end the RNA at appropriate point.

Besides these, RNAP must also recognize regulatory sites on both the DNA template and the RNA transcript and interact with the protein factors and small molecules that modulate the activity of the enzyme and the overall growth rate of a cell is dependent on the rate of expression of the regular housekeeping genes which are necessary for the energy generating metabolism and for making the cellular components directly or indirectly involved in cell growth.

The metabolism energy is transduced to make ATP. Other rNTPs and made from NDPs and dNDPs using ATP as energy-rich phosphate donor in reactions mediated by nucleoside diphosphate kinase (Ndk).

During the growth of a cell, all or some of these NTPs and dNTPs are heavily needed for the synthesis of DNA, RNA, protein and cell wall polysaccharides.

Depending on the small molecule energy source, a cell expresses its certain genes only when those small molecules are present in the growth medium.

RNAP can make one copy of mRNA from a single event of initiation, but it can be repeatedly translated to give several molecules of the relevant protein; this depends on the stability of mRNAs in the intracellular environment.

Subunit composition of bacterial RNA polymerase :

Subunit	Mol. Wt. Da	Function
β (1)	1,51,000	Binds rNTPs, inhibitors like Rifampicin, Streptolydigin, functions in initiation and elongation;
β (1)	1,55,000	Binds to template DNA, interaction with polyanionic inhibitors like Heparin; Heparin; functions in elongation;
α (2)	36,000	Interaction with transacting positive regulatory protein factors like CRP, Ada, IHF, OmpR, OxyR etc. and negative regulatory proteins like lambda repressor;

Sigma (σ) (1)	Mol. wt. varies with diff. σ s	Gives the core RNAP the ability to specifically recognize the promoters when associates with the core enzyme to produce holoenzyme and to initiate RNA synthesis.

Different Sigma factors specific for different promoter

Sigma-D (σ^{70})	(rpoD)	70,000	Transcription from promoters controlling the expression of regular house-keeping genes;
Sigma-H (σ^{32})	(rpoH)	32,000	Activates transcription from heat-shock promoters at extreme heat stock genes.

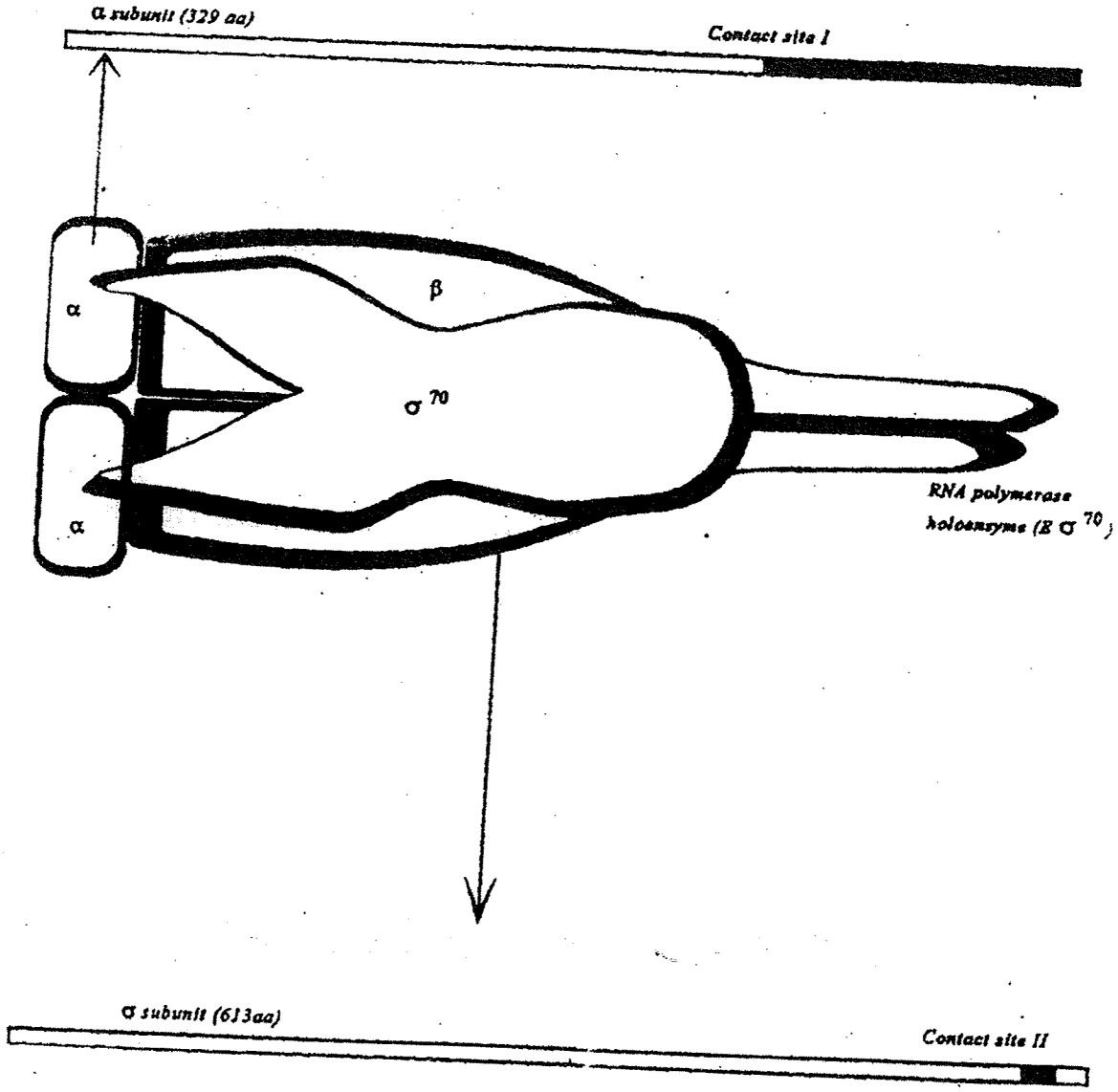


Fig.-1 : transcription factor contact sites on RNA polymerase the C-terminal contact site I on necessary for transcription activation by class I transcription factors and may be involved in direct protein-protein contacts. Two class II factors, PhoB and CRP (in the case of gal transcription), make contacts with RNA polymerase in the C-terminal region (contact site II) of the subunit aa, amino acids.

PROMOTER GENE

Promoter is generally defined as the segment of DNA that contains signals that direct the specific and irreversible binding of RNAP holoenzyme and its subsequent activation to a form which is capable of initiating specific RNA transcript.

There often occures within or immediately adjacent to the promoter, additional sequences of DNA-coded signals tht control the specific binding of accessory regulatory proteins (repressor or activators).

These accessory proteins modulate the effectiveness fo the promoter in bindin polymerase and activating transcription initiation.

Any cell has a large number of promoters.

The relative effectiveness in transcription from various promoters is referred to as promoter strength.

The promoter strength is determined by the intrinsic base sequences of the promoter itself and by accessory proteins that may modulate promoter-RNAP interaction.

Promoters are recognised by RNAP polymerease on DNA sequences.

***Promoter include consensus sequence :**

Any essential nucleotide sequence present in all promoter is called conserved. However an conserved sequence need not necessarily be conserved at every single position, some variation may be permitted. More than 100 promoters have been sequenced in E. coli and a striking feature is the lack of any extensive conservation of sequence over 60 bp associated with RNA-P. But some short stretches within the promoter are conserved. Just upstream at the start a 6 bp region is recognizable in all promoter. The hexamer is close to 10 bp upstream of the start point called - 10 sequence TATAAT.

The conservation of the base at each position of the sequence varies from 45% - 96%.

$T_{8}A_{95}t_{45}A_{60}a_{50}T_{96}$

the subscript denotes the persent occurence of the most frequently found base. (Capital letters used to indicate bases conserved >54% & small letters indicate bases not so well conserved).

Similarities of sequence also occur at another location, centered ~35 bp upstream of the startpoint. This is called -35 sequence. The consensus is TTGACA, in more detailed form, the conservation is

Factors determining promoter binding by RNAP :

(a) Promoters structures :

From analysis of several sigma -70 activable promoters of E. coli, it has been concluded that the promoters have a definite hexanucleotide sequences at around -10 and -35 regions at the upstream of +1 site. From such studies, following features of the promoters have been emerged :

1. **The promoter sequence is asymetric :**

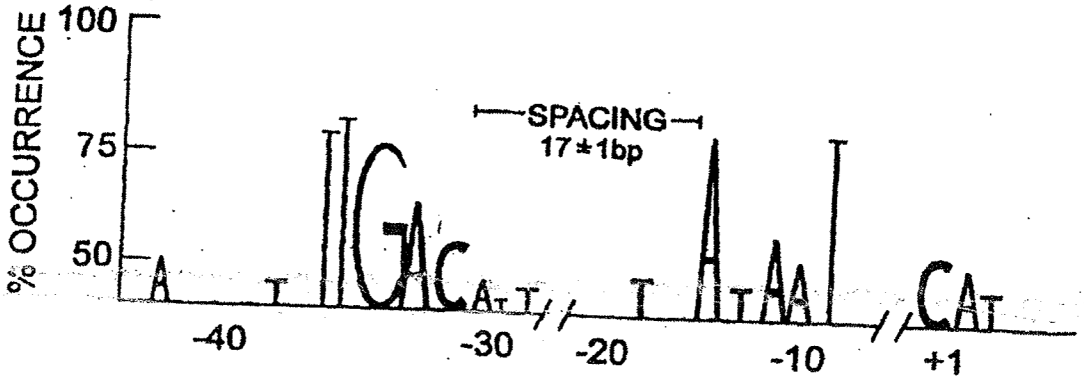
This reflects the inherrent asymmetry of the principle function of the promoter which is to initiate mRNA synthesis in the correct direction and on correct template strand.

2. There are highly conserved sequences around the -10 and -35 sequences is generally 17+ 1 bp with 17 resulting in maximum promoter strength.

(b) The association of specific sigma with RNAP core enzyme determines the specificity of the enzyme for transcription from specific promoters.

Structure of some sigma - dependent promoters

Fig. 2 : Sigma - 70 dependent promoters :



Sigma brings the specific recognition property to the RNAP specifically to the promoter sites σ and 70 binds both -10 and -35 sequences.

Extended -10 promoters :

TGn (-10 hexanucleotides) ---------- + 1

The extended -10 promoter is recognized by σ 70 RNAP and initiate transcription very efficiently even in the absence of defined -35 region.

An RNAP holoenzyme with truncated σ 70 having truncation of the C-terminus of the protein can effeciently initiate transcription from extended -10 promoters.

Sigma 54 dependent promoters :

-24 -12 +1

---------- GG ---------- GC ----------

These promoters do not have typically -10 and -35 elements.

σ 54 forms physically detectable closed promoter complexes (which is very stable) but it is unable to initiate transcription spontaneously.

The RNAP containing 54 is absolutely dependent on additional transcription factors like enhancer-binding protein (EBPs) to initiate RNA synthesis.

There is stringent requirement for the spacing between -12 and -24 elements of 54 promoters. A deletion of even a single nucleotide in the space region abolishes the promoter activity.

σ 24 (sigma E) dependent promoters :

-35 16 bp

---------- GACTT----------TCTG----------

Eukaryotic promoter

In eukaryotic cells, there are three different types of RNA polymerases, each having particular functions and properties. RNA pol I is found in the nuleolar region of the nucleus & is responsible for transcribing the large ribisomal RNAs. RNA pol II transcribes messenger RNA precursors and RNA pol III transcribes small RNAs such as transfer RNA, 5s ribosomal RNA.

At least six nuclear proteins have been shown to be necessary for the proper initiation of transcription by RNA polymarase II.

In the first step of RNA transcription, the TF II D complex binds to the TATA box. It is a multimeric protein, one of whose components – the TATA binding protein (TBP) – binds directly into the minor groove of the TATA sequences (Lee *et al.*, 1991). Another role of TF II D is to prevent the stabilization of nucleosomes in the promoter region. Histone H1 stabilizes nucleosomes and prevents transcription in the region where it binds. The addition of H1 prevents TF II D from finding the TATA sites and prevents transcription, however this inhibition is overcome if TF II D is added first. The binding of TF II D is fascilitated & stabilized by the transcription factor TF II A. Thus the decision to transcribe or not to transcribe a particular gene often depends on the balance between inhibitory factors such as histnes and TF II D & TF II A.

The TF II D/ TF II A complex can not form a stable complex directly with RNA pol II, rather TF II D binds factor TF II B. The binding of TF II B to TF II D appears to be the key rate limiting step in the transcription of numerous genes. This rate can be dramatically increased by certain promoter and enhammer binding transcription factors. The activator domain of these transcription factors bind directly to TF II B and fascilitates its assembly with TF II D (Lin *et al.*, 1991). Once TF II B is in places it can bind RNA pol II.

Either directly before or during its binding to TF III B, RNA pol II becomes associated with TF II F and TF II E (Conaway *et al.*, 1991).

TF II F has an enzymatic activity needed to unwind the DNA helix. TF II E is a DNA dependent ATP-ase & is probably needed for generating the enrgy for transcription. For transcription to occur, the RNA pol must be released from the promoter region. This releasing activity appears to be the function of TF II H. The RNA pol is tightly bound by its carboxyterminal domain (CTD) to TF II D. However TF II D will only bind the unphosphorylated form of the CTD. In mammels, the CTD contains 52 repeats of the seven amino acid sequence YSPTSPS. When the initiation complex is formed, the

completed complex activates the serine / threonine protein kinase activity of TF II H, TF II H then phosphorylates each of the 52 repeats (see Fig. 4). TF II D can not bind this heavily phosphorylated region and releases the RNA pol. while the first phosphodiester bond can be made without the phosphorylation of the CTD, this phosphorylation appears to be essential for the further transcription of messenger RNA (Akoulitchev' 95).

Functional map of σ 70 :

Sigma 70 is essential for the recognition of –10 and –35 elements of the σ70 promoter by RNAP holoenzyme and can spontaneously initiate transcription even in the absence of any activator protein when such activator is not needed.

Four major regions of sequence similarity have been identified in most sigmas.

a) The 4.2 subregion of σ70 is essential for the recognition of –35 element of the promoter;

b) The 2.4 subregion of σ70 is essential for the recognition of –10 element of the promoter;

c) The C-terminal end of subregion 2.3 around its junction with 2.4 is essential for DNA strand separation during open complex formation;

d) The 4.2 subregion which is essential for the 35 recognition, is not essential for the recognition of extended –10 promoter;

The truncated σ70 lacking the C-terminal portion containing the region 4 can efficiently initiate transcription from extended –10 promoter.

Steps in initiation of transcription :

a) Binding of RNAP holoenzyme at promoter site forming a close complex formation. At this step, RNAP-DNA interaction is reversible.

b) The close complex soon isomerizes to open complex formation which results in the undwinding of DNA from –9 to +3 positions of the promoter; during this process, the conformation of RNAP and DNA change. After isomerization, the RNAP--DNA interaction becomes irreversible;

c) Immediate after isomerization, initiation occurs with the formation of first phosphodiester bond.

RNAP does not relinquish its specific contact with open promoter until after an RNA chain 6 to 9 nucleotides in length has been polymerized.

d) Promoter clearence :

During this step, sigma is released and the complex enters elongation phase. Release of sigma is an essential step for promoter clearance. The rate of promoter clearance determines the rate of RNA eongation.

Any of the above 4 steps may be a rate-limiting one and determine the overall rate of transcription initiation.

Elongation :

The point of transcription at which the transition from initiation to elongation occurs can be defined as :

a) The point at which σ submit is released from the initiating RNAP-DNA complex.

b) Abortive initiation, if any, ceases living a stable ternary complex consisting of the core polymerase, the nascent RNA chain and the template DNA.

c) RNAP-DNA complex becomes salt resistant.

The Elongation Step :

a) Binding of rNTP substrates with DNA-bound core enzyme.

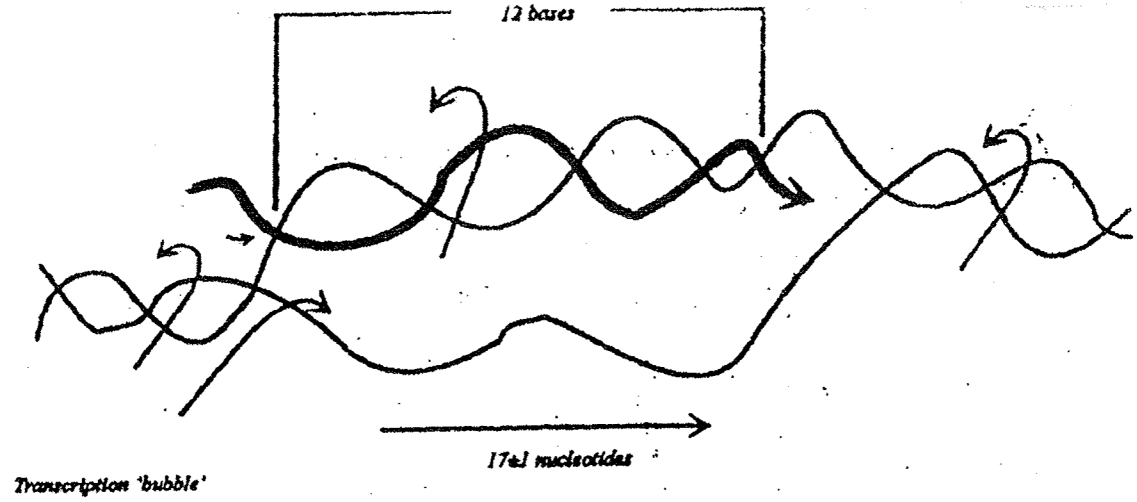
b) Transfer of a nucleotide residue to the 3'-OH of the nascent RNA chain and displacement of the pyrophosphate;

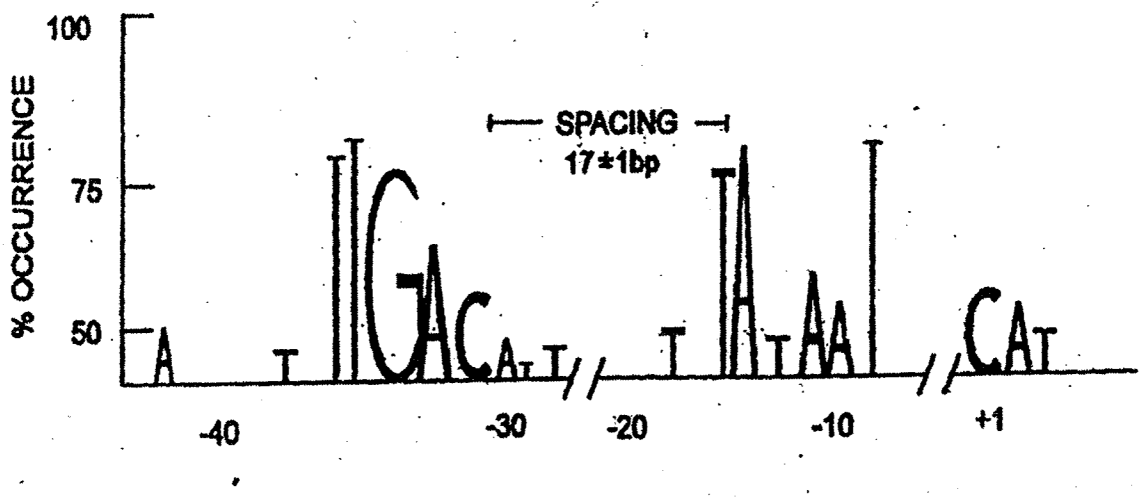
c) Release of Pi-Pi.

d) Translocation of the polymerase along the DNA template by one residue.

The movement of the polymerase involves melting DNA template in front and reformation of duplex DNA behind the transcription bubble and displacement of the 5' end of the RNA-DNA hybrid.

The RNA synthesis once enters elongation phase, becomes totally processive. Dissociation may occur on a time scale of hours.





Elongation rate :

E. coli RNA polymerase	:	Around 17 nucleotide / sec.
T7 RNA polymerase	:	100-200 nucleotide / sec.

Elongation rate is determined by several factors :

a) **Intrinsic factors :**

i) Base composition of DNA :

Presents of nucleotide sequence-dependent pausing sites within the template DNA which are GC-rich region around 10 bp upstream of 3' end pause site.

ii) Presents of a dyad symmetry elements centered around 16 bp upstream of the 3' end.

b) Extrincsic factors :

PP – G – PP

Protein factors : NusA protein

Process of Elongation is also regulated by :

1. The process of attenuation :

Example :

Amino acid bio-synthesis operon : Trp operon

2. **Termination and antitermination :**

Bacteriophage lambda operons.

PROPERTIES & FUNCTION OF EUKARYOTIC RNA POLS :

	Location	**Gene transcripts**	**Inhibit by-Amanitin**
RNA pol I	Nucleolus	5.8s rRNAs	
II	Nucleoplasm	mRNA, U-sn RNA	Sensitive fo low conc
		Uridine rich	(0.1 microg/ml)
		(small nuclear)	
III	Nucleoplasm	tRNA, 5s RNA,	Sens. to high conc.
		75Sc RNA	(10 micro g/ml)
		(small cytoplasmic)	

An RNA POL II promoter consists of a TATA BOX, a CAAT BOX and an Enhancer.

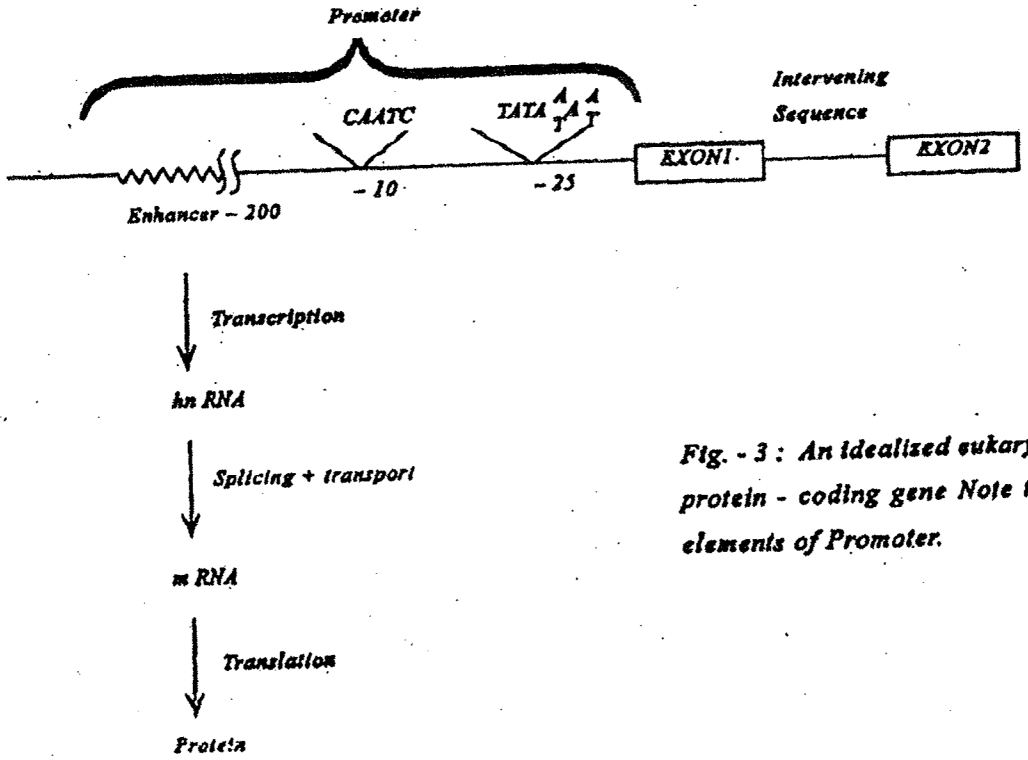


Fig. - 3 : An idealized eukaryotic protein - coding gene Note three elements of Promoter.

i) Conserved sequence TATA $\begin{matrix} A \\ T \end{matrix} A \begin{matrix} A \\ T \end{matrix}$ (called the Hogness - Goldberg box) occurs 20-30 before start. In prokaryotes A – T region is closer – 10. TATA is imp for transcription, its main function seems to be to position precisely the start of transcription.

TATA box sequences is bound by transcription factor.

ii) Many genes (not all) have conserved sequence CAAT between –70 and –80. When it is removed, the level of transcription is drastically reduced. It determines the efficiency with which a gene can be expressed.

iii) The enhancer is a segment of DNA that can increase expression of some gene & that has some puzzling hcaracteristics.

Globin expression greatly stimulated by transforming this sequence. The puzzling was that stimulation persisted whether enhancer DNA introduced in reverse orientation or positioned either before or after the globin gene. Thus enhancer can work at a distance from gene & regardless of orientation.

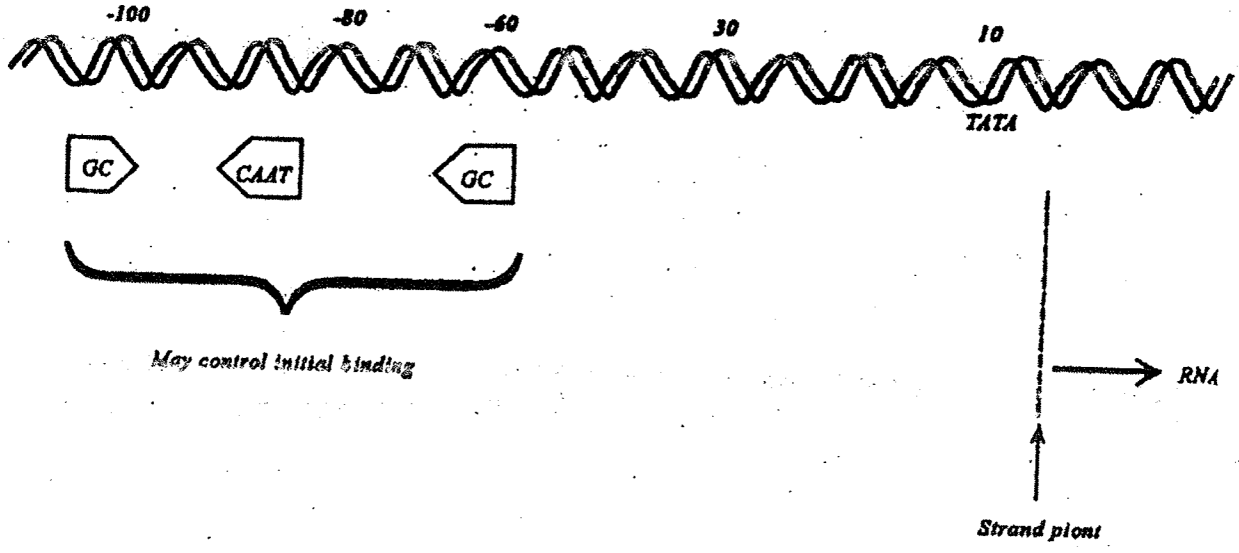
Ig	G T G G T T T G A	
Meloney sarcoma MSV	T G T G G T A A G	**Charecteristics :** i) The position of enhaneer need not be fixed.
Simian virus SV40	G T G T G G A A A G	ii) It can function in either orientation.
Polyoma	Py G T G T G G T T T	
Core nucleotides	(G)T G G A/T A/T A/T (G)	

Enhance Core sequence

The enhancer is much larger than this conserved core and in SV40 it has been mapped to a 72 bp (72 bp repeat) region located 100-170 nucleotides before early promoter.]

UAS=Upstream activator sequence in Yeast analogus to ehnacer.

The thymidine kinase promoter :



Encounting a TATA box, a GC box & then a CAAT box and a second GC box. The two GC boxes are in opposite direction & CAAT box is in reverse orientation.

RESPONSE ELEMENT : HSE, GRE, MRE

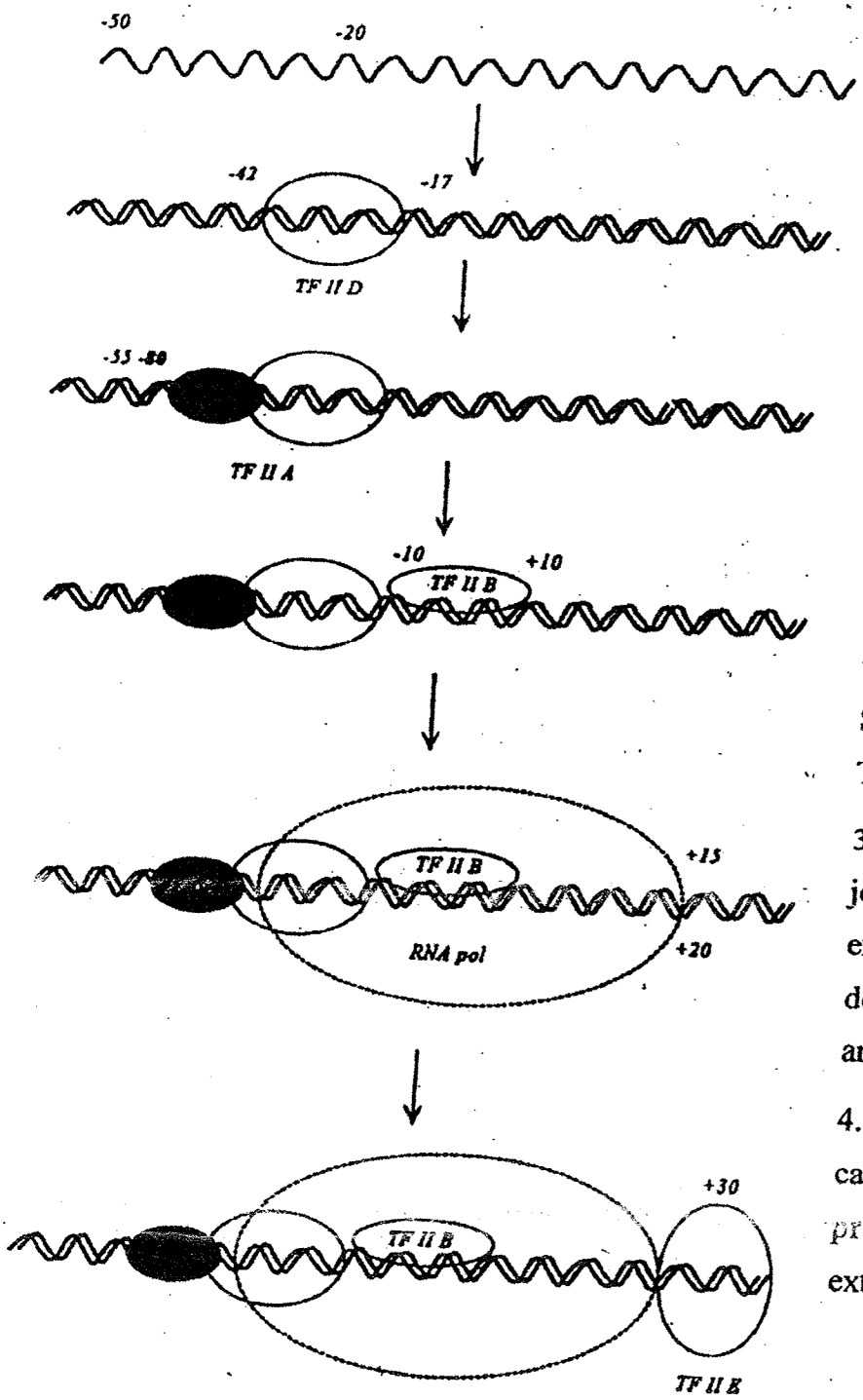
Response elements are recognized by factors that co-ordinate the transcription of particular group of genes.

The heat shock response is common to a wide range of prokaryotes and eukaryotes. Increased temparature turns off transcription of some genes, turns on transcription of the heat-shock genes & cause translation of mRNA.

1. The promoters of eukaryotic heat-shock genes possess an HSE in the form of consensus sequencer of ~15 bp upstream of stratpoint.
2. HSE recognised by HSTF which is active only in heat-shocked cells, it binds with heat-shock consensus sequence.
3. Activation of this factor provides a means to initiate transcription at the specific group of 20 genes.

Both HSE & HSTF leave been conserved in evolution.

Fig.-4 : RNA pol II binds to promoters during assembly of a transcription complex that contais several transcription factor :



1. The first Step - Binding of II D to a region that extends upstream from TATA hox, TATA box lies at 21-15 and the protected region extends from –42–17.

2. When TF II A is added, TF II D becomes able to protect a region further upstream extending from – 55–80.

2. Addition TF II B gives some partial protection of the region of the template strand form –10 to +10. So TF II B is bound downstream of TATA box.

3. RNA pol II becomes able to join the complex at this point. It extends the sites that are propeeted downstream to +15 on the template and +20 on the nontemplate strand.

4. TF II E can bind at this point & causes the boundary of the region protected downstream to be extended to +30.

Transcription factors have motifs that bind DNA and active transcription :

The common types of motifs can be found that are responsible for binding to DNA. The motifs are usually quite short and comprises only a small part of protein structure.

Several groups of proteins that regulate transcription by binding DNA :

i. The steroid receptor.

ii. THe zinc finger.

iii. Helox-turn helix–DNA binding domain of phage repressor.

iv. Leucine zipper.

v. Homeobox.

*** Some protein may bind DNA using zinc motif :**

A 'finger-protein' often has a series of zinc fingers, as seen below:

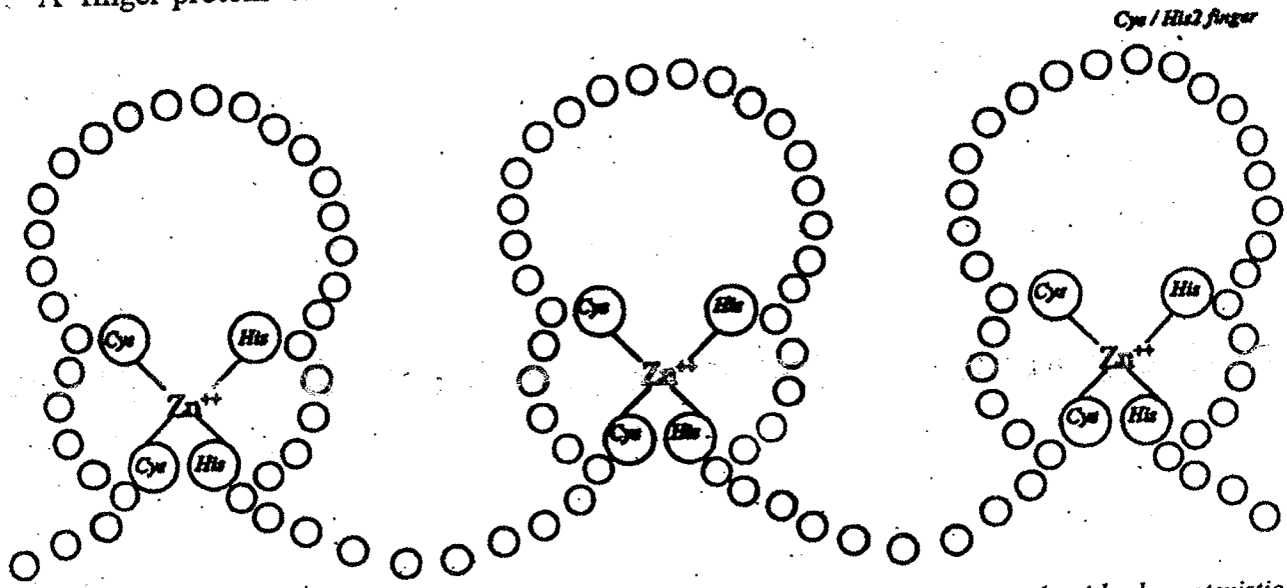


Fig. -5 : *Transcription factor SP1 (mammalian) has a series of three zinc finger each with characteristic pattern of Cysteine and histidine residue that constitute the zinc-binding site*

Homeobox :

is a sequence that codes for a domain of 60 amino acids present in many / all eukaryotes. Its

name derives from Drosophila homeotic lici (whose genes determine the identity bf body structures). Sequence similarity is pronounced in two regions –

(A ubiquitous transcription factor Oct 1 binds to Octamer to activate H2B genes. Oct 1 is the only octamer-binding factor in non-lymphoid cells. In lymphoid cells, a different factor oct-2 binds to octamer to activate immunoglobin gene).

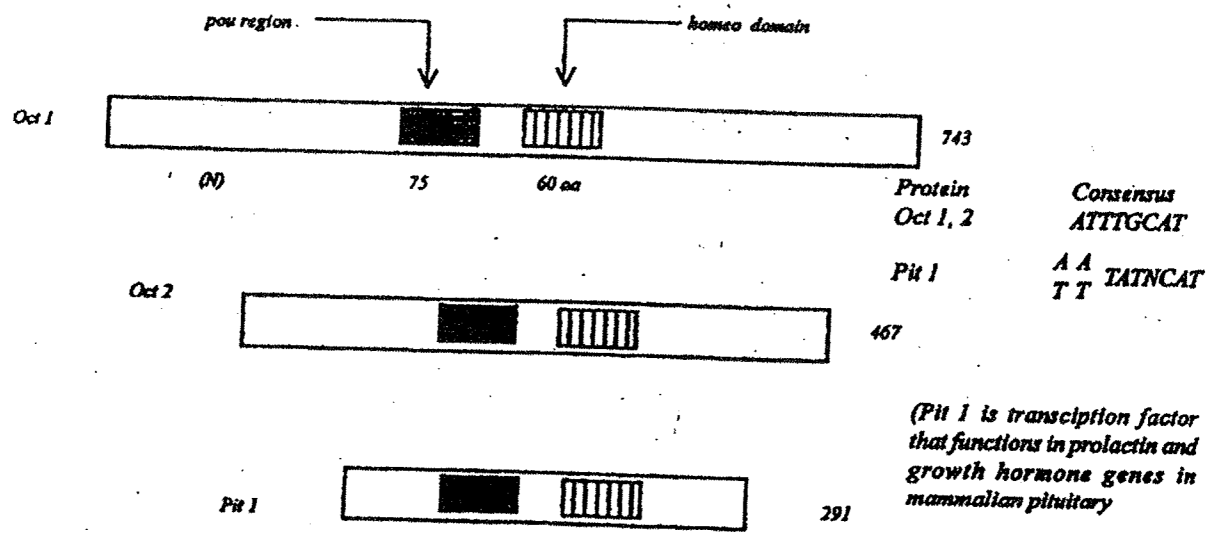


Fig. - 6 : Oct1, Oct2 and Pit1 have an extensive conserved region that comprises 75 residnes & 60 amino acid region velated to homeo domain.

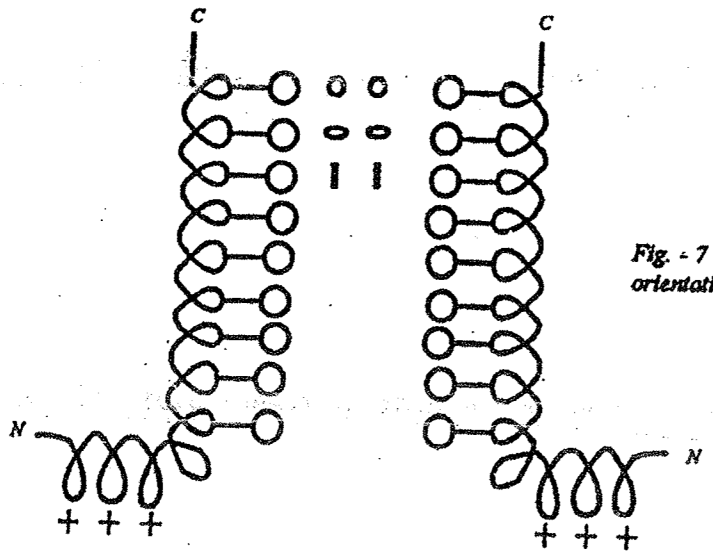


Fig. - 7 : Two leucine zippers in parallel orientation could form a dimeric structure

(The leucine zipper is a stretch of amino acids rich in leucine which form a α helix or coiled coil).

α helix has a structure in which hydrophobic groups (including leucine) face one side while charged group face the other side.

Original idea for the action of this motif was that the lucine, of the zipper on one protein could protrude from α helix and interdigitate with the leucine of the zipper of another protein in the reverse orientation.

The leucine zipper provides an important motif in the components of AP1, an interesting transcription factor that recognises the target sequences ATGA $\begin{matrix} G \\ C \end{matrix}$ *TCAT* TCAT (AP1 binds to the SV 40 enhaneer).

TRANSLATION OR PROTEIN SYNTHESIS

CENTRAL DOGMA states that information in nucleic acid can be transferred, but the transfer of information into protein is irreversible.

ROLE OF TRANSFER RNA (75-85 nucleotides in length) :

i) Transfer RNA provides the "adapter", with twin function of being used to recognise both the codon and the amino acid.

ii) Each tRNA can be charged with amino acid for which its anticodon is appropriate.

iii) The amino acid is linked by an ester involving its carboxyl gr. to one of the hydroxyl gr. of ribose of the last base of tRNA (which is always adenine).

iv) In the process of charging the tRNA is catalized by the specific enzyme amino-acyl tRNA synthetase.

v) The charging process involves a reaction between the amino acid & ATP to form aminoacyl ~ adenylate; then the activated amino acid is transferred to tRNA. This release AMp & PPi.

vi) The function of recognizing the codon is done by the anticodon of tRNA that is complementary to the codon.

Actually once aminoacyl–tRNA has been formed, it is the anticodon that is solely for recognizing the correct codon.

What is Polysome :

i) The interaction between tRNA & mRNA is sponsored by the ribosome.

ii) The ribosome moves along the mRNA, "reading" successive triplets by admitting aminoacyl–tRNA having corresponding anticodon.

iii) A complex of an mRNA associated with several ribosomes is known as polyribosome or polysome.

MECHANISM OF TRANSLATION

i) Initiation :

Involves the reaction between the first two amino acids of the protein. It requires the ribosomes to bind to the mRNA, forming initiation complex together with the first aminoacyl – tRNA This is a relatively slow step in protein synthesis.

Ribosomal site of action : (2 sites)

i) Messenger RNA (a stretch of about 30 bases) is associated with the small subunit of the ribosome.

ii) Only 2 mol. of tRNA can be in place of ribosome. An incoming aminoacyl-tRNA can be entered in A site (entyr.)

A tRNA carrying the entire polypeptide chain, called peptidyl–tRNA occupies the P site (donor). The codon representing the last amino acid to have been added to the chain lies in P site.

What initiates protein synthesis ?

Two features on mRNA are necessary for initiation reaction :

a) Actual coding region of mRNA functions as a single for the ribosome to bind.

b) The singal that marks the start of reading frame is a special codon, the triplet AUG (in bacteria, occasionally GUG)

The two signals that establishes the starting point of translation.

Note : The amino acid composition of E.coli proteins at their N-terminal and shows mostly methionine as the first amino acid.

There are two types of tRNA able to carry methionine in E. coli. One is used for initiation, other for recognizing AUG codons during elongation.

The initiator tRNA is known as $tRNA^{f}_{MET}$ After it has been charged with its amino acid to generate Met-tRNA, the formylation reaction occurs to block the free NH gr. The product is active initiator ^{f}Met-tRNA. It is used only for initiation & recognizes AUG/GUG.

FORMATION OF INITIATION COMPLEX : (See Fig. 1)

i) The initial contact with mRNA occurs by binding of a small sununit (30S/40S) to form an initiation complex at AUG.

ii) Only aminoacyl-tRNA has the unique property of being able to enter directly into partial P site to recognize its codon.

iii) Then the large subunit joins the complex to generate a complete ribisome, in which the initiator aminoacyl-tRNA in now-intact P site & the A site is available for entry of aminocyl-tRNA complementary to next codon.

iv) Active 30S sununit contain initiation factors (IF). There are 3 IFs in bacteria : IF1, IF2 & IF3.

The first stage involves the binding of 30S subunits specifically at initiation sites in mRNA. IF3 participates in this reaction.

The second stage is involved in ensuring that only the initiator fMet-tRNA is placed in the partial P site of the subunit-mRNA complex; IF2 is required for this reaction.

IF1 binds to 30S sununits only as integral part of the initiation complex. It may be involved in stabilizing the complex.

EUKARYOTIC INITIATION COMPLEX :

This proceeds through the formation of a ternary complex. This complex is formed in two stages :

i) GTP binds to eIF2, this increase its affinity for Met-tRNA which then is bound.

ii) The ternary complex association with free 30s subunit.

This association is stimulated by e1F2 & e1F4c, which probably stabilize it.

The binding of mRNA also requires e1F1, e1F4A, e1F4B & and a high energy bond (AtP) is hydrolyzed.

e1F2 + GTP ..... (1)

e1F2 – GTP + met-tRNA ..... (2)

e1F-2–GTP–met tRNA –40s..... (3)

eIF2 = eukaryotic initiation factor 2

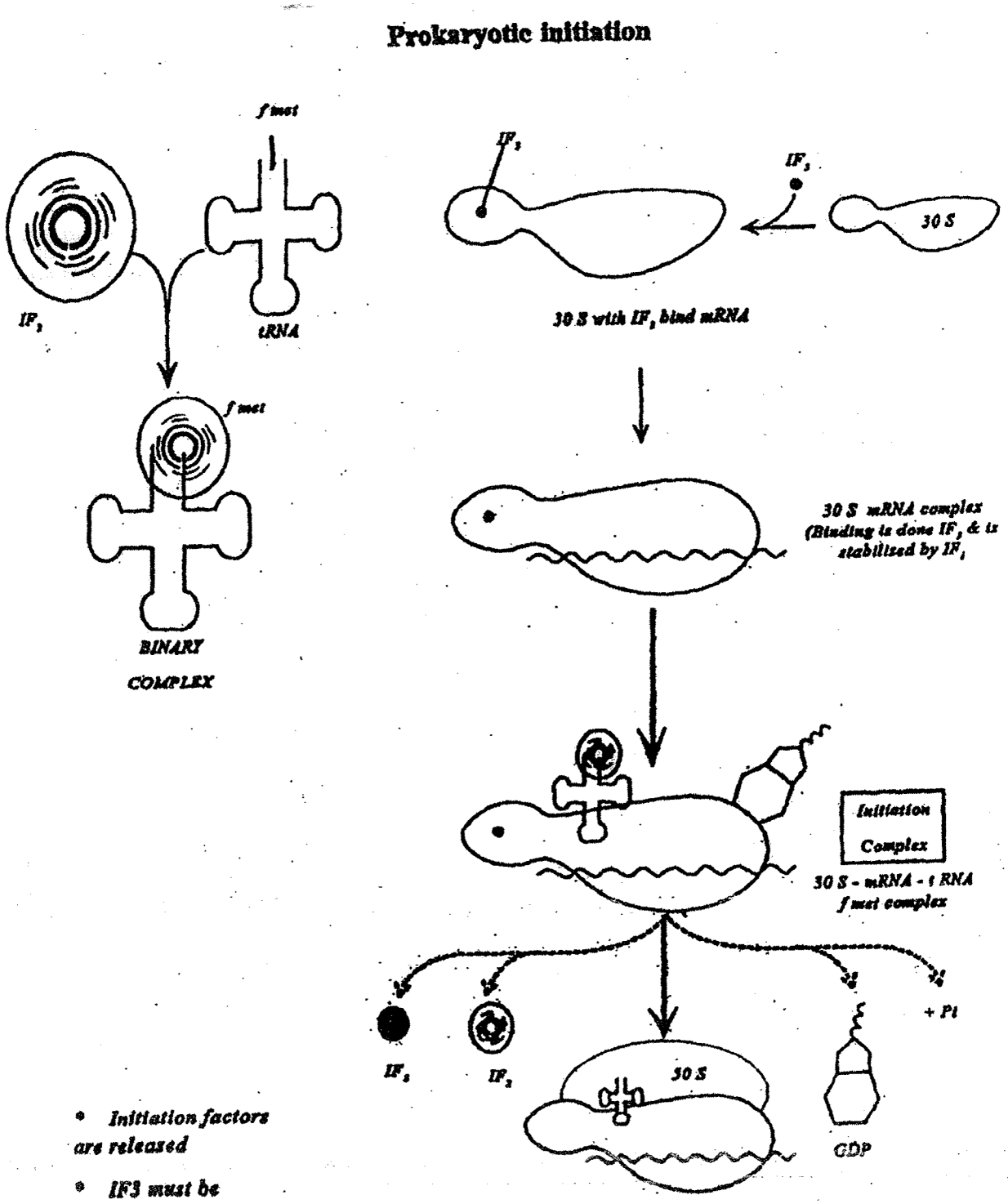
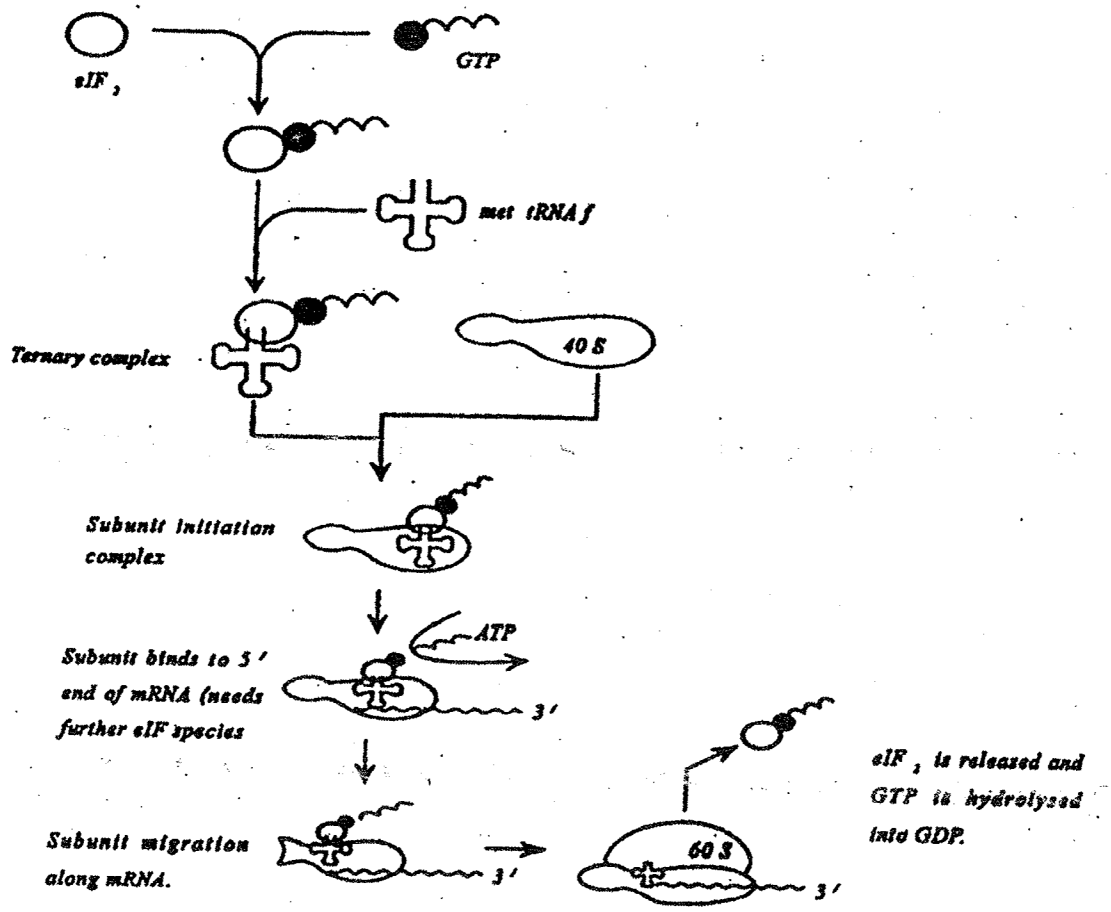

Prokaryotic initiation
f met
IF3
tRNA
f met
BINARY
COMPLEX
IF3
IF1
30 S
30 S with IF3 bind mRNA
30 S mRNA complex
(Binding is done IF3 & is
stabilised by IF1
Initiation
Complex
30 S - mRNA - t RNA
f met complex
+ Pi
IF3
IF2
50 S
GDP
• Initiation factors are released
• IF3 must be released before 50S can join

Fig. 1

Initiation factor (IF) in E. coli

Factor	Mass (dal)	Function
IF_1	9000	1. Probably stebilize the complete initiation complex. 2. Helps in the interaction between IF_2 & initiation tRNA.
IF_2	97,300	1. Initiator tRNA binding. 2. GTP hydrolysis.
IF_3	23,000	1. Binding of mRNA to 30s subunit. 2. Subunit dissociation.

Eukaryotic initiation



Three subunit for eIF_2

Subunit	Mass (dalton)	Func. in Initiation
α	35,000	Binds GTP controlled by phosphorylation
β	38,000	May be a recycling factor
γ	23,000	Binds met $tRNA_f$

b. Elongation : EF-Tu places aminoacyl tRNA on the ribosome during elongation in prokaryotes.

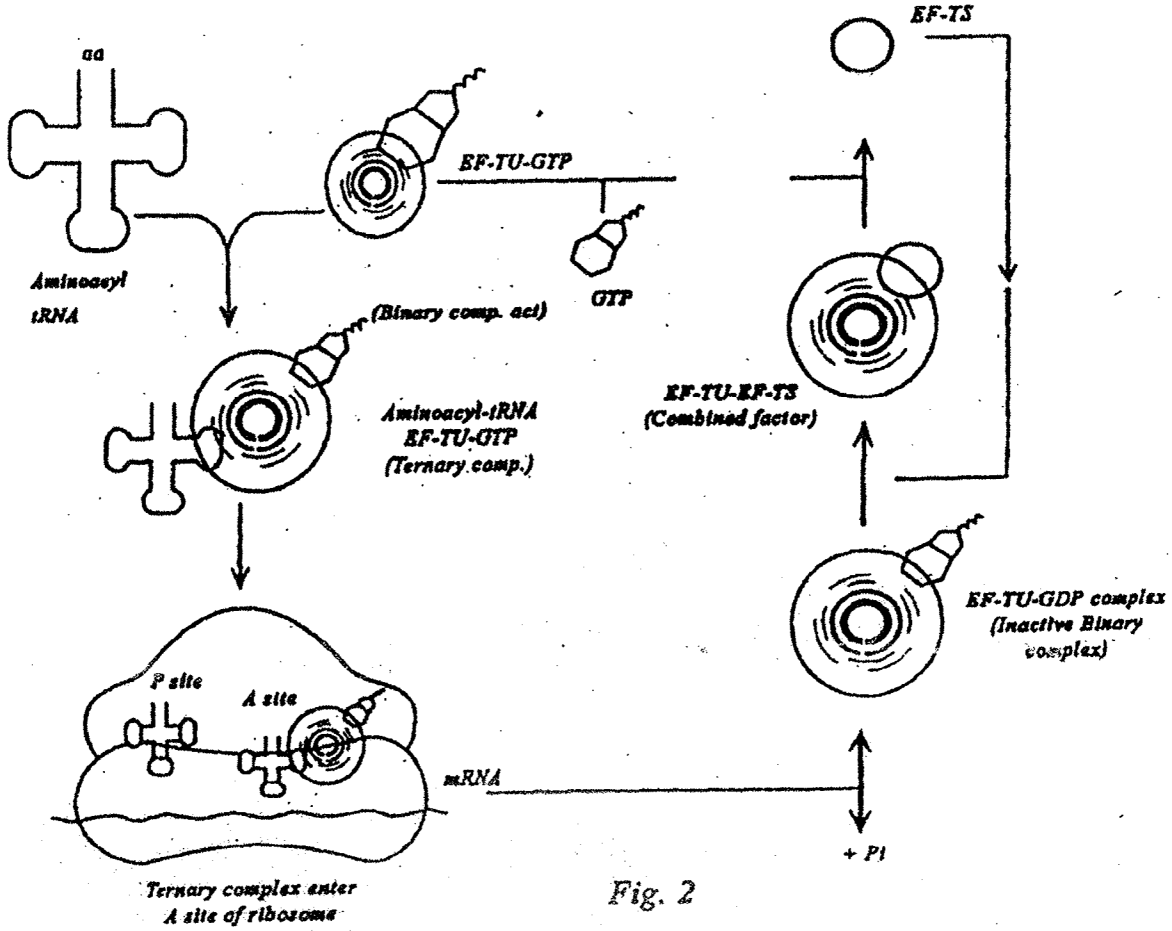


Fig. 2

Three subunit for eIF_2

Factor	Mass/Dalton	Function	Initiation
EF – TU	43,225	Binds aminoacyl tRNA & GTP	Kirromycin
EF – TS	74,000	Binds IF – TU by displacing GTP	

Note : In eukaryotes the factor eEF1 (like EF-Tu) is responsible for bringing aminoacyl tRNA to the ribosome. It also involves cleavage of high energy bond in GTP. The size & mass of the factor vary EF-T like component may be found.

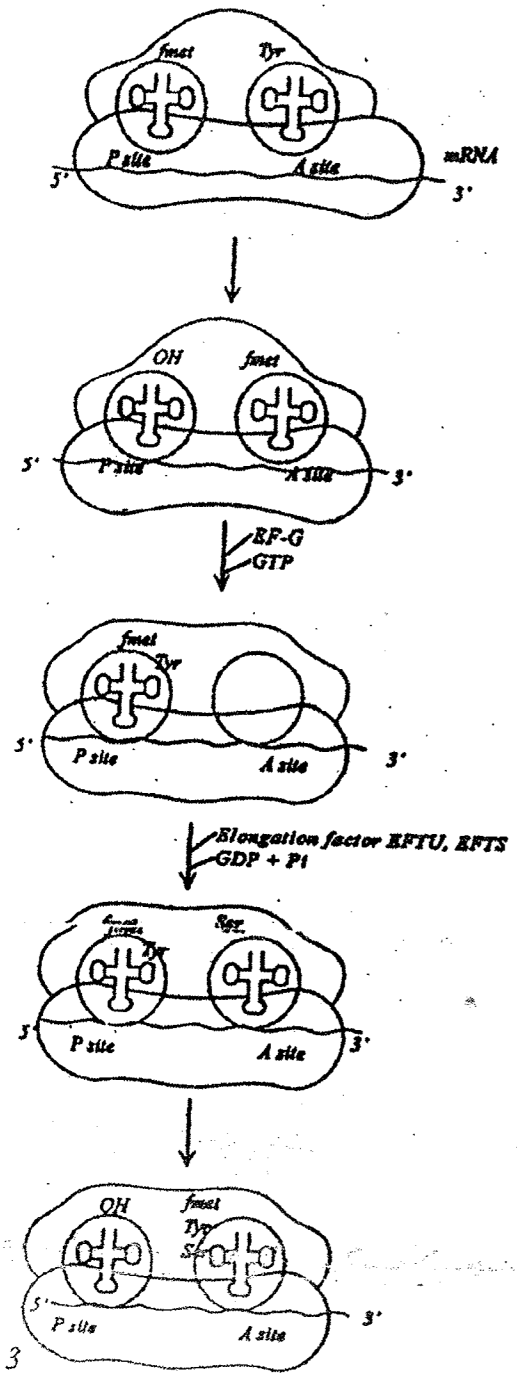


Fig. 3

i) tRNA moves from A site to P site.

ii) A site becomes free and welcomes another aminoacyl tRNA to come. Here A site is occupied by tyrosine (Tyr.).

iii) Peptide bond formation occurs between the amino acid of A site and the aa of P site with the aid of peptidyl transferase (at large subunit).

iv) Ribosome advanced nucleotides along with mRNA from 5' to 3'.

v) Uncharged tRNA is expelled from P site.

vi) With the advancement of ribosome peptodyl tRNA with fmet and Try. reaches P site.

vii) Another aminoacyl tRNA (here with ser) comes at A site.

viii) Ser makes bond with tyr. Fig. 3.

ix) Another tRNA becomes unchanged and expelled from P site.

x) New aa comes to elongate the polypetide chain.

The cycle of addition of amino acids to the growing polypeptide chain is called translocation in which ribosome advances three nucleotides along with mRNA. In the process EF-G and 4.5S RNA factors are required.

Translocation requires following factors :

Factor	Mass	Function	Inhibition
EF – G	77,444	1) Ribosome movement 2) Binds ribosome to GTP	Fusidic
4.5s RNA	114 bases	Unknown (found in E.coli)	

Note : The eukaryotic counter part to EF-G is the protein factor eEF2 which seems to function in a simioar manner.

c) Termination :

The chain elongation contains as the above described sequence is repeated for each codon of the mRNA (about 300 condons on the average) until a chain termination codon (UAA, UAG, UGA) is reached at the A site. None of the termination codon is represented by a tRNA but are recognised by protein factors or release factors (RF). As a result of termination, the nascent polypeptide, the tRNA in the P site, and the mRNA are released and the ribosomal sununits dissociate.

Two released factor in E. coli (prokaryote)

Factor	Mass Location	Function	Mutants
RF-I	35,911	Recognises terminator codon UAA/ UAG at A site	Suppress
RF-II	41,346	Recognises terminator codon UGA at A site	None

Note : In eukaryotic system there is only one RF called eRF.

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION
MIDNAPORE - 721 102

M.Sc. in Zoology

Part - I, Paper - II, Gr. A, Unit II (Molecular Biology)

Module No. 17

OPERON
A SYSTEM OF GENETIC REGULATORY CONTROL MACHINERY AND FUNCTION

The ability to switch the genes on & off is of fundamental importance to cells, since it enables them to respond to the changing environment & is the basis for the processes of cell growth & differenciation. The foregoing study will throw light on the regulation of gene expression in bacteria, specifically on a bacterium E. Coli whose primary nutrient for supply of energy is glucose, but occasionally the bacterium is seen encountering lactose, a dissacharide, that is utilised by means of a regulatory control on the enzyme system, that provides evidences for our knowledge regarding the functioning of genes. This developed system of co-ordinated gene expression in which groups of genes coding for proteins with interrelated functions (that requires the presence of such protein in the cell at the same time) are all controlled as a transcriptional unit in prokaryotes mostly, where as in eukaryotes they may also be controlled at the translational level. The evidences for such functional unit of transcription is now a matter of scientific history & stems mainly from ideas of Jacob and Monod who in 1961 postulated the term OPERON as a controlling element for the genes involved in lactose metabolism.

What then is operon

Operon has been defined in many ways so far of which some are -

Jacob & Monod (1961)

A complete unit of gene expression, including structural genes, a regulator gene (r genes) & control elements (the site at which the regulator proteins act). Each operon can be characterised by network of interactions between regulator proteins & their target sites.

Norman & Maclean ('76)

An operon is a sequence of genes situated together on the DNA, the transcription of which is controlled co-ordinately.

Goodenough ('78)

A group of related genes that is transcribed into a single molecule of mRNA is known as *Operon.*

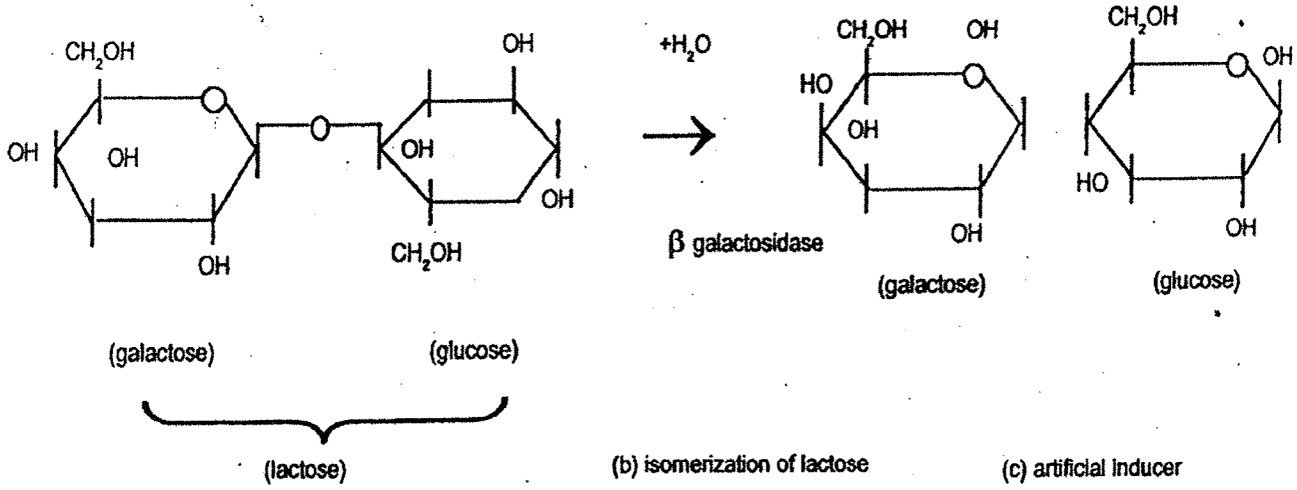
How the regulatory control is exercised?

The rate of expression of a bacterial gene is controlled at the level of mRNA synthesis through proteins called

repressors. The control is actually exercised by two systems:-

a) **Enzymatic Induction :** - The changes leading to activation of genes to synthesize true enzymes for the breakdown of a substrate that the cell encounters. It is seen in catabolic systems which degrade sugars, the best known induction system is of Lac-operon.

b) **Enzymatic Repression :-** Works in opposition to enzyme induction, i.e the process by which the synthesis of certain enzyme is selectively inhibited by the end product of its metabolic chain. The tryptophan operon, histidine operon are example of operon-repressible system.



LAC OPERON Machinery & Biochemical control

A) **Substrate :-** Lactose is composed of sugars, glucose & galactose joined with an O_2 atoms that links the first carbon of glucose to the fourth carbon galactose. The linkage is β linkage (Called the greek letter β), the formal name is

Lactose being glucose 1,4β galactoside.

This substrate is split into glucose & galactose by addition of H_2O at linkage site, H going with one sugar & OH withe other sugar the reaction is catalized by the enzyme β-galactosidase that specifically attacks the β - galactoside linkage of lactose.

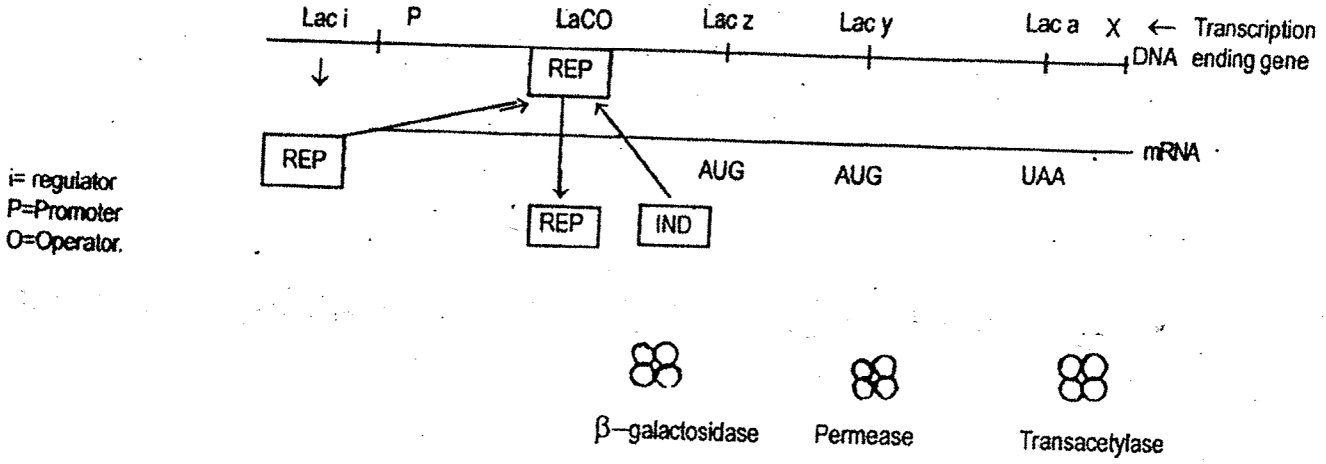
B. **Components of the Circuit :** are regulated structural genes; controlling elements (promotor & operator); regulator protein & its structural gene - the **Regulatory gene** & Effector or Inducer.

i) **Structural genes** that carry the codes for the synthesis of proteins and determine the primary structure of the polypeptides. By contrlling the amino acid sequence during synthesis (e.g. gene z, y, a in lac soperon).

ii) **Regulator genes** comprise of the promoter and operator genes that play the most important function.

iii) **Effector or inducer** - a molecule when added to the medium traps the repressor protein from operator that starts functioning & ultimately enzyme synthesis takes place. The effector is generally a small mol, a sugar or a nucleotide.

c) The operon skeleton proper



1. **A regulator gene** denoted by (i) or $(i)^+$ that produces the monocistronic mRNA that codes for the *'lac repressor protein'*. It is socalled because its function is to prevent the expression of the structural genes. The lac repressor is a protein with 4 protein subunits each of 40,000 daltons. There are about 10 repressor molecules in the E.Coli cells.

The REPRESSOR is an allosteric protein that has high affiinity for the operator & also forms a complex

with the inducer. The molecule has two binding sites (with notches) one for operator & other for inducer.

2. **Controlling Elements :-**

a) **PROMOTER** - denoted by P or (P^+) is the DNA segment to which RNA pol becomes attached. The promoter has two sites -

i) **CAP site :** that receives the CAMP -CAP complex during enzyme induction. The CAP is a dimeric protein with MW of 22000 (Haggis' 1974)

ii) **RNA pol interaction site** - has an AT rich region where the transcriptase enzyme binds. The lac promoter is under positive control.

b) **OPERATOR** - is the part of operron adjacent to a set of structural genes.

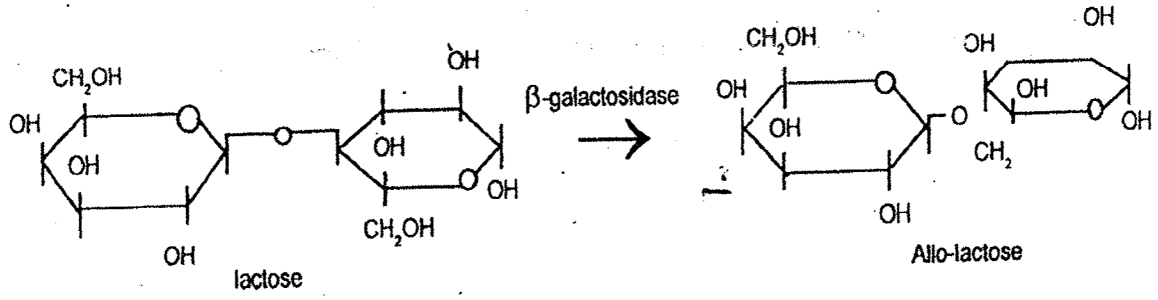
3. **Structural genes** - are z or z^+; y or y^+ and a or a^+.

a) z-codes for β -galactosidase enzyme (a tetrameric enzyme with subunits of 1,30,000 daltons each) that eleaves the dissachraride into galactose and glucose.

b) y-codes for galactoside permease or M protein located in the cell membrane that acts as a carrier for the entrance of lactose in the cell.

c) a codes for thiogalactoside transacetylase that occurs free in the cytoplasm. An enzyme of two subunits that catalyges the transfer of one acetyl group from acetylcoA to galactose.

4. **Effector or Inducer :-** Lactose though is the substrate for β -galactosidase is not an inducer of the operon, in fact it has slight effect as an anti-inducer (binds with repressor to improve its affinity for the operator).



The convoluted pathway for induction involves (in vivo) the uptake of a little lactose some of which is converted to *allolactose* by the enzyme. This allolactose then induces the operon. The strong inducing effect of the small amount of allolactose that continues to be made is sufficient to out weigh the anti inducing effect (weak of the lactose substrate).

NOTE : Experimentally, it is much easier to use an artificial inducer such as isopropylthiogalactoside (IPTG) which without further change binds to repressor and acts as an inducer.

i) **The enzymatic Induction** is effected by inducible system in which a series of events takes place one after another & production of monocistronic mRNA from the regulator gene (i) that codes for the repressor protein.

ii) Binding of repressor molecule in operator region due to its high affinity; blockage of transcription by RNA pol at the promoter.

iii) Now, if lactose is present, very soon the effector allolactose is produced that attaches at the specific site of the repressor mol. lead to a conformational change in it and make the operator free.

iv) CAP binds to CAMP in the promoter to free the AT rich region where the RNA pol. attaches and transcription follows.

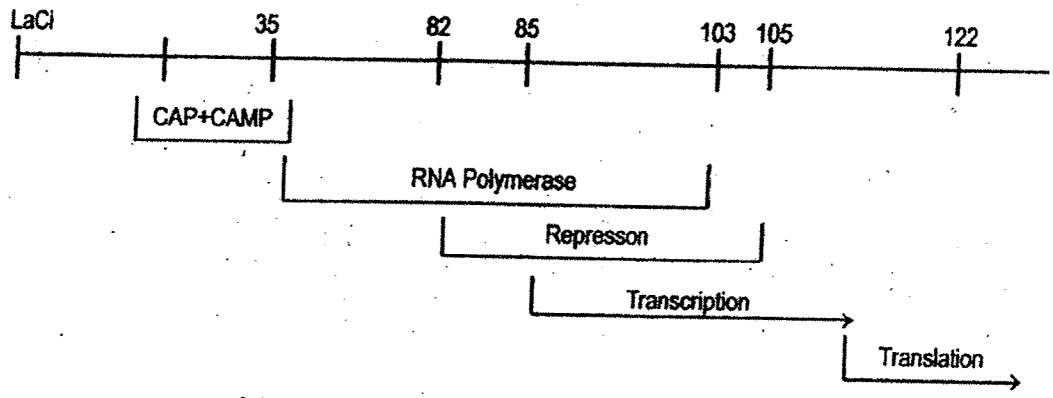


Fig. - 4

Comment : This induction is controlled by two almost simultaneous controls in the operon, one negetive & one positive. To understand these controls we ought to have a good knowledge of the structure of Lac operator and promoter.

THE PROMOTER OPERATOR REGION OF LAC OPERON (WOODS 1980)

On looking at promoter operator the following things are seen -

i) promoter operator region comprise of 122 base pairs.

ii) Lac repressor bound to DNA, covers the intitial region that must be transcribe into mRNA.

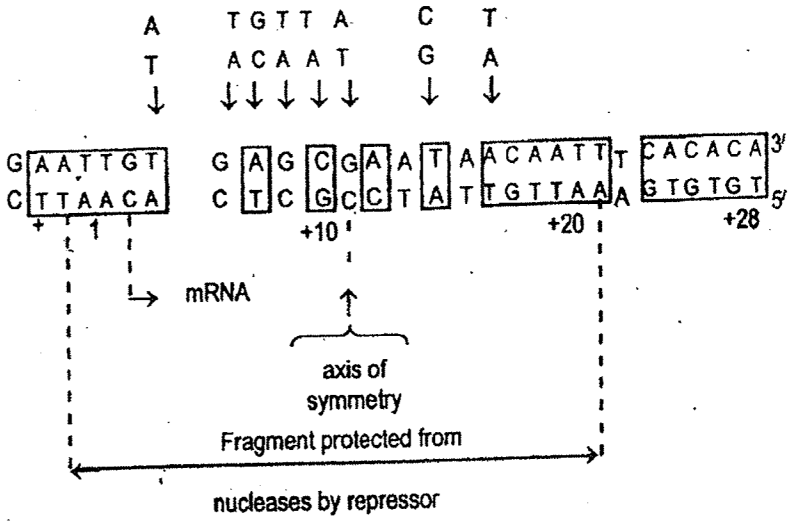


Fig. 5 : Nucleotide sequence of lac operator region showing its "palin dromic" symmetry : shaded box on the left of the central axis bear sequences (+1→+10) that are inverted repeats of the coloured sequences to the right of the axis (+12←+ 28)

iii) The binding sites for polymerese & repressor almost overlap, so transcription stops when the repressor is bound (can not be initiated)

OPERATOR - A DNA SEQUENCE THAT HAS TWO FOLD SYMMETRY AND IS UNDER NEGETIVE CONTROL.

i) The operator has a close linkage with the structural genes under its control.

ii) The Lac-represor binds to specific 21 pairs of bases within the operator. The sequnce contains two fold symmetry; some sequences on the left side of the operator are also present on the right side but on the opposite strand.

The two symmetrical sites probably represent recognition sites for different subunits of the repressor.

NOTE : The 21 base pair sequence is sufficient to blind the lac represser as demonstrated by the fact that a chemically synthesized fragment with the same sequence has operator activity both in vivo & in vitro.

The Negetive control

The process of binding of effector molecule to the repressor bound to the operator lead to a conformational change in the sepressor molecule This control is know as Allosteric control.

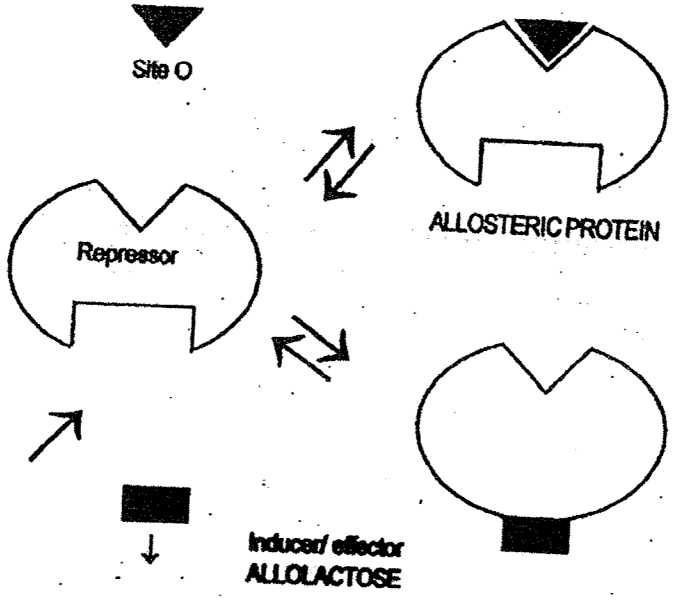


Fig. 6

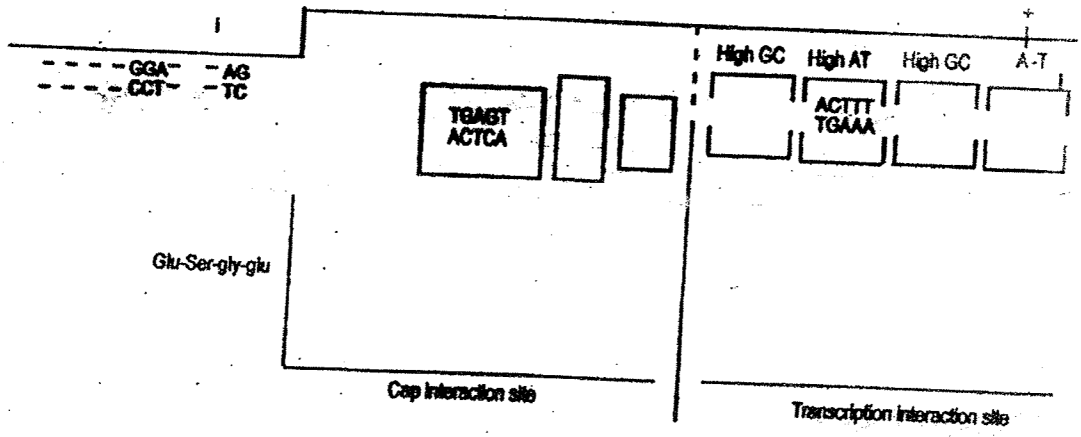


Fig. The promoter of Lac operon of E. Coli

PROMOTER - IS UNDER POSITIVE CONTROL

The promoter as said before has two sites, one concerned transcriptase interaction & has a repeat of 5 bp. This region containing an AT rich region is bordered on two sides by GC rich sequences. The transcriptase binds tightly to the AT rich sequences only when this sequence is denatured, by loosening the H-bonded GC rich sequences. The other region is thought to serve as a recognition site for a protein called 'CAP', (catabolite gene activator protein), a globulin Dimer. This region in the lac-promoter shows base symmetry close to one of the GC rich sequences & has hairpin loops that heep in anchorage.

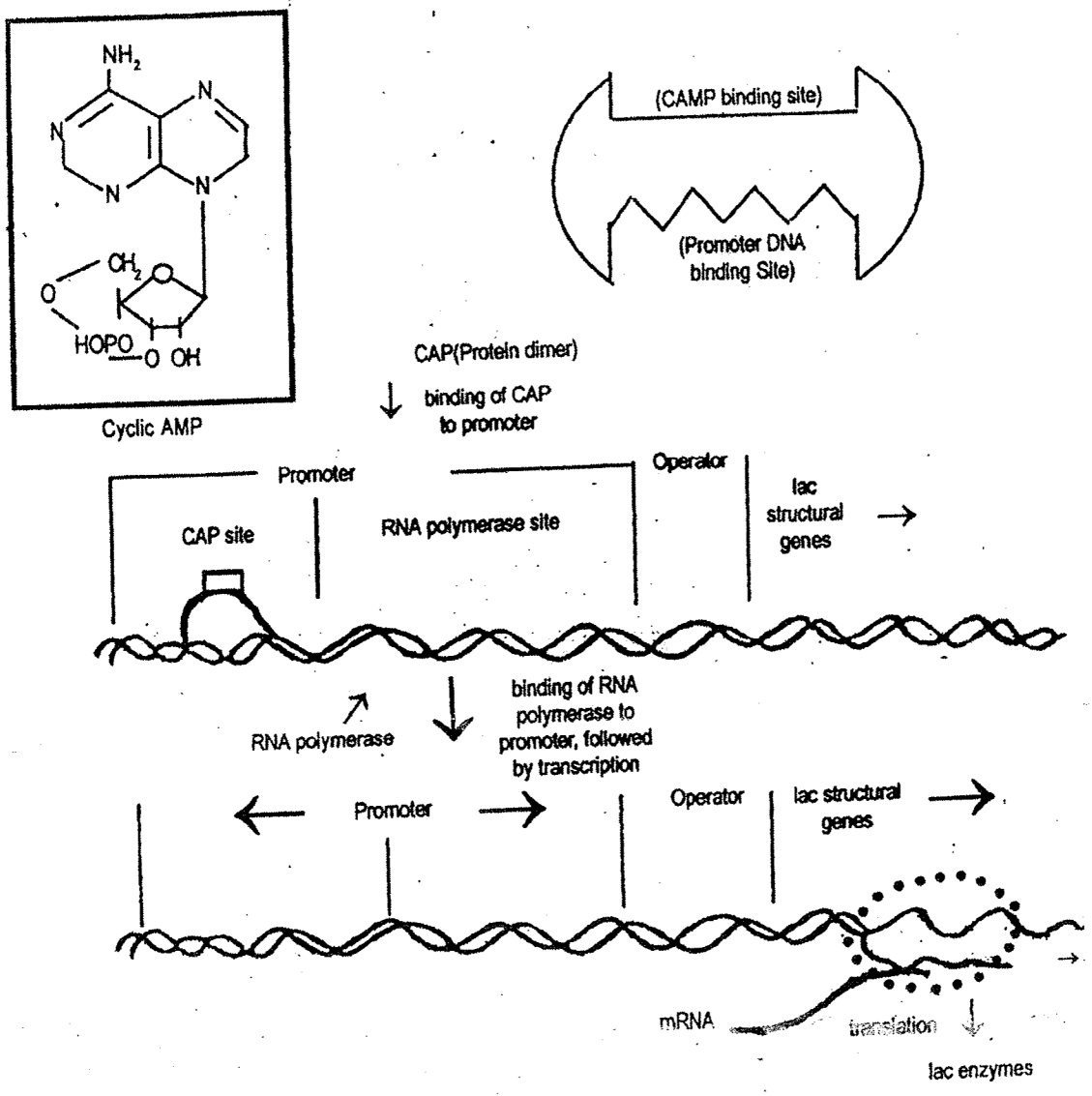


Fig. 8 Binding of cyclic AMP to the CAP protein enables the protein to bind, in turn, to its specific site on the promoter.

The operon function is repressed by products that results from the breakdown of carbon compounds such as glucose has become unown as *catabolite repression.*

The mechanism of catabolic repression also involves RNA polymerase function, but in contrast to negative regulation (the lac repressor protein is used to prevent transcription) catabolite repression affects a protein which is used to facilitate or activate RNA polymerase transcription. The mode by which this positive form of regulation occurs is through a dimesic protein called catabolite activator protein or CAP. There is a special site on the lac operon to which CAP attaches, and transcription is then facilitated because this attachment enhances the attachment of RNA polymerase to its own promoter site. CAP attachment, however, is not an independent event, since it relies on the presence of a compound cyclic AMP (adenosine cyclic monophosphate or cAMP). That is shown in Fig. (8), CAP attaches to its promoter site only when combined with cAMP. The concentration of cAMP, however is strongly affected by glucose breakdown or glycolysis; low concentration of cAMP are found during glycolysis and high concentrations occur when cells are energy starved. exactly how glycolysis determines cAMP levels is not yet known, but mutations that produce defects in either the adenylate cyclase enzyme (cya^-) that normally synthesizes cAMP from ATP, or in the CAP protein intself (Crp^-), significantly reduce the rate of enzyme synthesis in 'catabolite - sensitive" operons such as lac.

Thus lac enzyme synthesis is not a simple all-or-none affair, dependent only on the presence of lactose but, through cAMP can also be quantitatively tuned to the availability of other energy sources.

NOTE :

i) The binding of transcriptase to promoter is regulated by the concentrated glucose, the level of which if more in cell, caused a drop in cAMP level, that in turns prevent the binding of CAP to cap site. Therefore transcription occurs rarely in lac-operon.

ii) Also, the need of lactose splitting enzymes is raised, in the cell, since mostly the energy source is glucose.

iii) The presence of glucose also inhibits the synthesis of enzymes used in the catabolism of galactose and arabinose (catabolic repression) in most micro organism (including yeast).

iv) These examples of the effect of glucose also seems to involve the failure of the promoter of other operons to be bound by a cAMP complex.

The lac promoter is said to be under POSITIVE CONTROL because its function enhanced when, it combines with cAMP (a non protein molecule) - CAP complex & determines whether or not the protein can bind to the genetic material & activate it.

Lac operon is actually a catabolic sensitive operon that is dependent on the presence of cyclic AMP. Here the activator protein is CAP or CRP.

LAC-OPERON-GENETIC CONTROL

The concept of Lac operon was first introduced by F. Jacob and J. Monod who had explained this system on the basis of their brilliant studeis on the K_{12} *E.Coli* lactose metabolism. Jacob and Adelberg studied the lac by interrupted mating of conjugants lac^+ Hfr and F^- lac^- cells. The lac^+ colonies formed by the transferred F' lac^+ particles were unstable. They were termed '*partial diploids*' or heterogenotes or Merozygotes.

The presence of F' factors increases the amount of particular phenotypic product eg. the amount of β–galactosidase an enzyme involved in lactose utilization, increases 2-3 times because of F' lac^+ factors.

The discovery of a new class of mutations that affected the production of the three proteins by their synthesis from *inducible* to constitutive (production of enzymes in absence of inducer) led Monold and others (1956) to opine that, the constitutive production was controlled by a mutation (defective repressor production) in the regulatory gene called i^-, that is generally mapped at one end of the lac locus not far from z.

NOTE : i was called a regulator gene since it affected the inducibility of the enzymes & not their structure. The concept that, i-gene specifies a regulator (repressor) molecule is indicated by behavior of partial diploids, which have bacterial genes included by F' element.

Also that, in i^+/i^- diploids the normal repressor produced by the i^+ gene on an F element closely differs to and thereby regulates the lac genes on the main chromosome.

Moreover the regulation is normal, when i^+ gene is either *CIS* or *TRANS* with respect to the functional structural genes in the cell. Further expt. by JACOB and MONOD could classify the role of operator & other structural genes. This was based on the discovery of mutations and different combinations of merozygotes that produced β galactoside etc enzymes by induction or at random (constitutively) in spite of presence of i^+ genes.

EXPERIMENTAL FINDINGS:

TABLE - I

PRODUCTION OF B GALACTOSIDASE IN DIFFERENT *E.COLI* GENOTYPES, SOME OF WHICH ARE HETEROGENOTES BEARING F^+ LAC FACTOR.

GENOTYPE	β GALACTOSIDE PRODUCTION	
	NON INDUCED BACTERIA	INDUCED BACTERIA
1) i^-z^-	-	-
2) i^-z^+	+	+
3) i^+z^+	-	+
4) i^+z^-	-	-
5) $uz^+/F'i^-z^+$	+	+
6) $i^+z^+/F'i^-z^-$	-	+
7) $i^+z^-/F'i^-z^+$	-	+
8) $i^-z^-/F'i^+z^-$	-	+
9) $i^-z^+/F'i^+z^+$	–	+

TABLE II

GENOTYPES [β GALACTOSIDASE/GALACTOSIDASE TRANSACETYLASE]

		Non induced	Induced	Noninduced	Induced
1)	$i^+z^+a^+$	0.1	150	1	100
2)	$i^sz^+a^+$	0.1	0.1	1	1
3)	$i^sz^+a^+/F'i^sz^+a^+$	0.1	2	1	3
4)	$o^cz^+a^+$	25	95	15	100
5)	$o^+z^-a^+/F'o^cz^+a^-$	180	440	1	220
6)	$i^so^+z^+a^+/F'i^+z^+a^+$	190	219	150	200
7)	$i^+o^cz/F'i^+o^+z^+$	-	+	-	-
8)	$i^+o^+z^+y^+/F'i^+o^cz^+y^+$ Inducer not required for synthesis of both enzyme.				
9)	$i^+o^+zy^+/F'i^+o^cz^+y^-$ Require inducer for Acitylase only	+	+	-	+

10) $i^+o^+z^+y^-/F'i^+o^cz^-y^+$

Requires inducer for β galactoside synthesis

Findings in above tables - $i^+z^+o^+y^+$ are wild type genes.

i^s = mutation in regulator gene (super - repressor)

i^- = mutation in regulator gene.

$z^-a^-y^-$ = mutation in structural gene.

1) Wild type regulator (i^+) has two features -

   1) i^+ is dominant over i^- in partial diploids i^+/i^- eg. a mating between i^- Hfr x i^+F^- gives p galactosidase on induction.

   2) In merozygotes the entry of i^+ gene (wild type) into the constitutive mutants (in the cross i^+z^+ Hft x $i^-z^-F^-$) cause production of a regulatory substance that prevents constitutive synthesis in spite of the presence of i^- gene.

   3) Finding the combination no. 9 of table I, it is evident that the substance produced by z^+ gene prevents constitutive synthesis of β galactosides even when the wild type gene z^- for the enzyme is present on the recipient chromosome.

   4) In the mutation i^s (called the super repressor mutation) enzymes responsible for lactose splitting are not synthesized even in the presence of inducer (2 no. of table). Also, the i^s superrepressor substance is dominant over ordinary repressor substance produced by the i^+ gene (i.e. inducer can activate the normal repressor of i^+ but not of the i^s Superrepressor). Apparently i^s super repressor continues repression because it has lost the binding site for inducer attachment.

   5) O^c or the operator constitutive mutations are unique since they are seen to be affecting genes to which they are linked in *cis* position (see 5 of Table).

NOTE : O^c mutations present in i^+ cells are not fully constitutive & respond to inducer producing more enzyme than in non-induced cell. However induced O^c mutants will not respond to produce enzymes more than the induced wild type ordinarily.

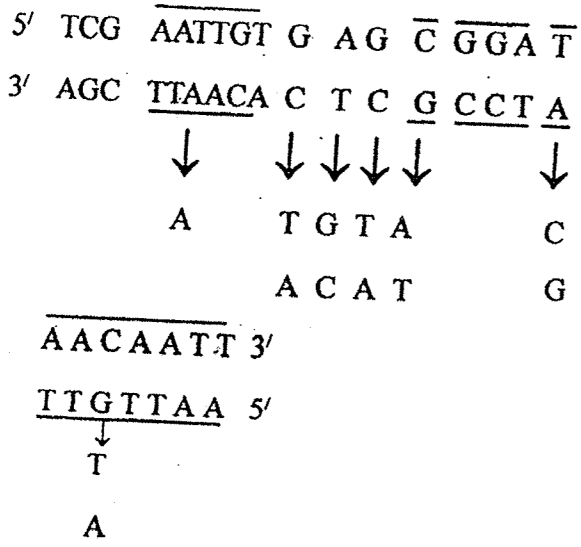


Fig 9 The Repressor molecule binding site of operator gene : the base pair changes that lead to the mutation of constituent operator gene.

NOTE : Alteration of a single base pair in the lac o^+ can effectively prevent the binding of repressor thus, the operon changes from normal inducible OPERON to OPERATOR CONSTITUTIVE STATE.

6) The properties of i^- and its mutations in short give clues how regulator & operator might interact. They also provide two kinds of evidences to support the idea that, the repressor is a proteins & not a RNA molecule.

   a) Mutant proteins are often known to be temperature sensitive and certain i^- strains produce a repressor that is inactivated at elevated temperatures.

   b) Protein synthesis is known to be inhibited by the amber (UAG) mutations & supressible i^- amber strains have been frequently isolated.

CONCLUSION

Operator gene is much more distantly related to the structural genes & can act both in *CIS* and *TRANS* condition (as seen by genetic mapping) operator is *CIS dominant*.

In fact, all the 3 enzymes in lactose region are controlled by a mutation in the OPERATOR GENE indicates that the linkage between operator and structureal genes was not only physical but also funtional.

The genetic integrated unit was called 'OPERON' by JACOB and MONOD.

TRYPTOPHAN - OPERON

Once RNA pol. has started transcription, the enzyme moves along the template, synthesizing RNA, until it meets a terminator (t) sequence. At this point, the enzyme stops adding nucleotides to the growing RNA chain, releases the completed product and dissociated from the DNA template. Termination requires that all hydrogen bonds holding the RNA-DNA hybrid together must be broken, after which the DNA duplex reforms.

At some terminators, the termination event can be prevented by specific ancillary factors that interact with RNA polymerase. Antitermination causes the enzyme to continue transcription past the terminator sequence, an event called read through. Antitermination is used as a control mechanism in both bacterial operons and phage regulatory circuits. In operons controlled by *attenuation*, it provides a link between translation and transcription, in which termination of transcription is prevented when the ribosome is unable to move along a leader segment of the m RNA.

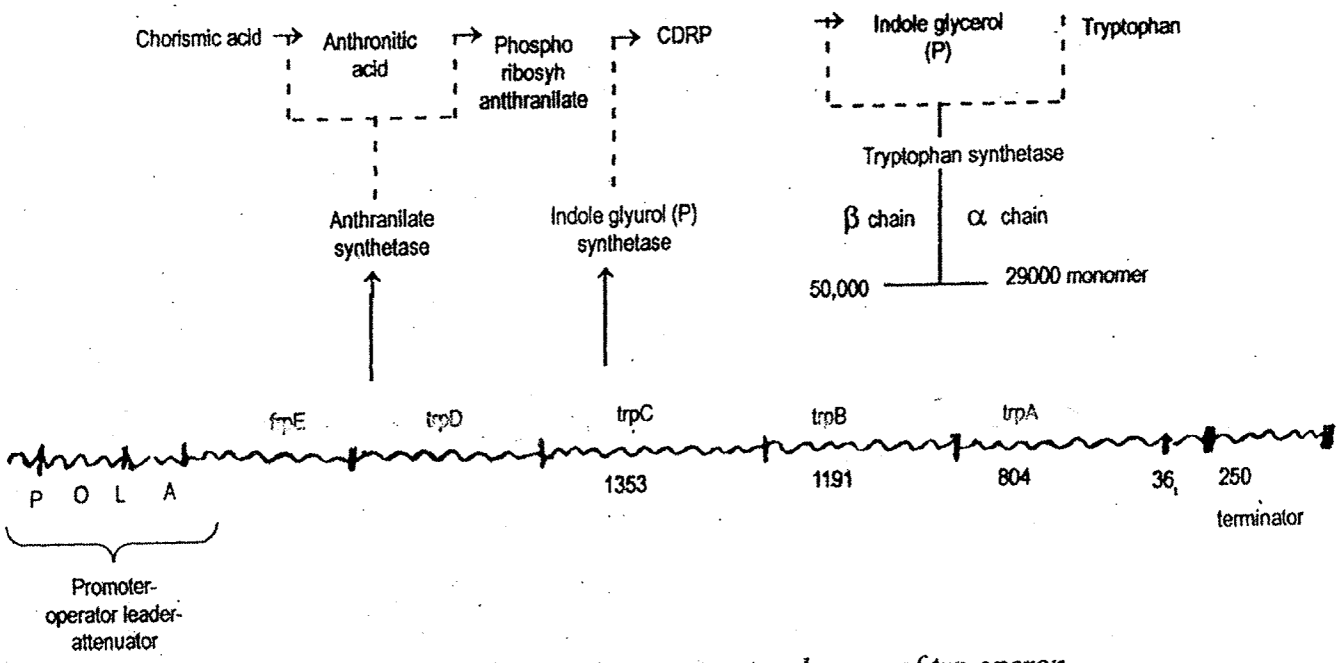


Fig 1 : Showing five contignous structural genes of trp operon.

Attenuation was discovered in the tryptophan operon, whose five structural genes are arranged in a contiguous series, coding for the three enzymes that convert chrosmic acid to tryptophen by the pathway given in the figure 1. Transcription starts at a promoter site at the left end of the cluster. Adjacent to it is the operator that binds the repressor protein coded by the unlinked gene trp R. A leader sequence lies between

the operator and the coding region of the first gene. Transcription of the structural genes is partially terminated at a rho independant site, trpt, 36 bp beyond the end of the last coding region. In addition to the promoter-operator complex, another site is involved in the trp operon. Its existence was first revealed by the observation that deleting a sequence between the operator and the trp E coding region can increase the expression of the structural genes. This effect is independent of repression. So this site influnces events that occur after RNA pol. has set out from the promoter.

The regulator site is called the attenuator. It lies within the transcribed leader sequence of 162 nucleotides that preceeds the initiation codon for the trp E gene. The attenuator is a barrier to transcription. It consists of a short G-C rich palindrome followed by eight successive U-residues. RNA pol terminates there to produce a 140-base transcript.

The termination event at this site responds to the level of tryptophan. In the presence of adequate amounts of tryptophan, termination is efficient. But in the absence of tryphophan, RNA polymerare can continue into the structural genes. This type of termination event is called *attenuation.*

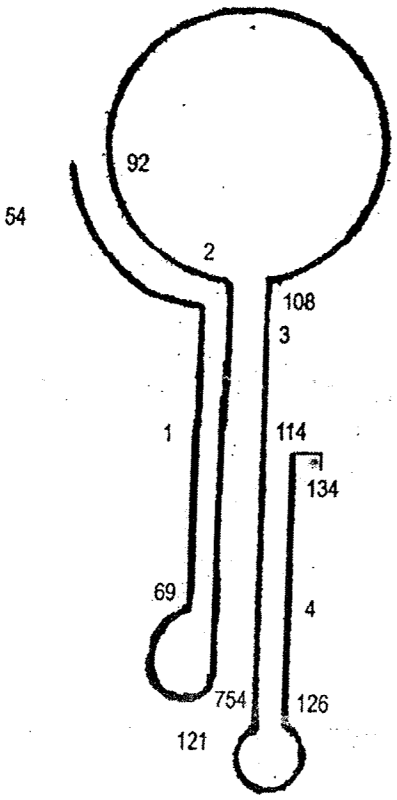


***Fig. 2** The trp leader region can exist in alternative base paired conformations. Region 1 is complementary to region 2, which is complementary to region 3, which is complementary to region 4.*

How can termination of transcription at the attenuator respond to the level of tryptophan? The sequence of the leader region suggest a mechanism. Leader peptide contains two trp residues in immediate succession. Tryptophan is a rare amino acid in E.Coli proteins, so this is unlikely to be more co-incidence. When the cell runs out of tryptophan, ribosomes initiate translation of the leader peptide, but stop when they reach the Trp codons. The sequence of the mRNA suggests that this ribosome stalling may influence termination at the attenuator. The leader sequence can be written in alternative base-paired structures. The structure determines whether the mRNA can provide the features needed for termination. Fig. 2 draw these structures. The pairing of regions 3 and 4 generates the hairpin that preceeds the U_8 sequences this is essential signal for termination.

A different structure is formed if region 1 is prevented from pairing with region 2. In this case, region 2 is free to pair with region 3. Then region 4 has no available pairing partner, so it is compelled to remain single stranded. Thus the terminator hairpin can not be formed.

When tryptophan is present, ribosomes are able to synthesize the leader peptide. They will continue along the leader section to the mRNA to the UGA codon, which lies between regions 1 and 2. As shown in fig.3 by progressing to this point, the ribosomes extend over region 2 and prevent if from base-pairing. The result is that region 3 is available to base-pair with region 4, generating the terminator hairpin. Under these conditions, RNA pol. terminates at the alternator.

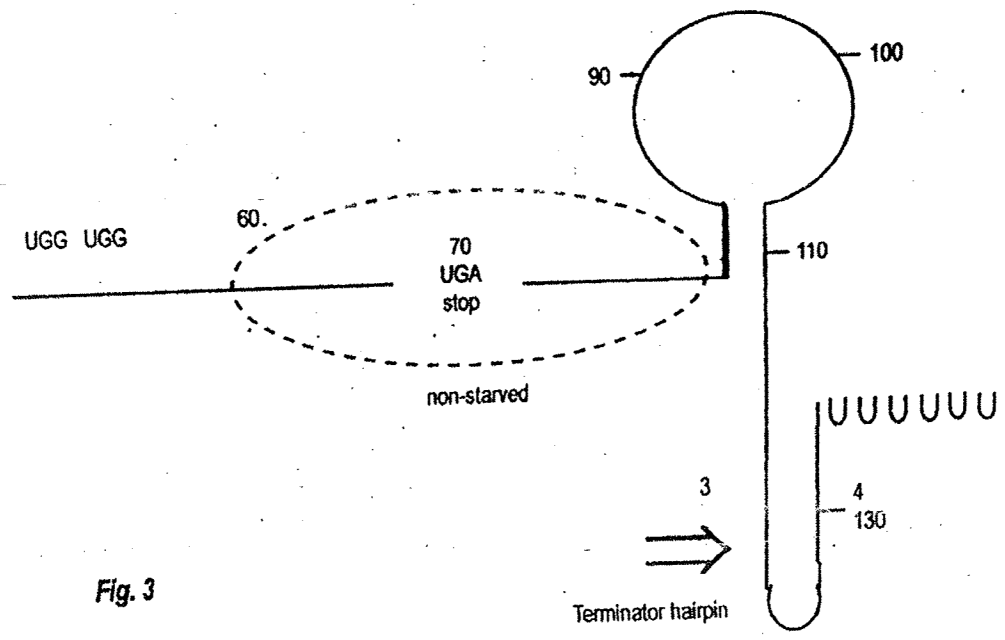
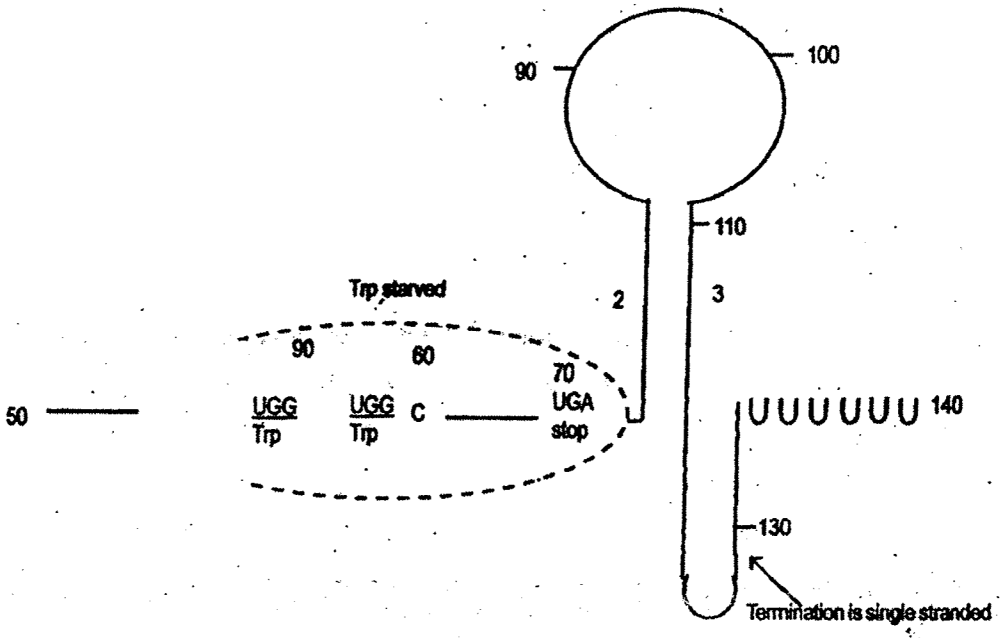


Fig. 3

When there is no tryptophan, ribosomes stahl at the Trp codons, which are part of region 1. Thus region 1 is sequestered within the ribosome & can not base pair with region 2. If this happens even while the mRNA itself is being synthesized, region 2 and 3 will be base-paired before region 4 has been transcribed. This compels region 4 to remain in a single stranded form. In the absence of the terminator hairpin, RNA pol. continues transcription past the attenuator.



MOLECULAR DISEASE

Sickle cell anaemia

In 1904, James Herric, a Chicago physician reported peculiar elongated and sickle shaped Red blood corpuscles in a case of severe anaemia patient. He noted the Chronic nature of the disease and the diversity of abnormal physical and laboratory finding : cardiac enlargement, a generalized swelling of lymph nodes, jaundice, anaemia and evidence of kidney damage. Indeed this is not a rare disease & is a significant public health problem whenever there is a substantial black population. The incidence of sickle cell anaemia among blacks is about four per thousand. The sickled red cells become trapped in the small blood vessels. Circulation is impaired, resulting in the damage of multiple organs, particularly bone and kidney. The sickled cells are more fragile than normal cells. They hemolyze readily & consequently have a shorter life than normal cells. The resulting anaemia is usually severe. The chronic course of the disease is punctuated by crises in which the proportion of sickled cells is especially high. During such a crisis, the patient may go into shock.

Sickle-cell anaemia is genetically transmitted. Patients with sickel-cell anaemia are homozygous for an abnormal gene located on one of the antosomal chromosomes. Offspring who receive the abnormal gene from one parent but its normal allele from other have sickle-cell trait. Such heterogygous people are usually not symptomatic. Only 1% of the red cells in a heterozygote's venours circulation are sickled in contrast with about 50% in a gomozygote. However sickle cell trait, which occurs in about one of ten blacks, is not entirely benign. Vigorous physical activity at high altitude, air travel in unpressurized planes and anesthesia can be potentially hazardous to a person who has sickle cell trait.

In fact, the haemoglobin in sickle cells is itself defective. Deoxygenated sickle-cell haemoglobin has an abnormal low solubility, which is about one twenty fifth of the solutbility of normal deoxygenated haemoglobin. Sickle cell haemoglobin is commonly referred to as haemoglobin S (HbS) to distinguish it from haemoglobin A (HbA), the normal adult haemoglobin.

Hemoglobin S has an abnormal electrophoretic mobility :

In 1949, Pauling & his associates examined the physical, chemical properties of hemoglobin from normal people & form those with sickle-cell trait or sickle-cell anemia. Their experimental approach was

to search for differences in these hemoglobins by measuring their mobilities in an electric field. This technique is called electrophoresis. The velocity of migration (v) of a protein in an electrical field depends on the strength of the electric field (E), the net electric charge on the protein (Z) and the frictional resistance (f). The frictional resistance is a function of the size & shape of the protein. The migration velocity is related to these variable by $V = \frac{EZ}{f}$

The different electrophoretic velocities of hemoglobins A and S could arise from a change either in the net charge Z or in the frictional coefficient f. A purely frictional effect would cause one species to move more slowly than the other throughout the entire pH range. This is not the case, because the slope of the plot of the electrophoretic velocity versus pH is the same for hemoglobin A & S.

These observations suggested that there is a difference in the number or kind of ionizable groups in the two hemoglobins. An estimate was made from acid-base titration curve of hemoglobin. In the neighborhood of pH 7.0, this curve is nearly linear. A change of one pH unit in the hemoglobin solution produces a change of about 13 charges.

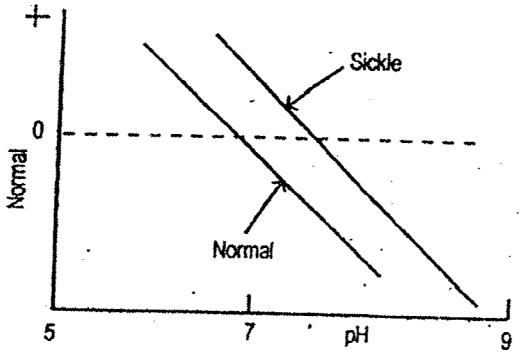


Fig 1. shows Electrophoretic mobility of sickle - cell hemoglobin and of normal hemoglobin as a function of pH.

The differences in isoelectric pH of 0.23 therefore corresponds to about three charges per hemoglobin molecule. It was concluded that sickle cell hemoglobin has between two and four more net positive charges per molecule than normal hemoglobin. (fig. 1)

Patients with sickle cell anemia (who are homozygous for sickle gene) have hemoglobin S but no hemoglobin A. In contrst people with sickle-cell trait (who are heterozygous for the sickle gene) have both kinds of hemoglobin in approximately equal amounts (fig.1 shows). Thus pauling's study revealed a clear case of change produced in a protein molecule by an alelic change in a single gene. This was the first demonstration of a molecular disease.

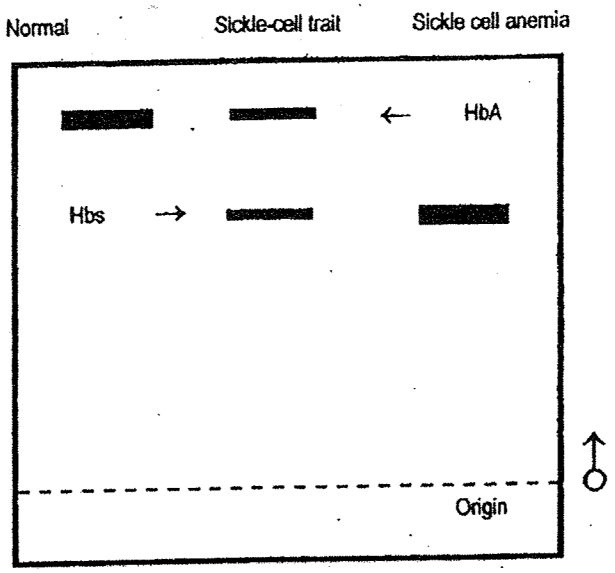


Figure 2 showing starch-gel electrophoresis pattern at pH 8.6 of hemoglobin isolated from a normal person, from a person with sickle cell trait & from a person with sickle-cell anemia.

Finger printing : detection of the altered amino acid in sickle hemoglobin

The electrophoretic analysis showed that hemoglobin S has two to four more positive charges than does hemoglobin A. A different of this kind could arise in several ways -

HbA	HbS
Neutral amino acid	Positively charged amino acid
Negatively charged aa	Positively charged = amino acid
Negetively charged aa	Neutral amino acid

The breakthrough in the elucidation of the precise change came in 1954, when Vernon Ingram devised a new technique for detecting amino acid substitution in proteins. The hemoglobin molecule was split into smaller units for analysis because it was anticipated that it would be easier to detect an altered amino acid in a peptide containing about 20 residues than in a protein ten times as large. Trypsin was used to specifically, cleave hemoglobin on the carboxyl side of its lysine and arginine residues. Because the αβ half of the hemoglobin contains a total 27 lysine & arginine residues, 28 different peptides were formed by tryptic digestion. The next step was to separate these peptides. This was accomplished by a two-dimensional procedure.

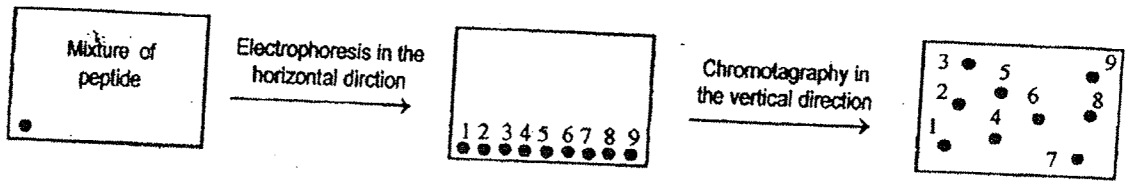


Fig. 3 : A mixture of peptide is resolved by electrophovesis in the horizontal direction, followed by partition chromatography in the vertical direction.

The mixture of peptides was placed in a spot at one corner of a large sheet of filter paper. Electrophores was first carried out in one direction, separating the peptides according to their net charge. However electrophoresis did not suffice to separate the peptides completely. A second separation technique, namely paper chromatography was carried out perpendicular to the direction of electrophoresis. In this procedure, the end of the filter paper closest to the peptides is immersed in a mixture of organic solvents & water at the bottom of a glassjar. The solvent ascends into the paper. Each peptide can either migrate with the solvent, which is quite non polar or cling to the hydrated cellulose support which is highly polar. This separatory technique is called *partition chromatography*. Paper chromatography and electrophoresis are complementary techniques because they separate peptides on the bases of different charecteristics -- namely their degree of polarity, & net charge respectively. This sequence of steps-selective cleavage of a protein into small peptides, followed by their separation in two dimensions is called *finger printing*.

The resulting fingerprint was highly revealing. The peptide spots were made visible by staining the fingerpring with ninhydrin. A comparison of the maps for hemoglobins A and S showed that all but one of the peptide spots matched. The one spot that was different was eluted from each fingerpring & then shown to be a single peptide consisting of 8 amino acids. Amino acid analysis indicated that this peptide in hemoglobin S differed from the one in hemoglobin A by a single amino acid.

A single amino acid in the beta chain is altered -

The α and β chains were separated by ion-exchange chromatography. Fingerprints of the separate chains showed that the change in hemoglobin S is in its β chain. In fact, the difference is located in the amino-terminal tryptic peptide of the β chain. Ingram determined the sequence of this peptide & showed that hemoglobin S contains *valine* instead of *glutamate* at position 6 of the β-chain.

Hemoglobin A	Val - His - Leu - Thr - Pro - Glu - Glu - Lys
Hemoglobin S	Val - His - Leu - Thr - Pro - Val - Glu - Lys
Hemoglobin C	

β 1 2 3 4 5 6 7 8

Thus sickle cell anemia, which is fatal to thousands of Africans & African Americans each year, is the result of the substitution of a single amino acid in two of the four long polypetide chains of hemoglobin. This single change is caused by a mutation in the gene that codes for the larger chain in hemoglobin. In this case a single codon must have been changed from either a GAA or a GAG, which codes for the amino acid Glu, to a GUA or a GUG, which codes for the amino acid Val. This substitution of a single nucleotide (A→U) out of the 438 used by the gene to code this polypeptide chain causes a mutation that is ultimately lethal to the individual. This single amino acid change can alter the entire structure of the cells.

Glutamic acid is a negetively charged amino acid & can hydrogen-bond with water. It is replaced by an amino acid that has no charge (Val) & is very hydrophobic (water-hating). Then at position 6, instead of a charged, water-loving side chain, there is a water hating side chain. This region of the polypeptide chain is exposed to the water in the cell & because of the change, now tries to hide from water. It does this by binding with a neighbouring hemoglobin molecule that has a similar hydrophobic patch. The result is that the hemoglobin molecules gather together in large bunches & can't carry oxygen well at all. So the person who has this disease literally suffocates, because he or she can not get oxygen out to the cells in the body.

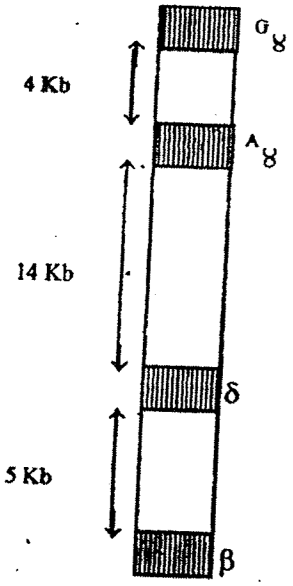
Thalassemias are genetic disroders of hemoglobin synthesis : β-thalasemia (defective β-globin chain)- occurs 1/400 among some Mediterranean populations. Investigations of the genes for hemoglobin & their mode of expression are proving to be sources of insight into enkaryotic gene function in general.

In development, embryonic hemoglobins are replaced by fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) and then by adult hemoglobin (HbA, α2 β2). Adults also produce a small amount of Hb A_2, which has the subunit structure $\alpha_2\ \delta_2$. It is known that Hb F has a higher oxygen affinity then does Hb A because it binds diphosphoglycerates less tightly. This higher oxygen affinity optimizes transfer of O_2 from the maternal to fetal circulation Hb F is actually a mixture of two species, one with glycine & the other with alanine at residue 136 of the γ chain. These subunits are designated γ^G and γ^A respectively.

All of the hemoglobin genes have been inapped. There are two closely linked α genes per haploid genome. These genes appear to be identical except in rare mutants. The other globin genes are elustered in the sequence ζ - γ -δ -β on a different chromosome.

The linkage of these ζ -δ -β genes may reffect their evolutionary history. The switch from the ζ genes to the 8 and δ genes in development depends on their proximity. The γ, δ and β gene, which became tandemly repeated & then diverged.

The thalassemias are a group of hereditary anemias in which the rate of synthesis of one of the chains of hemoglobin is diminished. Thalassemia major and minor refer to the homozygous and heterozygous states respectively. The prefix α or β designates which chain is synthesized at an abormally slow rate. Studies of globin mRNA and cloned globin DNA are beginning to provide an understanding of these disorders. The molecular defect in several thalassemias has been delineated.



Map of the human δ & β - globin genes.

1. Gene delection

- In some α–thalassemias, one or both α globin genes are deleted:

2. Instablility of mRNA

- In hemoglobin Constant Spring, the α chain has 172 residues instead of 141 because of a mutation from UAA (stop) to CAA (glutamine). Translation of part of what is normally a noncoding region somehow renders the altered mRNA susceptible to the action of nuclease.

3. Defective chain initiation

- In some β-thalassemias, initiation of translation is abnormally slow, probably because of a defect in the 5′ non coding region.

4. Premature chain termination

- A β-thalassemia arises from a single base change from AAG(lysine) to UAG (stop) at residue 17.

5. Underproduction of mRNA

- In many β-thalassemias, the β-globin gene is present, but very little β-globin in RNA is produced. The cause of this underproduction is being actively studied. An intriguing possibility is that intervening sequences are not properly excised in some β-thalassemia.

Reference Book

1. Geneties - Strickbeger.
2. Biochemistry- Stryer.
3. Genes V - Lewin.

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION
MIDNAPORE - 721 102

M.Sc. in Zoology

Part - I

Paper - II, Group -A, Unit-II

Module No. 18

Recombinant DNA technology :

In bacteria, the blueprint of life-DNA is found in the chromosome. Most of the time in bacteria, the DNA is found in a large circular, double stranded form. The DNA in the chromosome contains a no. of segments called genes, each of which contain the code for a particular protein. For this reason, the chromosomal material in a cell is often called the genome (gene + chromosome).

In addition to the main chromosome, another type of DNA is often found in bacteria. This DNA comes in small, circular structures called *plasmids*. Plasmid contains sex factor of bacteria and also genes that code for antibiotic resistance. Plasmids are of great interest be ause they are fairly simple in structure and easy to extract from bacteria. Probably more important is the fact that when plasmids are isolated they can be altered as needed and can be reinserted into the bacteria. The bacteria then treat these reinserted plasmid as part of themselves and plasmids are duplicated as the cells divide.

The plasmid is an ideal structure for genetic engineers for two reasons (1) it contains genetic information used by the bacteria and (2) the plasmid itself is not essential to bacterial functions. So it is possible to manipulate this DNA without upsetting the bacteria.

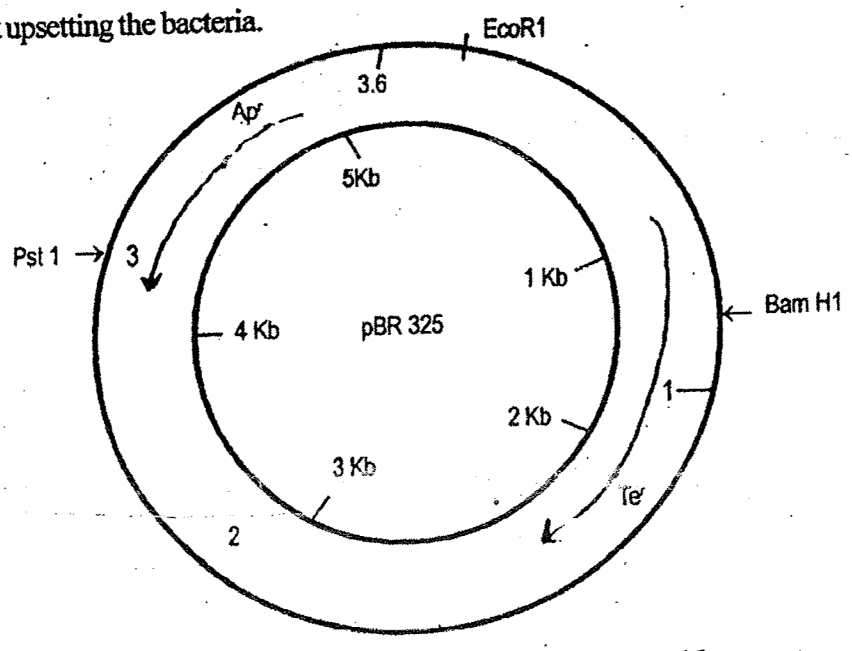


Figure 1 : The genetic map of plasmid.

Bringing new DNA into old bacteria

Bacterial cells go through a process called *conjugation* in which they share genetic material. DNA is transferred from one bacterium to other. During the conjugation process, a full plasmid or merely a piece of it may be transferred. In this way, bacteria can exchange resistance to antibiotics & other traits that will help them to withstand the rigors of their environment.

In addition to conjugation, plasmids can sometimes be inserted into bacteria just by being present in the culture medium. This is called *plasmid transfer*. These plasmids move into the bacteria through pores in the membranes. Once inside, the plasmids sometimes recombine with the bacterial chromosome. At some later time, the plasmid may be expelled from the DNA and form a small circular plasmid again. As it leaves, it may carry with it a portion of the cellular chromosome, or it may leave a bit of itself behind in the chromosome. In either case, the genetic information of both the chromosome and the plasmid is altered.

These kinds of genetic alterations give bacteria the ability to adapt to environmental stresses & changes which gives rise to wide variety of bacteria found. In case of antibiotic resistance, the alterations are acts of self-preservation for bacteria, but may present a problem. This is becoming increasingly apparent as more varieties of antibiotic-resistant bacterial strains are developing which make diseases they cause harder to treat (e.g. tuberculosis).

The Tools of genetic Engineering :-

Whenever free DNA is taken into bacteria from the surrounding environment, transformation occurs. In nature, exchange of genetic information occurs continually through both conjugation and transformation.

Genetic engineering of a bacterium takes place when a bacterial chromosome or plasmid is changed by design. In order to engineer changes in DNA & insert the changed DNA into bacteria, there are several things to learn. To place a piece of new DNA in bacteria the followings are to be done:

1. Extract plasmids from bacteria.
2. Cut the plasmids open in specific regions.
3. Insert a piece of DNA into the plasmid.
4. Fuse the new DNA to the old DNA & close the plasmid circle.
5. Insert the plasmid into the bacteria & have the bacteria make the new protein.

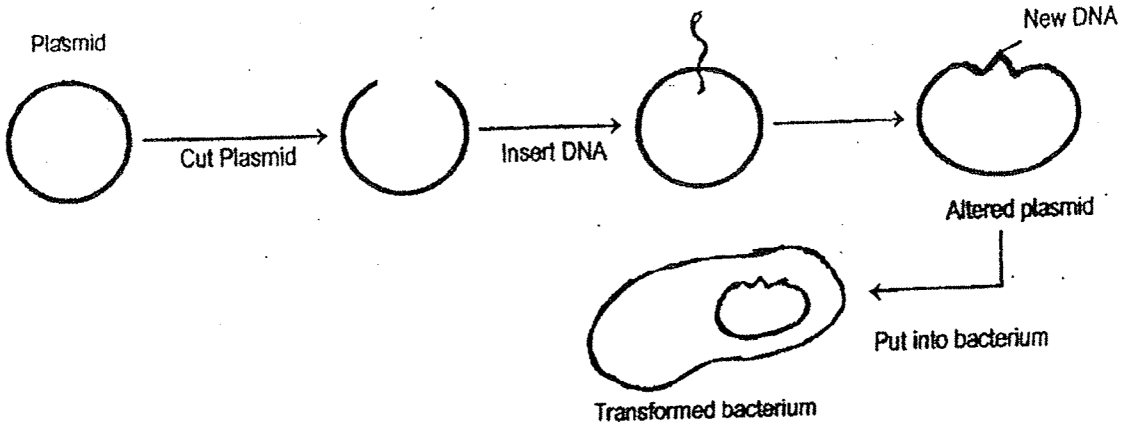


Figure 2 : *Diagrammatic representation of placing new DNA into a bacterium.*

1. Extraction of Plasmid :

Once the cells are broken open, the DNA is separated from the rest of the cell material, following which a solution of it is layered on top of a dense solution in a centrifuge tube. This tube is spun at high rates of speed in the centrifuge for several hours, during which the plasmid DNA separate from the chromosomal DNA because its density is different from that of the chromosomal DNA. These bands of DNA can be seen with ultraviolet light. The band of plasmid DNA can be extracted by poking a needle into the plastic centrifuge tube & sucking it out with a syringe.

The plasmid obtained can be precipitated out of the dense solution by using ethanol which caused all the DNA to clomp together.

2. Cutting plasmids open :-

Cutting is done by restriction endonucleases which were discovered in the 1950's and isolated in the 1960's. Each of these enzymes cuts DNA specifically at a unique site which depends entirely on a particular short sequence of nucleotides in the DNA.

EcoRI – The first restriction endonuclease that was discovered in *Escherichia coli* & hence its name. EcoRI specifically looks for regions in DNA that have the sequence :

5' – G – A – A – T – T – C – 3'

3' – C – T – T – A – A – G – 5'

Once the recognition site has been found, the engyme binds to that region & them cleaves the DNA in a

very specific way. The arrow in figure show where the cuts will be made. It is to be noted that the resulting DNA fragments have ends that are overlapping as shown in figure.

Often these overlapping ends are called sticky ends because they tend to hydrogen-bond to their counterparts really well. Many of he restriction endonucleases make these sticky ends, but the number & sequences of nucleotides in the sticky ends are different, depending on which restriction endonuclease was used to cut the DNA.

Hpa I – This endonuclease will only cleave DNA that has the following sequences :

5' – G –T – T – A – A – C – 3'

3' – C – A – A – T – T – G – 5'

This enzyme recognizes only the sequence shown & binds to that region of any DNA. Then cleavage occur. In this case, the product does not leave overlapping ends, but gives blunt or flush ends.

See fig. 3 for an illustration of what a small piece of DNA cut with HpaI would look like :

5'—— GTT AAC —— 3'

3'—— CAA TTG —— 5'

***Fig. 3** An example of a piece of DNA cleaved by a restriction engyme (HpaI) that recognizes the sequence GTT AAC & cleaves between the last T & the first A (in a 5' → 3' direction). The product is DNA that has blunt ends.*

Inserting New DNA into plasmids :

The next step in engineering DNA is to put a new piece of DNA into a plasmid & put it into working order again. But where do we get new DNA? Possibly from some other organism. It is possible to buy chemically synthesized DNA pieces that contain upto 100 nucleotides. These pieces of DNA are made from individual nucleotides and coupled in an instrument that forms the phosphodiester bonds between the nucleotides. These nucleotides are placed in the sequence that is needed. So it is possible to go to biochemical supply companies & buy short segments of DNA having any sequence. Therefore, depending on what we wish to do we must either obtain DNA from another source or synthesize the pieces we need.

If we are going to cut a piece of DNA from another source, we will have to know enough about that source to know the location of the appropriate restriction enzyme cleavage sites around the DNA we want. Suppose we knew that a portion of the DNA looked like that shown in figure below: (fig. 4)

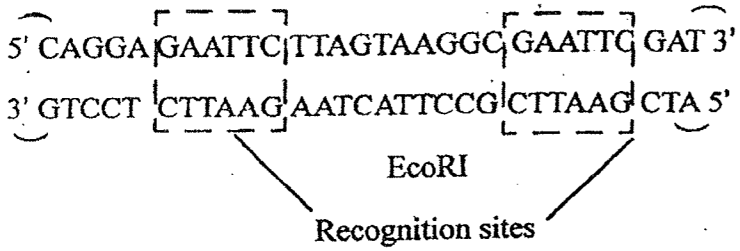


Figure 4 showing example using restriction enzymes to isolate a piece of DNA. Two EcoRI sites flank the fragment of DNA to be isolated. By cleaving the DNA with EcoRI, the desired fragment of DNA is removed from the longer piece of DNA (only a portion ofwhich is framed by dash line). Using gel electrophoresis the desired fragment can then be separated from the rest of the DNA.

By using that restriction endonuclease (EcoRI), we would get the piece of DNA that we want & would know its size. So by running the DNA fragments on an electrophoresis gel & using commercially available size markers, we should be able to identify the fragment we want. See figure below.

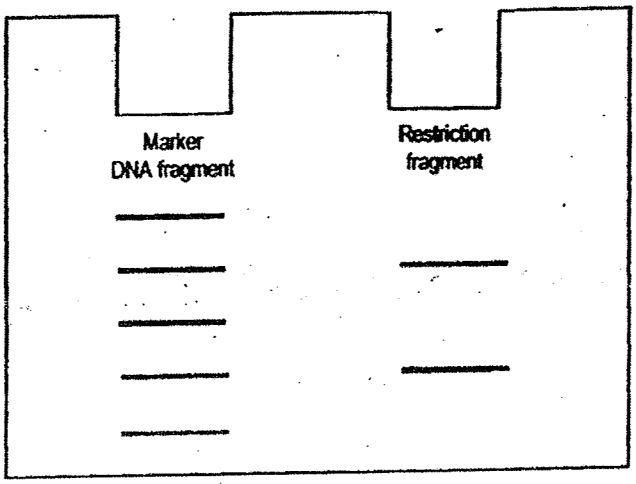


Figure 5 : Showing pattern of fragments of DNA obtained by digestion of DNA using a restriction enzyme. In the left hand lane is a set of marker DNA pieces, obtained commercially, which have known lengths. By comparing the unknown restriction fragments with the DNA markers, fragments of size expected can be identified & then isolated by extracting them from the gel.

When we have identified the band (fragment) we want the DNA can be removed from the gel by finely mincing the gel, dissolving the acrylamide & then precipitating the DNA out of the solution using ethanol. In this way we can purify a particular band of DNA.

The isolated DNA could be a gene for a protein, but for the moment it may be just any piece with a given sequence. If we have isolated this piece of DNA, using EcoRI, we would have the following structure.

AATTCTTAGTAAGGCG

GAATCATTCCGCTTAA

Gluing Genes Together:

How do we attach this piece of DNA to another piece of DNA or better yet, insert it into a plasmid? When we insert a piece of DNA into a plasmid, it is called an insert. One of the important considerations is that both the insert & the plasmid pieces have ends that are compatible. For instance, it would be difficult to attach an insert containing blunt ends to a plasmid containing sticky ends. The easiest way to see that the ends fit together properly is to use the same restriction endonucleases to cut out the insert and to cut the plasmid. Then we will have correct ends on both pieces.

We now place both pieces of DNA into the same vial and then incubate for a short period. The sticky ends hydrogen-bond (anneal) together & the new piece of DNA is inserted into the plasmid. See the figure 6.

Even though the sticky ends are hydrogen bonded, the covalent phosphodiester bonds are still not formed. So we have to add an enzyme, called ligase which makes new phosphodiester bonds. Ligase is able to form the bond between the 3' and 5' ends of adjacent sugar molecules as shown in figure (6). The product is called a *chimeric DNA* because it contains DNA from two sources.

All this works as outlined in theory, but when we actually do the expt. by placing both the cut plasmid and the DNA insert into the same vial & adding ligase we can have a no. of products. In some cases, our DNA will be inserted as we wanted it to, in others, the plasmid will attach its own ends. Sometimes the new pieces of DNA will attach to themselves.

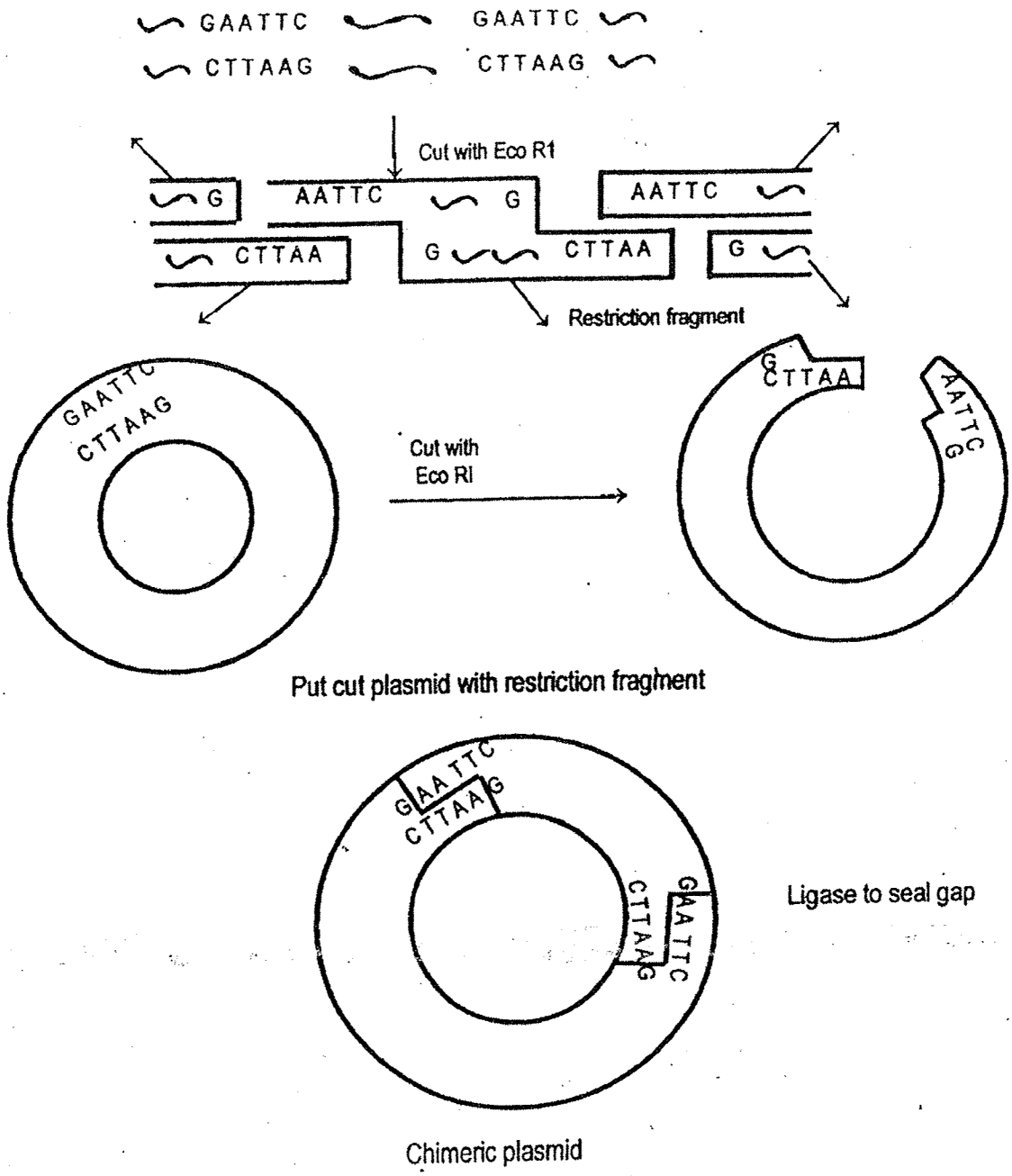


Figure 6 : *Diagram of cutting a plasmid & inserting a new piece of DNA. By cutting plasmid with same restriction enzyme, similar ends are formed in the plasmid that are found in the fragment. By placing both the fragment & cut plasmid into a vial & allow it to mix. New phosphodiester bonds between plasmid & fragment are formed by ligase.*

Initially, we would not know which of the possible end-products have occurred. It could be anything from the original plasmid reformed to a multiple insert placed in the plasmid. It will ultimately be necessary to separate these products, but it is much easier to do it later, after the plasmid have been put into bacteria. For now, we just need to realize that many kinds of products are possible.

If we use bluntend restriction endonucleases for the experiment, the game is much the same, but we do not need to worry about the overlapping ends. However, the ligation of the blunt ends generally takes more time & material & often is less successful.

A final comment on the use of plasmids for use in bringing new DNA into bacteria. Plasmid can only "carry" so much DNA. If they become too large, the bacteria do not use them and the plasmids will not be reproduced properly. So there is a limit to the size of the insert that we can put into a plasmid.

Starting the Engineering Process:-

Engineering the plasmid :-

The two regions of the plasmid are marked Tc^r (tetracycline-resistant) and Ap^r (ampicillin-resistant). Resistance in both cases means that the bacteria will continue to grow in the presence of the antibiotic. For reasons that will become apparent as we proceed, it is important that we cut this plasmid using Pst I, which cuts the plasmid in the Ap^r region. See the figure next.(7)

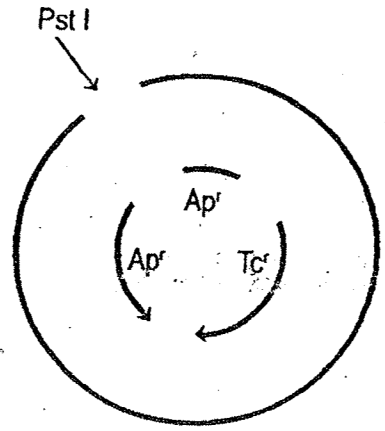


Figure 7 : *The plasmid has now been cut with Pst I. Note that by cutting it with this restriction enzyme, the ampicillin-resistant region (Ap^r) is broken, making the bacterium carrying the broken plasmid sensitive to ampicillin (i.e. it will die if put is contact with the antibiotic ampicillin).*

We will use the opening in the Ap^r region to insert a new piece of DNA that we have obtained either by chemical synthesis or by cutting from another piece of DNA. It is important that Pst I be used to cut the new DNA out of the original host or that the proper "sticky" ends have been synthesized on the synthetic DNA, so that it will stick when placed with this cut plasmid. See next figure.

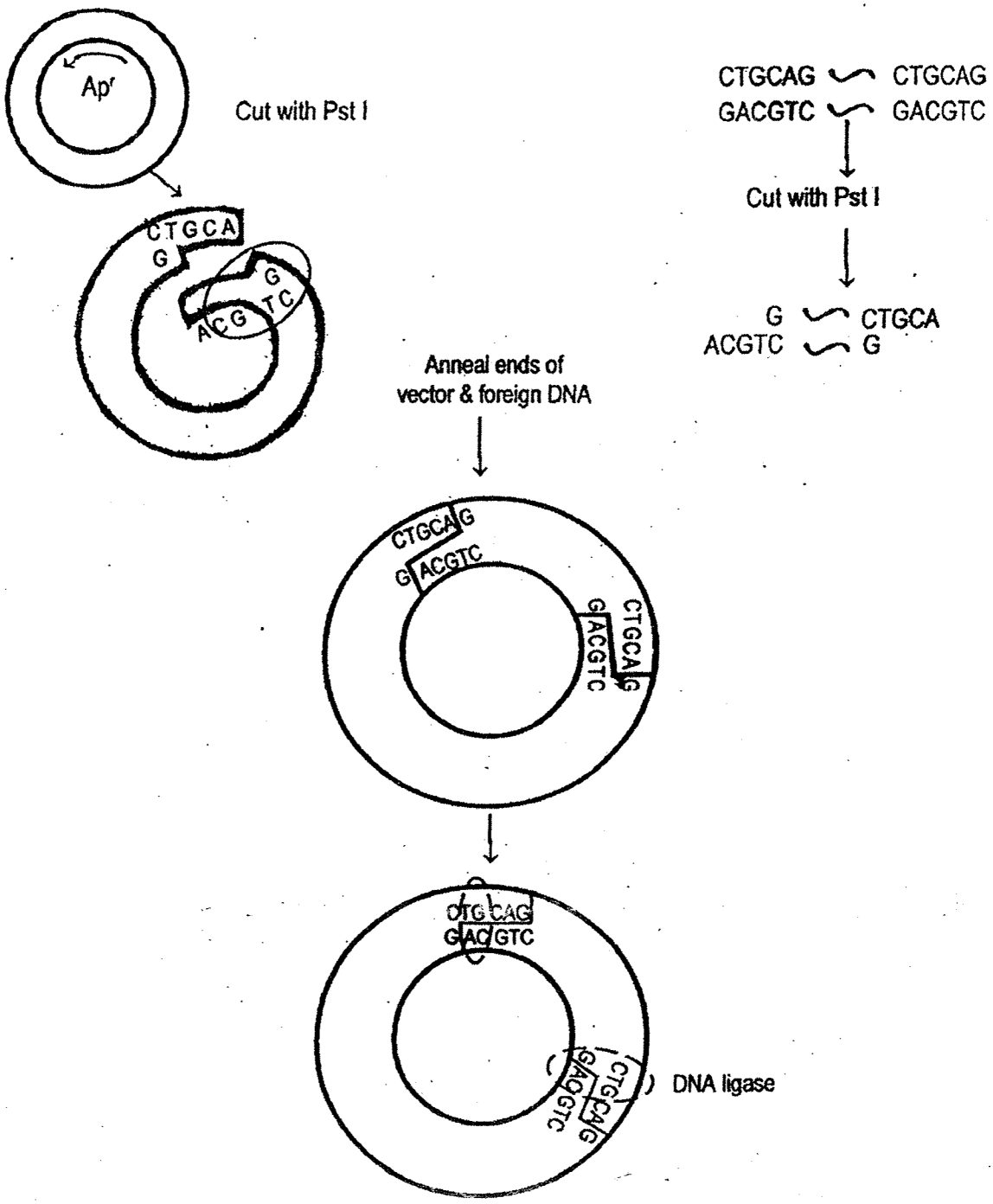


Fig. 8 : *The use of Pst I to insert a fragment into a plasmid. The process is exactly the same as that described in Fig. Except that Pst I is used. The reason we use Pst I is because this restriction enzyme cleaves the plasmid in the Ap' region, which provides a way to identify which bacteria have the modified plasmids present.*

We then take solution containing the cut plasmids and add the new DNA to the solution. We incubate this for a period of 1 to 4 hours at 16°C, so the pieces can find each other. The enzyme ligase is added, which will attach the new DNA insert to the plasmid DNA. The result is a plasmid that has a new insert in it. The insert is bonded to the DNA with phosphodiester bonds. It is now an integral part of the plasmid.

Not all the plasmids will contain the insert, because some just combine with themselves, giving the original plasmid. Others may have multiple copies of the plasmid or multiple inserts. We need to segregate these various forms later.

Getting plasmids into the Bacteria :

The next step is to get the plasmid containing the insert into the bacteria. This can be done in several ways. One method is to place bacteria in a medium containing calcium sulfate. When this is done, the pores in the cell membranes open up, allowing the plasmids to enter the bacteria. Once this is done, all we need to do is let the bacteria grow on a Petri dish & look for the bacteria that contain the new DNA.

Screening the Bacterial colonies :

Plasmids came about in nature to give cells added features, such as resistance to antibiotics. We have chosen a plasmid that contains resistance to both ampicillin and tetracycline. So, with the plasmid containing both the Tc^r and the Ap^r regions, the bacteria would be able to grow on petri dishes with nutrients containing both tetracycline and ampicillin. All bacteria that did not contain the plasmid insert would die.

If the plasmid inserted had been cut in the Ap^r region, as we wanted, it would desrupt the ampicillin resistance. The cell would then be susceptible to ampicillin, giving an Ap^s (ampicillin-susceptible) cell. Then if we grew the bacteria on Petridishes containing some ampicillin in the agar, we would kill all the cells that had our insert in them. This does not do us much good, because we would much rather kill all cells except those that contained the insert. So we need to do something different.

Replica plating :

Initially we grow the bacteria that we have transformed in a medium containing tetracycline. We will spread the bacteria on agar on petri dishes. On incubation, these bacteria grow into colonies.

Because the medium contains tetracycline, all bacteria that do not contain the plasmid will die, because only bacteria containing plasmids with a Tc^r region will live. So we know that all these colonies contain bacteria that contain the plasmid, but it could be either the original plasmid or one that we modified. What we want to do now is make several copies of the colonies on the petri dish. To do this, we take a piece of velvet, cut it into a circle of the size of petri dish & then sterilize the velvet. By placing this sterile velvet on the original petri dish, some of the bacteria stick to it and can be transferred directly to other petri dishes. We can then grow the bacteria on these petri dishes. These new petri dishes will have the same pattern of colonies as the original. This is called *replica plating.*

Suppose we make two replica plates of the original, one on agar containing ampicillin & the other on agar containing tetracycline. By comparing the bacterial colonies, we can see those colonies that were not resistant to ampicillin & thus died. This tells us that the plasmids in those bacterial colonies contained an insert, since the insert destroyed the ampicillin resistance portion of the plasmid & made the bacteria susceptible to ampicillin.

Isolating the strain :

On the other replica plate, all the original colonies are grown, all of which have plasmids inserted & some of which are the modified plasmids. By taking samples of the bacterial colonies that died on the Petri dishes containing ampicillin but lived on the tetracycline agar we have isolated strains of bacteria that contain our insert. This approach to determining which bacteria contain the inset is called *screening* and the antibiotic resistant sections of the plasmid are called *markers.* Fig 9.

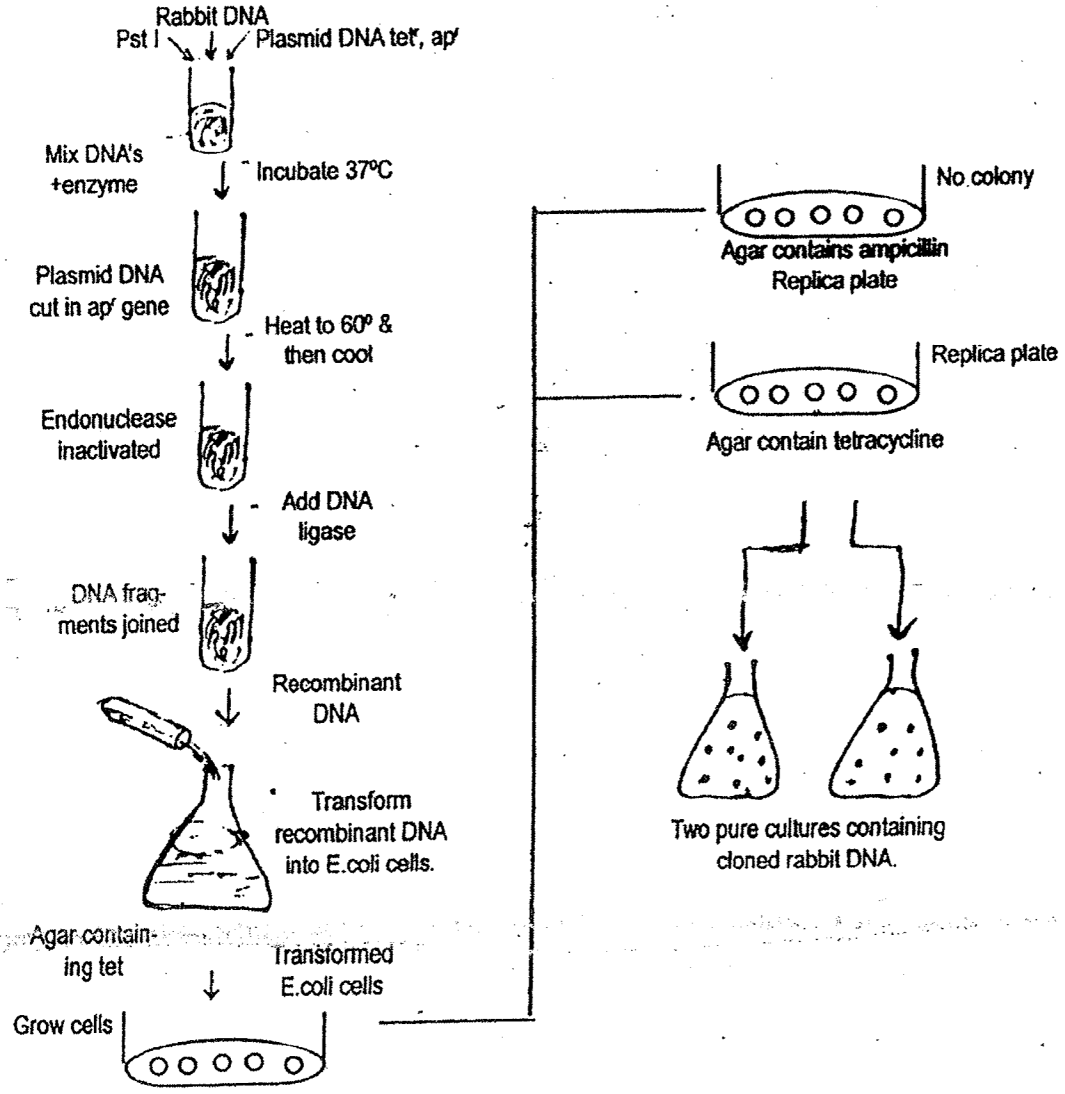


Figure 10 : *Showing an illustration of the complete technique for inserting a piece of foreign DNA into bacteria & screening those bacteria that contain it. In this case, rabbit DNA & plasmid DNA are both cut with Pst I incubated together joined using DNA ligase & the plasmid solution mixed with bacterial cells. To screen for bacteria that contain the plasmids with the rabbit DNA inserted, some of the bacteria are then transferred to petri dishes that contain tetracycline. Only bacteria that contain plasmids will grow on these. Replica plates are made from the petri dishes on agar containing ampicillin & tetracycline. The colonies that die on the ampicillin contain plasmids that have been cut in the Ap^r region, suggesting that these bacteria might contain the new DNA. These colonies are identified by their position & then removed from the other replica plate that contains tetracycline only. The bacteria are then cultured & grown.*

We have now placed engineered DNA into a living organism when we put these bacteria in a culture flask, they will continue to grow & reproduce, replicating the plasmid DNA which hopefully contains DNA insert.

But still there is a question. We know that we have altered the plasmid in Ap^r region, but we are not certain that the insert we want is really there. We need to perform some further tests to make sure the insert is really there.

Screening DNA in bacterial Colonies :

We know that transformation has taken place by using the screening method previously outlined. If we cut a nitrocellulose paper to fit in the petridish & place the paper against the bacterial colonies, we can lift off some of the bacteria from each colony. By heating these bacteria on the paper, the bacteria are lysed & the DNA within them sticks to the paper.

Hybridization :

We now want to find out if our insert is contained within any of the DNA molecules on the paper. This may seem as if we are looking for a needle in a haystack, but the nucleotide sequence complementarily (i.e. the fact that A sticks to T & G to C) of theDNA strands themselves comes to the rescue. Perhaps one of the most powerful tools in genetic engineering is that of *hybridization*. This term merely means that two complementary strands of nucleic acids tend to find each other & hydrogen-bond when they are in the same vial.

Making a Probe :

We first make a short piece of DNA that is complementary to a portion of the DNA that we have insert into the plasmid. See figure 10.

This short piece of complementary DNA is called a *probe* & should be able to hybridize to the portion of any DNA to which it is complementary, provided that the strand of DNA is available for hybridization.

Heating the double-helical DNA on the nitrocellulose paper breaks the H. bonds between strands & opens up DNA in a proper fashion. To be useful in this search, the probe must be labelled, often with a redioactive label such as 32p (radioactive phosphorus). This will allow us to find the probe when we use it to search for DNA.

The minimum probe length needed for hybridization is about 10 nucleotides, but the size can vary, depending on hybridization condition. The longer the probe, the more stable the hybridization. To ensure that the hybridization is unique for the gene for which we are looking, probes of 20-25 nucleotides are generally used.

The nitrocellulose paper containing the lysed colony of bacteria is then put in a plastic bag & a solution containing the redioactively labelled DNA probe is also put in the bag. This probe has a sequence complementary to the sequence of the new DNA insert in the plasmid. The bag is sealed and the solution is

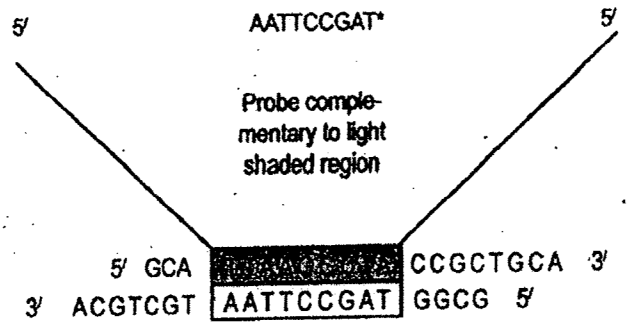


Fig. 10 : showing hybridization. Whenever a new piece of DNA is hydrogen-bonded to a complementary piece of DNA, the product is called a hybrid. Short pieces of DNA can be synthesized chemically with any desired sequence of bases in them. These new, short pieces can be added to existing DNA strands & hybridize with them in those locations in which they are complementary, as shown here. If the existing DNA is in double-stranded form, the new DNA can still hybridize with it, forming a triple strand. Often the short DNA probes are made radioactive to allow identification.

swirled around. The nitrocellulose paper is then removed & the solution is washed off to remove all probes that are not stuck to complementary DNA. The paper is dried & then placed on top of a piece of X-ray film & put in a light-tight container and allowed to stay there for a few hours. The radioactive DNA probes will expose the X-ray film, causing a dark spot on the film wherever they are bound to the DNA of the plasmid insert. Thus the pattern of spots on the X-ray film will reveal which, if any, colonies of bacteria contained the new insert. This is called *colony screening techinque.*

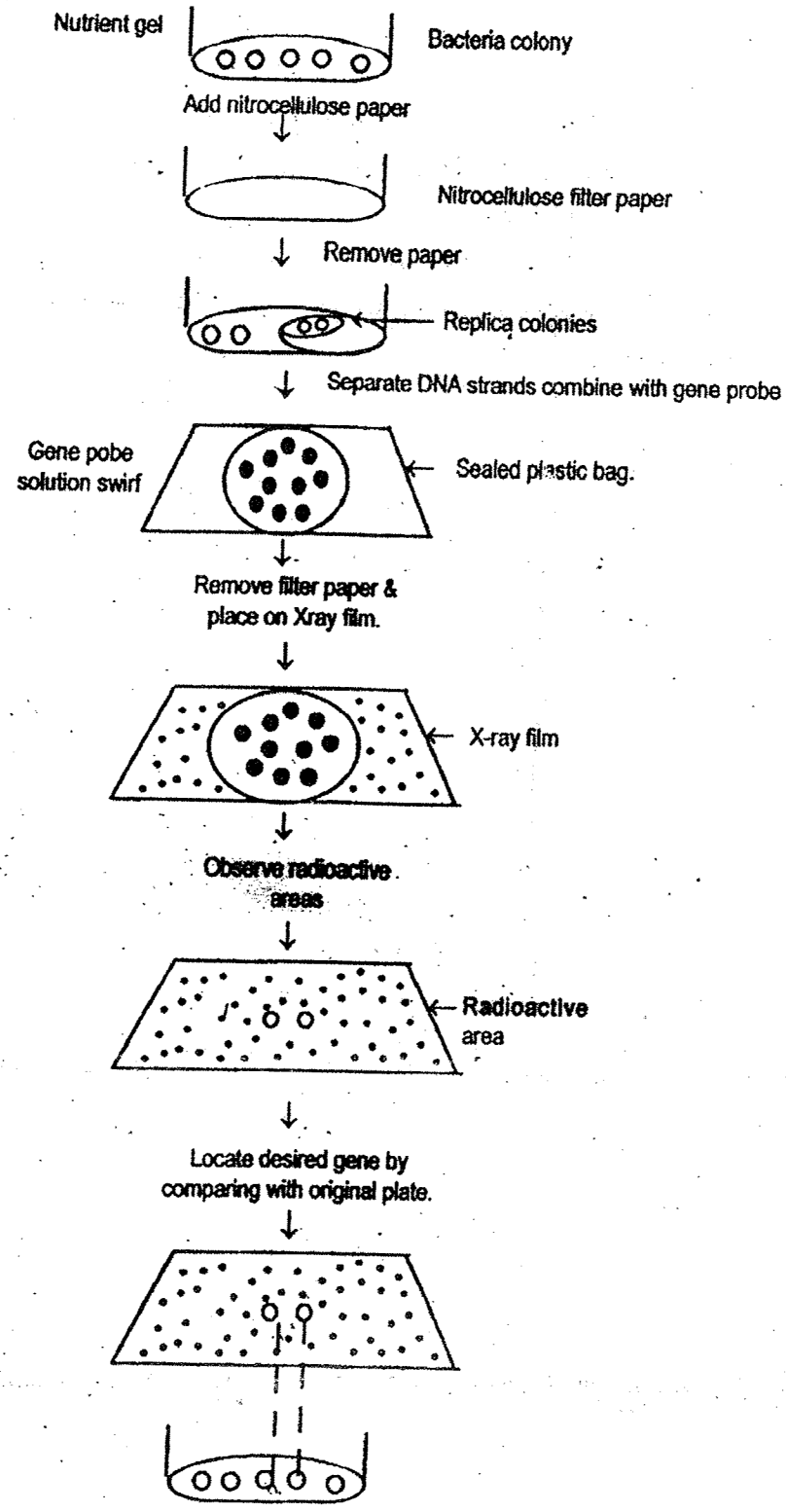

Nutrient gel
Bacteria colony
Add nitrocellulose paper
Nitrocellulose filter paper
Remove paper
Replica colonies
Separate DNA strands combine with gene probe
Gene pobe solution swirf
Sealed plastic bag.
Remove filter paper & place on Xray film.
X-ray film
Observe radioactive areas
Radioactive area
Locate desired gene by comparing with original plate.

Fig. 11 showing the colony screening of techniques. This method is designated to identfy bacteria that contain the new DNA. See previous figure. After the bacterial colonies are grown, nitrocellulose paper is used to lift off some bacteria from each of the colonies, giving a replica of the pattern of colonies on the dish. The nitrocellulose paper is heated to lyse the bacteria & separate the DNA strands. It is then put in a sealed plastic bag along with a solution containing a short piece of redioactively labelled DNA, which is complementary to some portion of the inserted DNA. After mixing for a short time, the nitrocellulose paper to removed & washed & then placed on X-ray film. The film is exposed wherever the radioactive DNA probe has hybridized with new DNA the bacteria. In this way, the colonies of bacteria containing the new DNA are screened & identified.

After the colonies that have the correct insert are identified, these bacteria can be obtained from the original Petri dish and put in culture & substantial numbers of these engineered bacteria grown. Using some of these bacteria, the plasmids could be isolated directly & the sequence of the plasmid determined to certify the absolute presence of the correct insert.

Screening specific DNA fragments (Southern Blotting)

Another way to identify a specific gene in a piece of DNA is to screen the DNA particles directly. Each of the different bacterial colonies can be isolated, grown up & lysed and the DNA in the bacteria extracted. This DNA is then cut, using one or more restriction endonucleases. The fragments are placed in an electrophoresis gel apparatus & separated according to size. Because DNA in the gel itself is not really available for hybridization with a probe, a piece of nitrocellulose paper is placed on the gel & the DNA is "blotted" out of the gel onto the paper.

The paper is then heated to open the double–stranded DNA fragments, after which it is inserted into a plastic bag and swirled with a solution of radioactively labeled DNA probes. These probes are complementary to the piece of DNA that was inserted into the plasmid. Upon washing the paper & exposing the X-ray film, the radioactive label will expose the X-ray film at those sites where it binds to the plasmid fragments containing the new inserts. This techniques is called *Southern blotting*, named after its inventor, Dr. Edward Southern Fig 12.

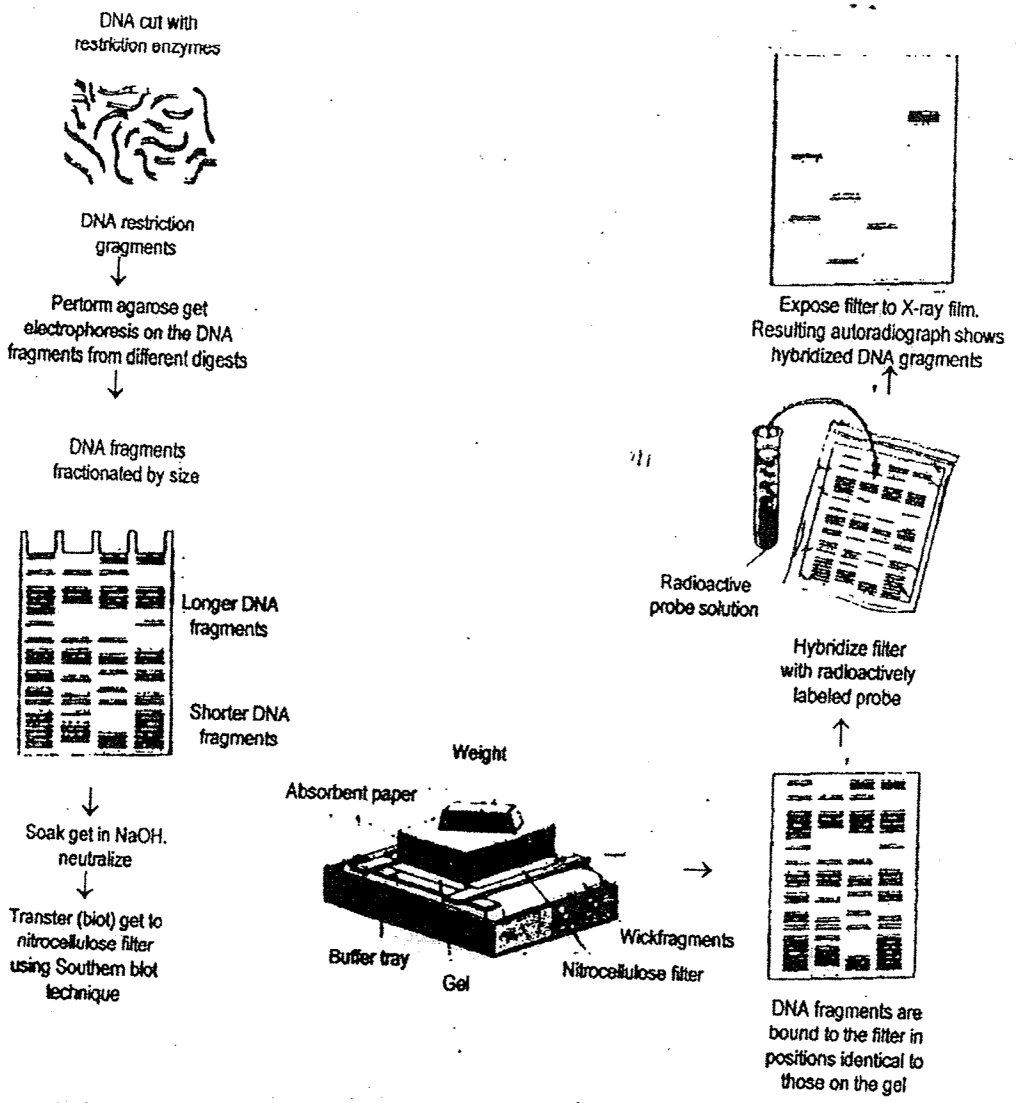


Fig 12 : The Souther blotting technique. This technique is very useful to identify fragments of DNA containing a known sequence of nucleotides. The DNA is initially extracted from a cell and then digested using one or more restriction enzymes. Gel electrophoresis spreads the fragments according to size. The DNA fragments are "pulled" from the gel by placing the gel on a platform with a piece of nitrocellulose filter paper above it. Absorbent paper is placed above the nitrocellulose paper to "pull" the solution and DNA through the gel and to the nitrocellulose paper. The result is a pattern of the bands from the electrophoresis on the nitrocellulose paper. This is now heated and swirled in a bag with the radioactively labeled DNA probe, which is complementary to regions of the DNA fragments. The fragments containing the right sequence can be identified by placing the nitrocellulose paper on a piece of X-ray film and letting the radioactivity expose the film in the bands containing the probe.

Producing proteins from genetically engineered bacteria

One of the major purposes of engineering bacteria is to make large amounts of a specific protein. Identical bacteria that are replicating without conjugation or any other means of altering their genetic information are *clones*. So whenever we take some bacteria & spread them around on a petri dish until there are only single cells left, the colonies that grow from that single cell are clones of the original bacterium. If there is a plasmid with an insert in the original bacterium, we then have clones of a single, engineered bacterium.

Suppose that the DNA we inserted into plasmid is really the gene for a specific protein, such as insulin. Insulin is used to treat diabetes, so lots of insulin is used throughout the world. Although insulin from other sources, such as pigs has been used to treat diabetes, it does not always work well. Human insulin is highly desirable. So suppose that the human insulin gene is inserted into a plasmid & the plasmid is put into bacteria that are cloned. When that gene is translated into protein (expressed), the insulin we wanted will be made by the bacteria as well as the usual bacterial proteins. Multiple copies of the gene may be inserted as well, giving what is called a ***high copy number*** plasmid. This allows numerious identical proteins to be made by the bacteria. They literally become "protein factories". So we can harness bacteria to do our work for us.

In principle, we should be able to take any piece of DNA within reasonable size limits and put it into bacteria. Therefore, if we were to take a gene that makes any protein we want, we could place it in a plasmid and insert that plasmid into bacteria. Thus we should be able to make enormous amounts of that particular protein. Unfortunately it does not always work that way.

There are some proteins, especially those used by humans, that bacteria do not make well. So we need to find other ways to make this process work.

Applications :

Several genetically engineered proteins, notably insulin, interferon & tissue plasminogen activator (TPA) are being made by bacteria & are now sold in large quantities. The quality and quantity of these engineered proteins are high & the cost is low, compared with earlier costs when they had to be extracted from tissue. For instance, the cost of interferon was enormous – about $10 million per gram before it was cloned Now the cost is but a few hundred dollars per gram.

Genetically engineered pharmaceutical product -

Product	Originator	Sales ($ billion)	
		US	World
Hepatitis vaccine	Biogen	260	724
Human growth hormone	Genetech	270	575
	Biotechnology General		
Human Insulin	Genetech	245	625
Erythropoietin	Amgen, Genetics Inst.	600	1125
Tissue plasminogen activator	Genetech	180	230

Ref. Books

1. Genetics : Strickberger 3rd edition.
Genetic Engineering – Walter E. Hill A primer.

CELL FUSION

We know that immediate product of cell fusion (two cells) is a heterokaryon – successor of heterokaryon is hybrid cell. It has been observed that chromosome may be lost from hybrid cell following fusion.

Utilising this phenomenon, we can identify the particular chromosome & impression of genes as well. Thus preferential chromosome loss provides a technique for mapping structural genes to chromosomes.

EXPLANATION OF DIFFERENT TERMS :

Heterokaryon – The immediate product of fusion between two cells is a heterokaryon that contain two parental nuclei.

Hybrid cell – Next step of heterokaryon formation is hybrid cell in which single nucleus contains both the diploid complement.

Stable and Unstable cells – Generally homospecific hybrid cells are stable and with the few exception heterospecific hybrid cells are also stable. Stable means descendent hybrid possesses intact parental chromosome.

Some heterospecific hybrid cells are unstable so that their descendents possess only some of the parental chromosomes.

As chromosomes of one parental genome are lost during fusion, after some generations the resulting cells contain intact set of chromosome from one parent with addition of a few chromosomes from the other.

Prove that "only human chromosomes are lost gradually following fusion" –

d Green (1967) suggested that when human & mouse cells are fused, hybrids which form ucleus in first appearance & possess all the chromosomes of both parents. But after a few of human chromosomes are lost, although the hybrid retain all of the mouse chromosomes.

After 20 generations of growth they already lost many human chromosomes, with from 2-15 remain

eased with the loss of human chromosomes. Hybrids with 10 human the doubling time fell to 25-30 hrs when the human chromosome containing only a single human chromosome doubled in 23 hrs. cells doubled in 16-19 hrs.

This effect may therefore establish selection for hybrids that have lost increased no of human chromosome.

Selective Advantage

Specific human chromosomes appear to be retained more frequently than expected, it is always possible that their presence confers a selective advantage.

Groce, Girardi & Kapowshi (1973) found that human chromosome of SV 40 transformed cells tends to be retained in hybrids with mouse cells. A possible reason is that this chromosome carries the integrated viral genome & may increase the growth rate of the cells carrying it, thus confirming a selective advantage upon them.

An imp. technique to distinguish or identify chromosomes :

Heterospecific fusion in which the chromosomes of one parent are lost can be used to correlate the loss or retention of a particular chromosome with the disappearance & maintenance of a given gene.

A prerequisite for this analysis is the ability to distinguish the chromosome of the two parents & to identify these remaining in any given hybrid.

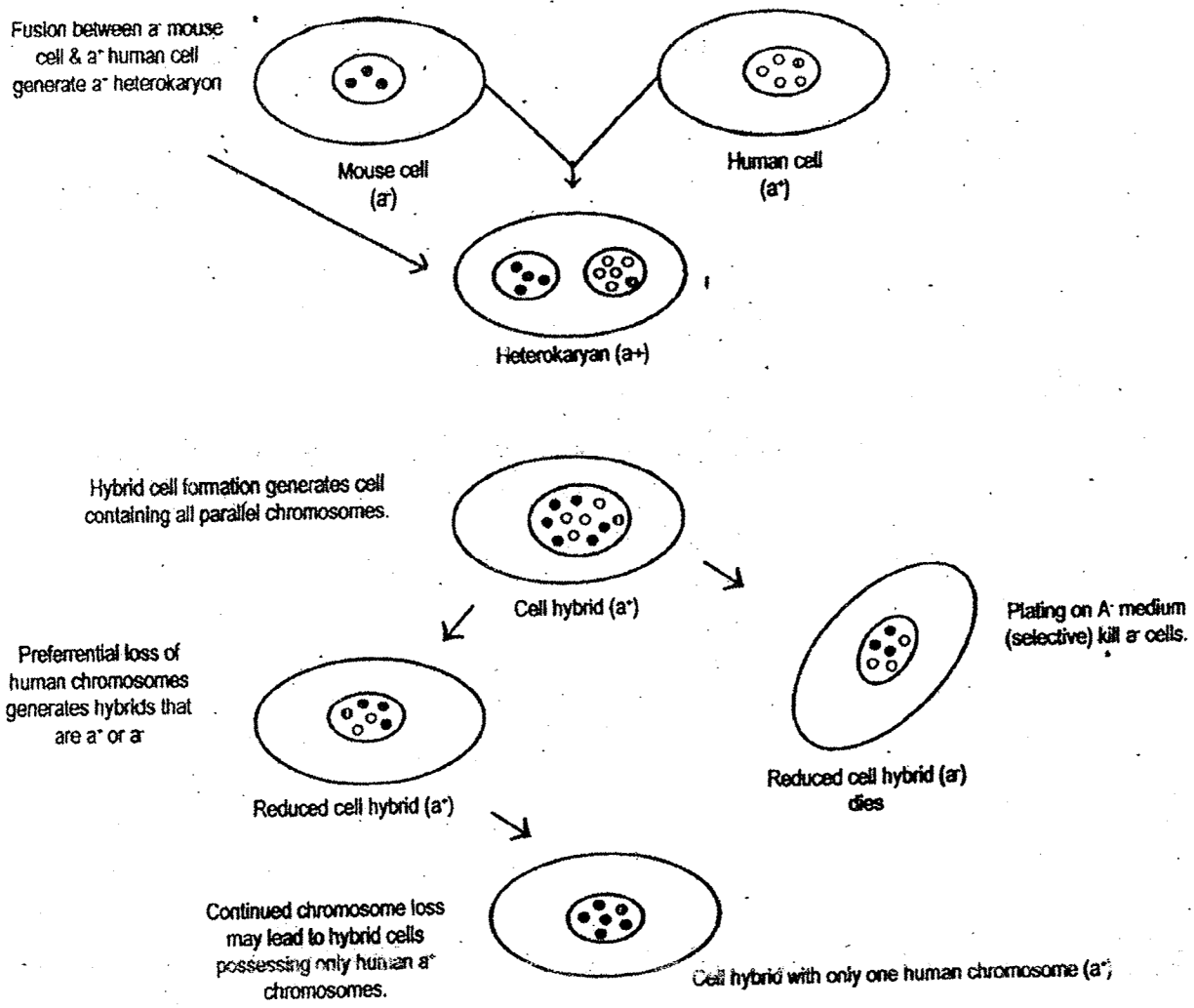
The technique of chromosome banding with reagents such as giemsa make it possible to distinguish all the chromosomes of the mammalian complements.

NOTE : When hybrid cells first began to be used for chromosome mapping. This was difficult because it was necessary to rely upon gross morphological difference.

Genetic mapping in Cell hybrids :

A general procedure – (technique)

1. The principle of the approach is to fuse human cell which possess the gene coding for some function with a rodent cell that lacks the function.
2. Then the hybrid cells are transferred to medium in which they can survive only if they possess this enzyme activity.
3. The chromosome which contain the gene responsible for necessary enzyme activity is carried by the hybrid.
4. As increasing numbers of human chromosomes are lost from the hybrid cells in successive growth cycle, the survivors come to retain only the single human chromosome.



Location of gene coding for human thymidine kinase

Weiss and Green (1967) fused human diploid fibroblasts which synthesize the enzyme with a mouse cell line lacking it.

Human mouse hybrids were grown for 4 days in a standard medium & then transferred to HAT medium in which thymidine kinase activity is essential for growth.

The parental mouse cells die in this medium & human parents grow only rather poorly; hybrid cells that synthesize thymidine kinase grow cell.

In hybrid cells examined after 20 generations, all or nearly all the mouse chromosomes were present, but only a few (2-15) of the human chromosomes remained.

But all the cells grown in HAT medium retained at least one human chromosome of the 'E' group.

Cells which lost the thymidine kinase gene occur with high frequency in each generation.

Chromosomes of the 'E' gr. are rare in the hybrids. These results prove that the thymidine kinase gene is located on the 'E' gr. (See figure 2).

More specific result with the help of banding technique :

Using the fluorescent banding, Miller et al (1971) were able to show that the 'E' gr chromosome retained in the hybrids is no. 17.

Protocol for mapping gene on human chromosome

Fuse a^-b^+ rodent cell with a^+b^- human cell.

↓

Allow heterokaryon to form hybrid cells.

↓

Plate on A^-B^- medium to eliminate both types of parental cell & allow growth only of hybrids.

↓

Allow continued growth to eliminate human chromosome.

↓

Charecterize surviving clones by

a) **Karyotype analysis** to identify common human chromosomes.

b) **Enzyme analysis** to confirm that a^+ character is due to presence of human enzyme & not reversion or suppression of rodent enzyme.

Some valuable Information :

1. During growth on HAT media, only three human enzyme markers HGPRT, G6PDH and PGK are invariably retained; these must all be carried on the same (the X) chromosome.
2. Correlation of enzyme with chromosome – Examining all eight lines for the presence of uridine monophosphate kinase (UMPK) showed an unequivocal correlation with chromosome I.

From the supplied data it can be suggested that WA-11a, AIM-3a, AIM 8a & AIM 11-a do not have UMPK activity & in this chromosome no. 1 is absent. Again JFF, 14b, etc. show enzyme activity (UMPK) and contain chromosome no. 1

From the above findings we can conclude that activity of UMPK enzyme is somehow correlated with the chromosome No. 1

Cell-line	UMPK	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
WA-IIa	-	-	+	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	+
JFa-14b	+	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	+	+	+	-	-	-	-	+
WA-Ia	+	+	+	-	+	-	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+
J10H-12	+	+	-	+	-	-	+	+	-	-	+	+	+	-	+	+	+	+	+	-	-	+	-	+
AIM-3a	-	-	+	-	+	+	-	+	+	-	+	+	+	+	+	-	-	+	+	-	+	-	-	+
AIM-8a	-	-	-	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AIM-11a	-	-	+	+	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+
AIM-23a	+	+	+	+	+	+	-	+	+	-	+	-	+	-	+	+	+	-	+	-	-	+	+	+

Fig. 3A *hybrid clone panel that allows mapping of enzymes to individual chromosome.*

All cell lines retained the chromosome indicated by (+) with a frequency of 5% or more each chromosome is present in a unique subset of the clones. Examining all 8 lines for the syntentic presence of Uridine Monophosphate kinase (UMPK) for example showed an unequivocal correlation with chromosome 1.

Syntentic genes :

Sometime two or more genes else on the same homologue or chromosome are said to be syntentic genes.

Mapping of the human X-chromosome by fragmentation

Principle :

1. This general technique relies upon the fragmentation and rearrangement of chromosomes that is induced by gamma irradiation.

2. Gross and Harris argument (1975, 77)

   They argued that the probability that two loci will be separated by a radiation – induced break should be proportional to their distance apart.

General technique :

a. How distances between diff. markers is worked out ?

   i) Human cells were irradiated & fused with chinese hamstor Wgzh cells lack HGPRT.

   ii) Growth on HAT medium to identify the human enzyme.

   iii) Other sex linked unselected marker such as G6PDH, PGK & α-galactosidase can be identified.

iv) The above three unselected markers are linked to the selected HGPRT.

v) In controls, in which the human parent was not irradiated, 95% of the hybrids containing HGPRT contained all three of the unselected markers.

Remark : The above findings prove that the 95% of the hybrids contain intact human X-chromosome.

vi) After irradiation there is a decline in the frequency with which three unselected marker are found in the hybrid surviving on HAT medium.

vii) As the maximum level of irradiation(Krad), G6PDH was retained in 42% of the clones – that means surviving hybrids of 42% contain two genes HGPRT and G6PDH, α-galactosite was present in 34% & PGK 22%

viii) We know that frequency of co-survival as for e.g. frequency of HGPRT and G6PDH is inversely proportional to the distance of unselected markers to the selected marker (HGPRT).

ix) Thus G6PDH should be closest to the GHPRT, α-galactosidase somewhat further and PGK the most distance locus.

Remark : The abovesaid technique helps us to determine the distance between diff. markers.

b) Another technique for the relative distance between loci -

If a single hit is sufficient to separate the loci & events fall on a poisson distribution, the probability of no break is

$$P_0 = e^{-t}.f.(D) \ldots\ldots (1)$$

Where 't' is the size of the target & f (D) represents the appropriate function of radiation dose. We know that probability of no break = frequency of co-suvival (F)

$$\text{So } F = e^{-t} f(D) \ldots\ldots (2)$$

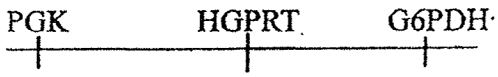
The nature of f(D) can be assigned only empirically. Here value of t is taken as 1,6. (As a dose eponent between 1 & 2).

From the above formula we can calculate the value of t, which corresponds to the distance between loci

gives $t = \frac{-\ln F}{D^{1.6}}$ As $F = e^{-t}D^{1.6}$ .....(3)

ORDER OF FOUR LOCI

i) Experiments have been suggested that if two unselected loci lie on opposite sides of the selected marker [illegible] ly.



As for e.g.

ii) On the other hand, if two unselected markers are present on the same side of HGPRT then they should not segregate independently or there will deviation of obs. frequency from exp. value.

Date – Selected marker	**Exp. frequency**		**Obs. Freq.**		**Obs./Exp.**
PGK^+			0.22		
$G6PDH^+$			0.42		
α-gal^+			0.34		
PGK^+ $G6PDH^+$	.22x.42 =.09	.54	.13	.62	1.15
PGK^-G6PDH^-	.78x.58 =.45		.49		
PGK^+G6PDH^-	.22x.58=.13	.46	.09	.38	0.83
PGK^-G6PDH^+	.78x.42 =.33		.29		
PGK^+ α-GAL^+	.22x.34 =.07	.58	.16	.76	1.31
$PGK^-\alpha$ gal^-	.78x.66 =.5		.6		
PGK^+ α gal^-	.22x.66=.15	.42	.06	.29	0.57
$PGK^-\alpha$ gal^+	.78x.34=.27		.18		

Fig. *Mapping X-linked loci by chromosome fragmentation.*

Conclusion :

i) Observed frequency of PGK & G6PDH is 0.62 & their exp. frequency is 0.54. Here little or no deviation exist between expected & observed frequency.

Here value of obs/Exp. frequency is 1.15 (near 1) which confers that the two unselected markers degregated independently.

Remarks : As the above two unselected markers segregated independently so PGK and G6PDH lie on the opp. side of HGPRT.

ii) Data signifies that PGK and α-galactosidase show related segregation. Because a great deviation exist between the obs. frequency of PGK and α-gal.

Remarks : Value of obs/exp. frequency greater or lesser than 1 as for e.g. 1:31 and 0.57. So PGK & α-gal must be on the same side of HGPRT with α-galactosidase constituting the central marker since it is closer to the GHPRT & G6PDH must lie on the opp. side.

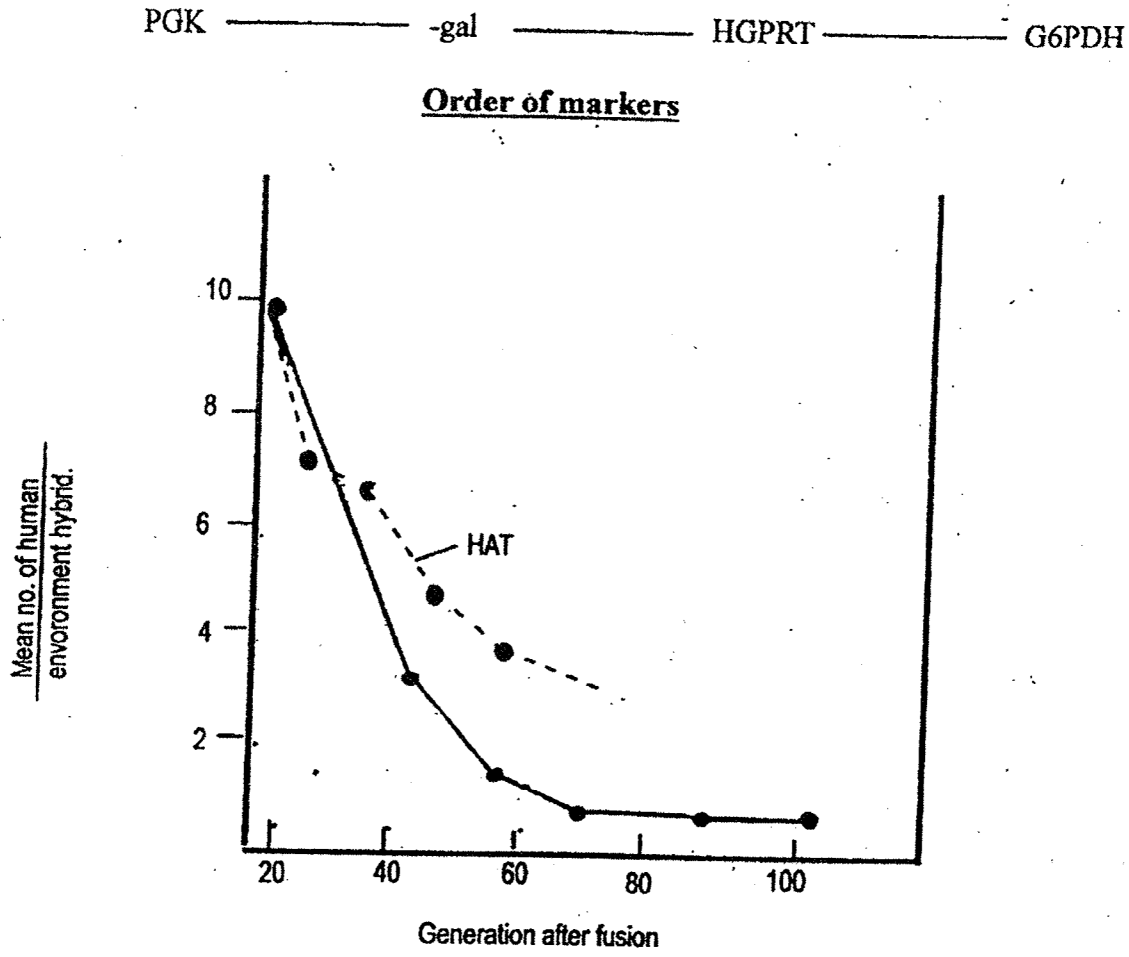


Fig. 2 : *Loss of human chromosome from human-mouse hybrid cells. On standard medium (ST) all human chromosomes may be lost over 80 generations. On HAT medium, retention of the human gene for thymidine kinase is necessary for survival, after 80 generations only 2- 3 human chromosomes are present.*

After 18 genes, when the cells were first examined, this population showed an average of 10.2 human chromosomes per hybrid cell. The human chromosomes continue to be lost & by 80 generations only 3 may be present. The value may fall to zero when the cells are grown on ST medium so that selective pressure for cells that retain the human thymidine kinase gene is removed. Cells which lose the thymidine kinase gene occur with high frequency in each generation. Although they die in HAT medium, they can be selected by growth in medium containing BudR, when the presence of thymidine kinase becomes lethal. Chromosomes of the E group are rare in these hybrids. These results therefore suggest that the thymidine kinase gene is located on one of the chromosomes constituting this group.

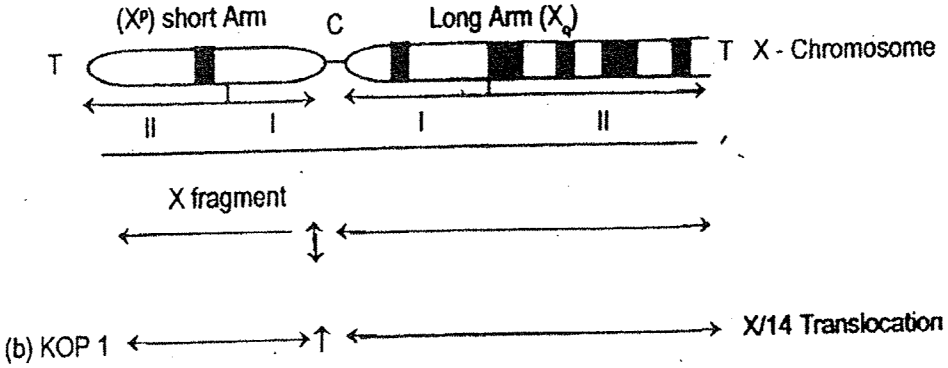


Figure 3 : *Cells with a translocation between theX-chromosome & chromosome 14 have been obtained from a patient with a balanced exchange. X-chr. is broken in its long arm, close to centromere, with the short arm fragment forming an independent Xp chr. & with the long arm becoming attached to the end of chr. 14 to generate the large chr. T (14q, X p). These cells (KOP-1) possess one copy each of Chr. 14, a normal X-chr., the Xp short chr., & the t(14q, Xq) tanslocation.*

I. In hybrid, between the human KOP-1 cells & mouse cells, the Xp and t (14q, Xq) chromosomes may be presumed to segregate independently. In two independent series of experiments to select for HGPRT by growth on HAT medium, Grzeschik etal & Ruddle (1973 a b) formed that PGK and G6PDH almost always also are retained. In the first set of expts, all 3 X-linked markers were retained in 36 of 37 human-mouse hybrid clones and 22 of 24 human-hamster hybrid clones, in the second series, all 3 markers retained linked in 19 of 20 primary human-mouse hybrid clones & in 64 of 66 secondary clones derived from them. This immediately suggests that all three genes are carried by the same X-chromosomes fragment, i.e. either all lie on Xp or all lie t (14q, Xq). Ricciuti and Ruddle showed that in these clones, the X-linked genes also are linked to the autosomal gene for nucleoside phosphorylase. This suggests that nucleoside phosphorylase is coded by chr. 14 and that PGK, HGPRT and G6PDH lie on the long arm of theX-chr. that is part of t (14q, Xq).

II. The separation of either PGK or G6PDH from HGPRT must be caused by the occurrence of a chromosome break or translocation in the hybrid cells. In the expts. Of Grzeschik et al, the aberrant clones always represented the segregation of PGK from HGPRT and G6PDH, which remained linked together. Coupled with karyotypic analysis of the chromosomes remaining in these clones,

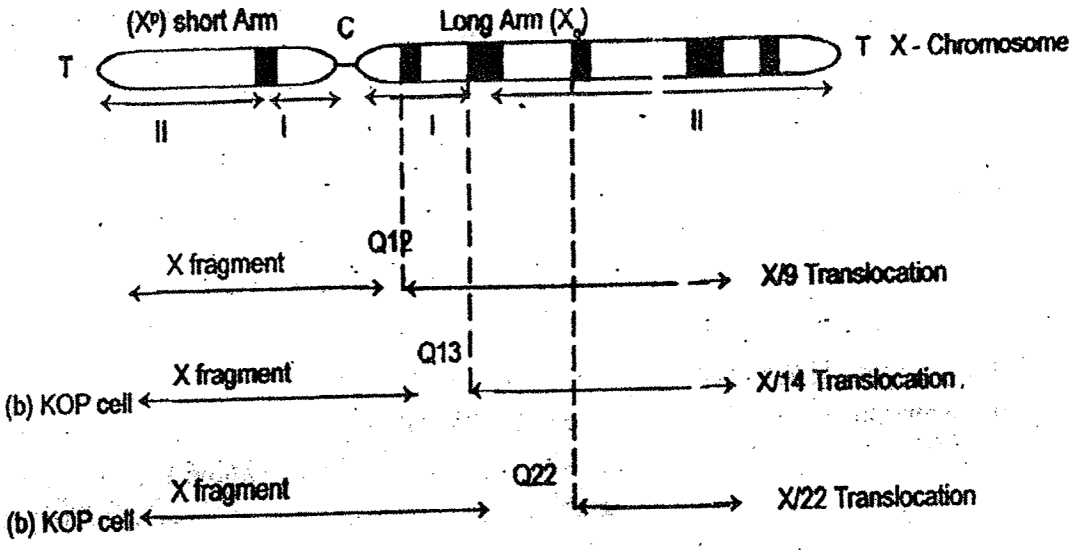
this led to the suggestion the HGPRT & G6PDH lie on the short arm of theX-chr. while PGK lies on the long arm. But the idea that all 3 loci lie on the long X-arm fits better with the overwhelming preponderance of clones in which they show common inheritance. The segregation only of PGK from HGPRT & G6PDH, observed also to be the aberrant form in the expts. of Ricciuti & Ruddle is better explained by supposing that PGK is more distant from HGPRT than is G6PDH. This is supported by their karyotype analysis to show an association between the presence of t(14q, Xq) and 3-x-linked markers and nucleoside phosphorylase.

III. In the two exceptional clones in which PGK was segregates from HGPRT and G6PDH, nucleotide phosphorylase was present in the PGK^+ $HGPRT^-$ $G6PDH^-$ clone and absent from the PGK^- $HGPRT^+$ $G6PDH^+$ clone. This suggests that PGK lies closest to the nucleoside phosphorylase gene; & since this lies on chr. 14, PGK must lie closest to the site of the break on the long arm of the X chr. i.e closest to the centromere. From these data, all that can be said about the location of G6PDH is that it must lie sufficiently close to HGPRT so that breaks occur much more frequently to separate PGK from HGPRT. Contrary to some suggestion these data do not distinguish on which side of HGPRT the G6PDH locus is to be found.

IV. The properties of two further translocations allow the positions of these genes to be defined more closely. One involves the translocation onto chr. 9 of the long arm of the X chr. from bandk q^{12} to the terminus; in the other, the region of the long arm from bandk Q^{22} to the terminous is translocated onto chr. 22. Shows and Brown (1975) used cell lines carrying these translocations as the human partners for fusion with RAG or A9 rodent cells. Human HGPRT was selected for or against by growing the hybrids on HAT. Medium. When the active sex linked genes were provided in the form of the X/9 translocation, all 3 of the enzymes HGPRT, PGK & G6PDH were retained or lost together. With the X/22 translocation, HGPRT & G6PDH always segregated together, while the segregation of PGK was independent. In both cases, the translocated chr. Carrying the long arm of the X-chr. was associated with HGPRT activity.

As summariged in Fig. this suggests that the genes for HGPRT & G6PDH lie between bandk Q^{22} and the terminus of the long arms of the X-chr. while PGK must lie between bands Q^{12} and Q^{22} since it is present on the X/9 translocation (carrying the region from $Q^{12\text{-ter}}$ and on the t(14Q, XQ) translocation (carrying $^{13\text{-ter}}$); but is absent from the X/22 translocation (carrying only $^{22\text{-ter}}$). These locations are consistent with the order suggested by chr. fragmentation mapping.

X-chromosome mapping by translocation



t =(14Q XQ)

KOP 1 Cell = Chr. 14; X chr, Xp, t (14Q XQ)

Expt 1 : 1) Human KOP 1 x mouse cell.
Ref. 1 - Grazeschik et al. Ruddle'73

Fig. 4

Ref. Book Gene Expression - Lewin Vol. I, II & III.

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION
MIDNAPORE - 721 102

M.Sc. in Zoology

Part-I
Group-B Paper-II Unit-I
Module No. - 19

FIXATION:

Fixation is a chemical process by which tissue organ or organic material is stored in such a manner by which antolysis or breakdown of material or sensitive to microbial action is prevented as well as tissue is rendered to certain suitable reaction.

The fixation process should perserved the str. of the cells in as near a life like condition as possible and prepared them for subsequent treatment. The first requirement for preservation of protoplasmic structure is to interrupt the dynamic processes of the cell as promptly as possible and to fix structure with a minimum change. In effect we wish to render or maintain as much as possible of the structure in an insoluble from with a minimum distortions.

One of the most important effects of fixation is the coagulation of tissue proteins and constituents, thus minimizing their loss or diffusion during tissue proceessing.

PURPOSE OF FIXATION:

1. To provent autolysis and bacterial decomposition.
2. To coagulate the tissue as to prevent loss of easily diffusible substances.
3. To fortify the tissue against the deleterious effects of the various stages in the preparation of sections.
4. To leave the tissues in a condition which facilitates differential staining with dyes and other reagents. An idea fixative should have the ability to;
   i) penetrate the material rapidly,
   ii) kill the cell and prevent post mortem disintegration,
   iii) preserve the physical structure of the cell,

iv) convert the cell contents into stable compounds which will not be destroyed by subsequent processing,

v) harden the material without making it brittle, so that, it will withstand handling and subdivision,

vi) increase the refractive index of the material differently, so that, the differences among both cellular and tissue constituents will be enhanced,

vii) Render the material receptive to strain.

Classification of fixative:

Most of the fixatives are aqueous solution. According to reaction with soluble proteins fixatives are grouped into two types.

(A) COAGULANT FIXATIVE:

They cause the protein material to separate itself from water, in the form of an opaque clot. Example - Ethanol, Picric acid, Marcuric Chloride, Chromium trioxide etc.

The fixatives usually transform cytoplasm and nuclear sap into a microscopic-sponge-work of protein with empty spaces between the strands. This fascilitates the entry of paraffin and other embedding media and also of dyes but destroy very fine structures. For this reason, coagulant fixatives are usually avoided in studying the electron microscope.

(B) NON-COAGULANT FIXATIVE:

They do not coagulate the solution of egg albumin (protein).

Example - Formaldehyde, Osmium tetraoxide, Potassium dichromate and acetic acid.

Formaldehyde and Osmium tetraoxide are more important fixative. Osmium tetraoxide gives faithful stabilization of delecate cell constituents.

Non-coagulant fixatives leave the tissue in a state in which paraffin can rarely entire.

The fixatives are also classified on another basis:

I. ADDITIVE FIXATIVE:

Add themselves in whole or in part to the paraffin in which they fix. Eg.- Formalin. Certain atoms of the fixative combine chemically with some part of the protein and remain in combination. In additive fixation a part or the whole of the fixative molecule adds itself to the substance that it fixes by making ionic or covalent links.

In the fixation of proteins, the link is usually with the side-groups of one or more particular amino-acids. Additive fixation is sometimes coagulant.

An additive fixative need not necessarily alter the reactions of a protein very profoundly. For instance, it might act only on tyrosine side groups, most of the amino-acids might be able to retain their character and show their usual responses to reagents.

II. NON-ADDITIVE FIXATIVE:

It impacts its effects without making a chemical compound with protein. Eg.: Ethanol. The nature of the protein is, however, profoundly changed by its action, and the substance is therefore said to be denatured. The most obvious change is the loss of solubility with resultant coagulation. When a denaturing fixative is added to a solution of albumin coagulation follows so quickly that it appears to be instantaneous.

Mode of Action of Fixative:

According to modern concept of protoplasm the elongated protein molecules are held together at different points giving the protoplasm are structural continuity and elasticity. The molecules contains many different active group, at the surface and side chains. Some of the active groups are bound with other protein chains, while many are surrounded by bound water.

Fixation is known to be the denaturation of the protein. It is generally assume that denaturation is due to the unfolding of globular protein through disruption of some forces which hold the chain closely folded and subsequent formation of new links between molecules with resulting coagulation. Active groups which are not available for reaction in the folded molecule become available for reaction in the folded molecule become available for reaction in the folder molecule become available for cross linkage and also for reaction with the compound in denaturing steps.

Some Examples:

A. Formaldehyde (HCHO):

It is very soluble in water to a maximum extent of 40% by weight and is sold as such under the name formalin. It contains 10-14% added methanol as a stabilizer.

It is virtually without effect on Carbohydrates although preserving glycogen when this is hold by fixed proteins. Lipids are generally will preserved.

Formalin favours the staining of acidic - structures with basic dye.

The reaction of formaldehyde (HCHO) with tissue protein are numerous and complex. It can bind with a member of different functional groups of the protein and form a bridge – like structure between the two protein molecules forming hydroxymethyle compound (methylol).

Pearese (1980)

$$RH + HCHO \rightleftharpoons R.CH_2(OH)$$

According to ***French*** (1945) the compound is reactive and condences with further H atoms to form a methyline bridge $(-CH_2-)$

$$R-HCHOH + HR' \rightleftharpoons R-HCH-R' + H_2O$$

Later by Kallen and Jenks - 1960 an alternative possibility was suggested. They found the product of the condensation of aquous formaldehyde (HCHO) as methlin glycol with an aliphatic primary amine was a dehydroxy methyle derivatives formed by he addition of a second molecule of formaldehyde to the initially formed hydroxymethyle.

$$RNH_2 + CH_2(OH)_2 \rightleftharpoons \underset{\text{Hydroxymethyle derivatives}}{.R.NH.CH_2 + H_2O}$$

$$RNH_2 + CH_2(OH) + CH_2O \rightleftharpoons RN(CH_2OH)CH_2OH$$

$$RNACH_2OH \rightleftharpoons RNCH + H_2O \text{ (slow rate)}$$

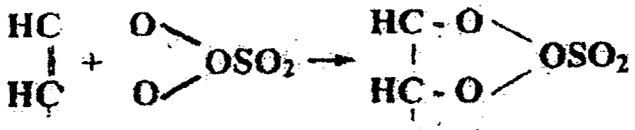
B. Osmium Tetraoxide (OSO_4)

Commonly referred as to as 'Osmic acid', like formalin, it forms additive compounds with protein. It is soluble in water at about 7%.

On solution, it takes up a molecule of water to become H_2OSO_5

Reaction with Protein

The exact site of attachment of to proteins is not known. It is capable, in certain circumstances of simultaneous reaction at both ends of double bond present in many substances.



Action of the amino acid tryptophan and histidine react strongly, forming dark precipitates.

C. Ethanol (C_2H_5OH)

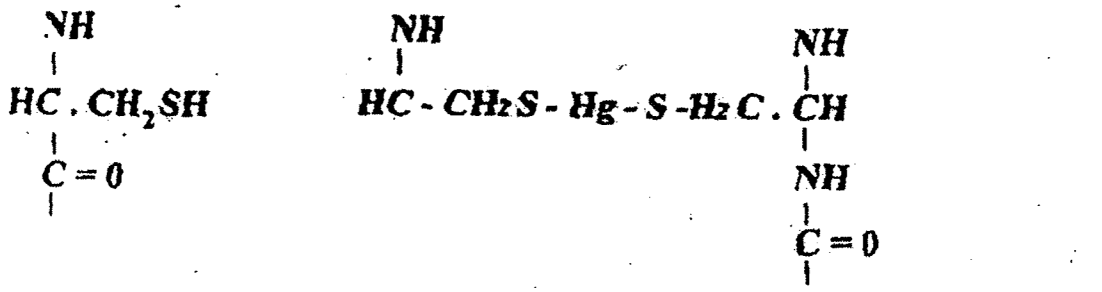
A non-additive or denaturing coagulant of many proteins. Ethanol is a powerful unmasking agent for lipids; that is to say, it sets them free from combination with protein, with nuclic acid.

It precipitates but not fix with lipids. 70% - 100% ethanol is used alone or mixture for the fixation of nucleus along with certain basic dyes. It does not precipited protein but dissolve lipids. It makes the cell hard but cause shrinkage.

D. Mercuric Chloride ($Hgcl_2$)

Soluble in water at about 7%, readily soluble in ethanol, benzene.

An additive, coagulant fixative, very poisonous. The reactions depends on the pH at where the fixative is used. $Hgcl_2$ is generally used at a pH below the isoelectric point of proteins and consequently the $-NH_2$ groups of their basic amino acids will mostly be ionized as NH_3. There will therefore be electrostatic attractive between this positively charged group and the negatively charged ion [$Hgcl_4$] = The mercury thus adds itself to the protein in the form of an ion.



Cysteube as a part of protein chain.

mercuty formins a link between cysteine side groups in two protein chains.

E. Bouins fixative:

It is a microanatomical fluid.

(a) Alcoholic bouins fixative

Composition:

(i) Picric acid (saturated in 70% alcohol) - 75 ml.

(ii) Formalin (40% formaldehyde) - 25 ml.

(iii) Acetic acid (glacial) - 05 ml.

The fixation time varies from tissues i.e., for overnight or 12 hours to several days.

Advantages:

Penetrates rapidly, staining very good, excellent fixative for animal tissue and glycogen.

Disadvantages:

Some tissue become brittle if kept in the fixative beyond 24 hrs. causing difficulty in cutting. It left beyond 3 days nucleii fail to stain properly in alum haematoxylin.

F. Carnoy's fixative:

It is a Cytological fixative

Composition:

(i) Absolute alcohol - 60 ml.

(ii) Chloroform - 30 ml.

(iii) Glacial acetic acid - 10 ml.

Advantages:

Rapid fixation, preserves glycogen, nuclear staining is greatly improved. Dehydrates as well as fixes.

Disadvantages:

Causes excessive shrinkage, destroyes red cells; small pieces of tissue only should be used for rapid fixation.

G. Susa's fixative:

Composition:

(a) Solution A:

(i) Mercuric Chloride - 4.5 gm.

(ii) Sodium Chloride - 0.5 gm.

(iii) Distilled Water - 80 gm.

(b) Solution B :

(i) Trichloro acetic acid - 2 gm

(ii) Formalin - 20 ml

(iii) Glacial acetic acid - 4 ml

These solution 'A' and 'B' mixed together before it is used. Fixatino is usually carried for an 12 hours. followed by washing 9n 90% alcohol. Fix tissue should be treated with iodine.

STAIN:

Stains are defined as colourful substance which have ability to impart their own saturation in the tissue. Most of them are aromatic substances, technically known as dye.

Principle of staining:

I. Change the refractive indices of the different organs of a cell to make visible under microscope.

II. To identify the localised certain differential chemical constituents of these cells.

These two basic requirement in the study of histology is fulfilled by the use of dye.

Definition of dye:

According to Barker 1969 for the purpose of microtechnique, dyes may be defined as "they are aromatic, salt like, crystalline solids, that dissolve in water or any aqueous solution in the form of ions either the cations (+ve) or anions (–ve) or both. The ions can link themselves chemically with proteins when linkage takes place.

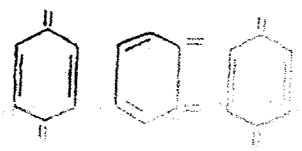
In other words, dye is an organic compound which containing as integral part - the ***Chromophore*** (colouring part) and a linging part that is ***auxochrome*** so dye is an auxochromophoric system.

Types of chromophore:

Depending upon the chemical structure the chromophore are divided into the following types.

1. **Quinoid chromosphore:**

Such chromosphore have a quinoid ring which is unsaturated because rings with double bonds (=) they can exist in two forms Quinoid A and Quinoid B.



Quinoid A

Quinoid B

There two structure are basically same but relative orientation of double bond (=) along the ring are different in position. It is important in histology, the chromophore containing quinoid ring are most common and show different colour variation due to such orientation.

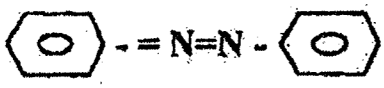
There are about 200 quinoid dyes in histology.

Example: Haematoxylin, Eosin, Methylin blue, basic fuchsin, light green, crystal violet etc.

2) Azo Chromophore:

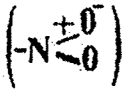
Such type of Chromophore have no quinoid ring, the nitrogen atoms link the two ring together (–N=N–). These Chromophore commonly used in textile industry & there are about 20 types of azo chromophore.

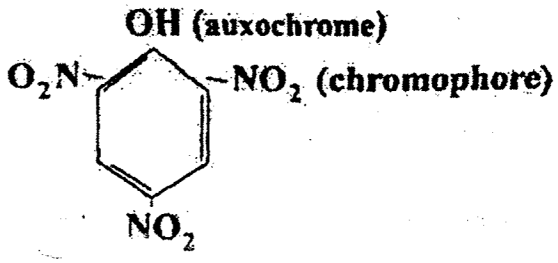
Example : Orange G, Genus green.

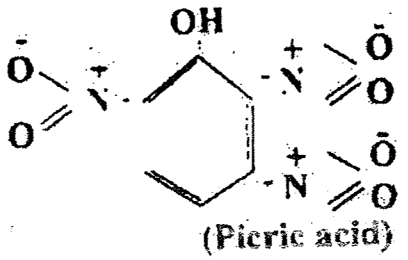


3) Nitro Chromophore:

Such type of Chromophore contain effective radical as $-NO_2$. It is particularly found only one dye, i.e., Picric acid which has 3 $-NO_2$ groups and an –OH group. It is primarily a dye but secondarily a fixative (non-additive).







Dey Diversity:

Basically there are 3 main types of Chromophores in 3 different types of dye but they produce different colours which is much considerable in number than the basic types - this is known as dye diversity. This may be due to the following reasons.

a. Resonence:

Change of intramolecular configuration with the changing effective radicals in a unsaturated conjugated molecule to form a new one with different colour responses in known as resonence. Due to resonence a single dye can exhibit several centigaration as well as several bathcromic property due to special shift.

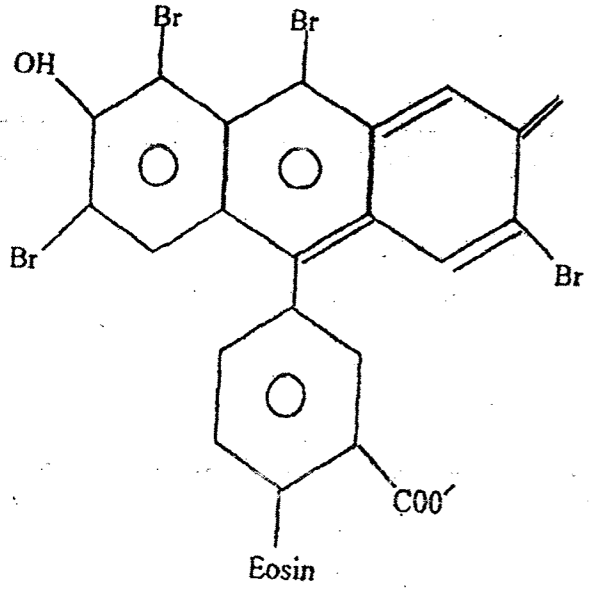
b. Number and types of Chromophore:

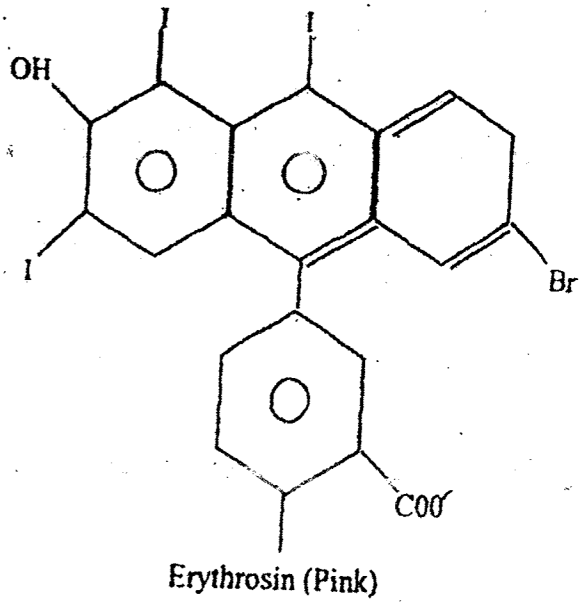
Colour diversity depends upon the nature and number of Chromophore present in dye. If introduced more than one Chromophore into dye molecule it causes dye-diversity. The colour of a dye changes in relation to the No. of Chromophore so change the No. of Chromophore causes special shift.

c. Substitution of radicals:

Generally in organic compound the halogens can be easily substituted by another with the change of radicals which leads to spectral change in terms of colouration.

Example:





When the 4 Bromin groups are persent in eosin then it shows a particular colour but when Br. group is substituted by the Iodin, then the bathocromy of eosin is changed and spectral shift occurs and subsequently a new dye known as Erythrosin involved.

d. Addition of Radicals:

Addition of extra molecule in the str. of dye molecule cause the change of bathocromy, subsequently changes the colouration.

AUXOCHROME:

The auxochrome is responsible for the attachment of Chromophore with the substrate. Chemically these are the ions and such ions portion form bond with the substrate and totally Chromophore clinged with the substrate by these auxochrome.

On the basis of ion auxochrome are two types.

a) Cationic auxochrome:

The auxochrome with a net positive charge is called cationic auxochrome. The +ve charge are responsible for basic characteristic of the dye. The basic auxochrome are amine which ionising thus -

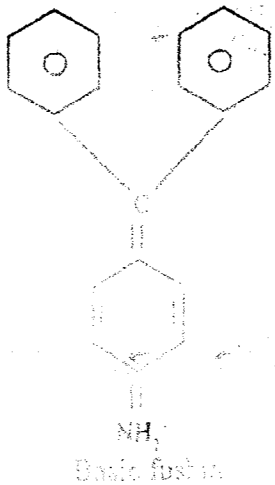
Primary amine - $NH_2 - H \rightleftharpoons -NH_3$

Secondary amine - $\geq NH + H \rightleftharpoons \geq NH_2^-$

Tertiary amine - $\geq NH + H \rightleftharpoons \geq NH^-$

Quarternary amine N exist only in ionised form.

Example:



b) Anionic auxochrome:

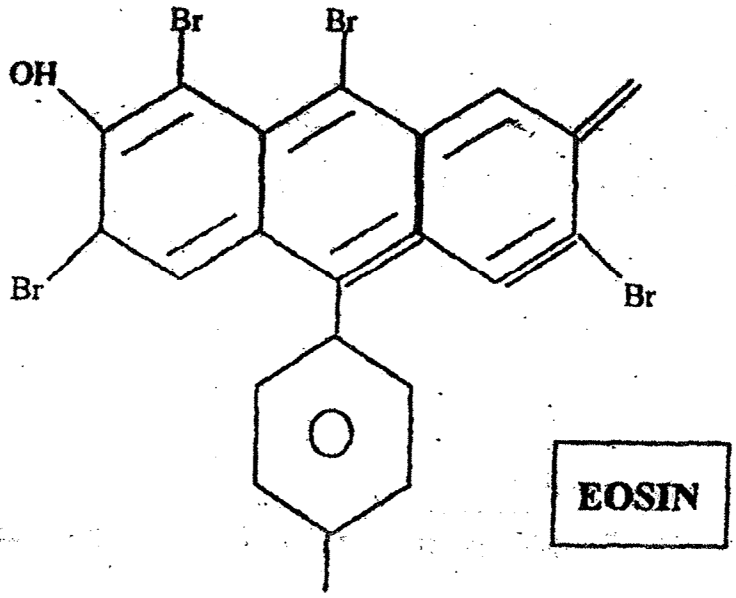
The auxochrome with a net negative charge (-ve) is called anionic auxochrome, the -ve charge are responsible for acedic characteristic of the dye. The acid auxochrome are derived from $(-COOH),(-SO^3H),(-OH),$ $(-H_3PO_4)$ etc.

$$COOH \rightleftharpoons COO^- + H^-$$

$$SO_3H \rightleftharpoons SO_3 + H^-$$

$$H_3PO_4 \rightleftharpoons H_2PO_4^- + H^-$$

Example:



COOH (acid group)

Classification of dye :

1. Physical mode of classiciation:

According to the ionic nature of the auxochromophoric system i.e., what type of auxochrome is attached to the chromophore,

On this basis dye may be of two types.

a) Acidic dye:

When the Chromophore are associated with the acid radicals from acidic dye or anionic dye. Hence, staining property of the dye depends on such type of radicals present in auxochromophoric system. Which part of the tissue or cell stain with the acid dye is known as acidophilic. So acid dye stain the basic part of the tissue/cell. Most of the acid dye are salts of Sodium. The anionic part remain associated with the auxochromophoric system and Sodium remaining as true ions in the whole dye molecule and those $COOH^-$ / COO^- and $Na+$ balance the total molecule as a nutral natural Salt.

Example: Eosin, Methylin blue.

b) Basic dye:

When the Chromophore are associated with basic radicals form basic dye or cationic dye. Hence the staining property of the dye depends on such types of radicals present in the auxochromophoric system which part of the tissue/cell stain with the basic dye is known as basidophilic. So, basic dye stain the acid part of the tissue/cell.

In most cases basic dye are Chloride Salt. Beside Chloride it may be Nitrate or acetate.

Example: basic fuchsin, Crystal Violet, Methyle green.

c) Amphotic dye:

This type of dye may be either cationic or anionic.

Example: Haematin is Cationic below the pH 6.6 but anionic above this pH.

2) Chemical mode of classification:

Depending upon the chemical nature of the Chromophore the dye may be of three main types.

A. Quinoid:

Chromophore having quinoid ring depending upon the resonence the Quinoid type further classificied into following types:

I) Haematin:

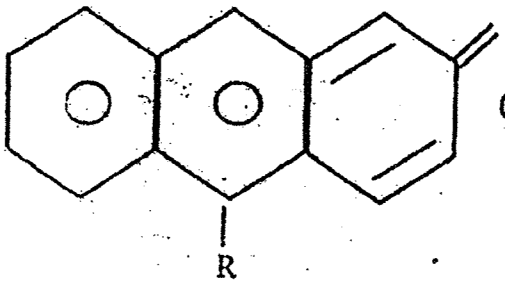
In this type at dye the two benzene rings remain attached side by side and quinoid remain attached to the one benzene ring.

Example: Haematoxyline.

II) Xanthene:

The Chromophoric system contain R, R', R'',

Eg. Eosin, Pyronine.

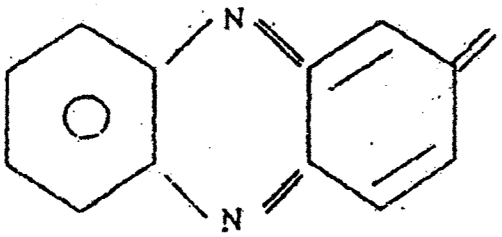


(Quinoid B)
Eosin

III. Azine:

In this type of dye the Chromophoric system is a Pyrazine ring between two aromatic system.

Azine may be two types:



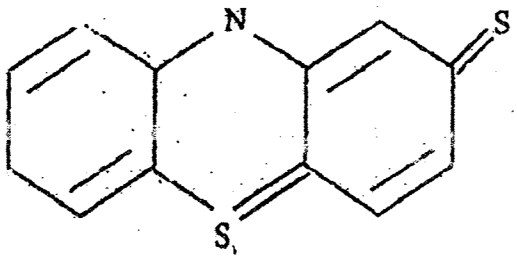
Quinoid B

a) Oxazine:

The Oxazine Chromophore has very much similar with that of azine and exist in O - quinoid form.

b) Thyazine:

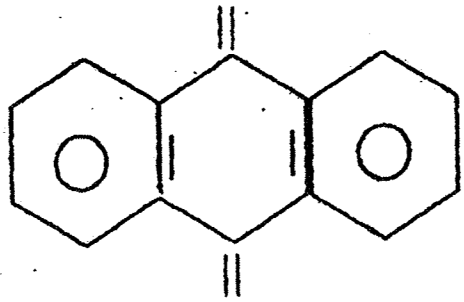
The Chromophore of thiarine is like that of the oxazine with Sulphar instead of Oxygen. These are metachromatic dyes.



IV) Anthroquinon:

These are also quinoid A type dye where a quinoid ring present between the two benzene ring and produce anthroquinoid structure.

Eg.: Carmine.



B) Azo dye:

It is very large groups of dyes which is important to the textile industry and in biology. The Chromophore (–N = N–) of this group containing two aromatic rings. This aromatic ring usually Benzene or Napthalene. The azo linkage can exist in two isomeric forms cis and trans.

N = N
/ \
C is form

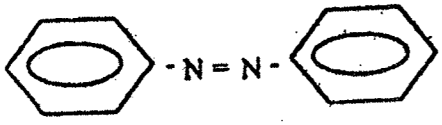
N = N
/ \
Trans form

1) Amino azo dye:

It is mono aro compounds with Sulphonic and Phenolic substituents.

Example: Orange green, Genus green.

Example - Bismark browny



2) Modant azo dye :

These group of dye are capable of chelating with Cromium (cr) or similar metals.

3) Cationic azo dye:

These are not used in histology but mainly used for colouring of leather.

Example - Bismark browny.

4) Nitro dye:

Chromophore of this dye contain - NO_2 group. It shows resonance variety.

Example - Picric acid.

MORDANTING:

This is a process by which a colour dye is connected into a strong colour dye is connected into a strong colour dye by using strong Cationic multivalent metals present in the double salt. These double sale are called mordants.

Mordants:

i) **Ammonium alum**: aluminium ammonium sulphate.

ii) **Iron alum**: ammonium iron sulphate.

iii) **Crome alum**: Potassium aluminium sulphate.

Mordants are used in two ways:

a) **Single bath method:** directly mixed with haematin.

b) **Double bath method:** First tissue section treated with mordants and then the complex is mixed with haematin.

Chelation:

If there are two adjacent off group in a benzene ring, the strongly Cationic Polyvalent metal is changed their orientation due to special bond between OH and Polyvalent metals. This type of bond is known as Chelate bond and this process is known as Chelation.

After the attachment of the strong cationic metals the whole dye molecule act as a basic dye which attaches to the negative group on negatively charged molecule of the tissue.

Example: Chelation bonding generally occur in the mordanting as haematin to haematoxylin. In such case aluminium and iron generally used as Polyvalent Cationic mordant element in single and double bath method respectively.

Advantage of mordanting

i) The colour is not easily removable by nutral fluid (liquid).

ii) Progressive and regressive staining become possible.

iii) Slow dehydration is possible.

iv) Counter staining also possible.

CARMINE:

Carmine has so much interest than any other dyes, in this respect that it is only the dye which is extracted from animal source. All other dyes are plant product. Carmine is extracted from the animal *Dactylophious cocti*, popularly known as scale insect coccid insect.

The dye produce from the fat body or ovary of the female insection.

Technique of extraction: Nearly 1000 female insect taken and dried, this dried specimen known as "cochineal". These are then boiled in water to extract the dye. Then add subsequently lead adequate, ethanol, sulphuric acid into the extract. During addition of H_2SO_4, Carmine dye is precipited down at the bottom and then carmine isolated from it.

Carmine + borax – Borax Carmine used for whole mount preparation of invertebrate areas.

Carmine + acetic acid – acetocarmine which is used for Chromosome staining.

HAEMATOXYLIN:

This is a natural dye that is obtained from the heart wood of the tree *Haematoxylene campechianum*. It is a native of the Mexican State of Campeche after which tree is named, but it is mainly cultivated in West Indies.

Haematoxylin is extracted from the log wood with hot water and precipitated out in the presence of urea (Lamb - 1974).

Since 1840 it has become the most used and adaptive histological stain despite its poor staining qualities when used alone.

To obtain a suitable staining solution with haematoxylin, two condition must be fulfilled; first its active constituent haematin must be produced, secondly the stain must be used with mordant. In alum haematoxylin the mordant is incorporated in the staining solution, usually Potassium, aluminium Sulphate. In iron haematoxylin the mordant is a feric salt (usually feric Chloride or feric ammonium Sulphate) and is often prior to the dye.

Haematin is produced by the oxidation of haematoxylin. This is brought about by the use of chemical oxidising agents.

As for example Sodium idate or by the traditional method of exposure to light and air, turned ripening. Instant ripening by using agents gives good results and sodium iodate is very satisfactory for this purpose.

Oxidation by exposure takes up 15 weeks.

Haematoxylin are divided into two groups.

I) **Alum haematoxylin**: which utilise aluminium ions (Potassium ion etc.)

Example: Mayer's, Ehrlichs, Harris and cole's haematoxylin.

II) **Iron haematoxylin**: which employ feric ions (feric ammonium Sulphate and feric Chloride).

Example: Heiden hain's and weigerts haematoxylin.

MODEL QUESTIONS

1. What is dye? Describe the chemical basis of classification of dye with characteristic and examples.
2. What is fixation? Why is it necessary? Discuss its chemistry. Mention the effectiveness and a few demerits of Bouins fluid.
3. a) What are the purpose of fixation?
   b) Describe brief the mechanism of fixation taking example of formaldehyde and picric acid.
4) WRITE NOTES ON
   a) Autolysis
   b) Effects of fixative
   c) Additive & non-additive fixative
   d) Spectral shift
   e) Mordanting
   f) Bathychromy
   g) Carmine
   h) Haematoxyline

SUGGESTED READING

1. Practical section cutting and staining

   by

   E.C. Clayden

2. Histology by Pearse

   Volume-I

3. Histology by Michael H. Ross

4. Histopathological stains and their diagnostic

   used by

   (John D. Bancroft and Alan Stevers)

5. Introduction to functional Histology

   by

   TELFORD, BRIDGMAN.

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION
MIDNAPORE - 721 102

M.Sc. in Zoology

Part - I
Paper - II, Unit - II, Group - B

Module No. 20

MEMBRANES :

The structural and functional unit of any living organism is a cell. Two kinds of cells are recognised - Prokaryotic and Eukaryotic. Both the two types of cells are bounded by a membrane that separates the internal environment of the cell from the external environment. However, the prokaryotes has a minimum of internal organization with no membrane bound organelles. Whereas eukaryotic cell has a considerable degree of internal structure with distinctive membrane enclosed organelles. All membranes are made up of lipid and protein molecules. Carbohydrates are present as glycolipids and glycoproteins. The semipermeable barrier between the internal and external environment of a cell is the plasma membrane. It is a thin, continuous film-like, semifluid, lipoprotein membrane. Organelle membranes posses similar structure and they separate the internal environment of the organelle from the external cytoplasmic environment.

MODEIS :

Artificial membranes have been prepared with lipids and proteins. Their properties like thickness, refractive index, capacitance, dielectric constrant, membrane protential, resistivity, water permeability etc. have been studied and compared with such properties or characteristics of natural membranes. Moreover, the structure of the natural and artificial membranes was studied under electron microscope using conventional techniques as well as some special techniques like Freeze-fracture, freeze etching etc. Basing on such studies different models have been proposed to explain the structure of the membrane.

LIPID BILAYER MODEL OF GORTER - GRENDEL :

In 1925, Gorter and Grendel proposed that the erythrocyte membrane is composed of a lipid bilayer. It is made up of two monolayers of amphipathic lipid molecules. The lipid molecules are specifically oriented in each monolayer. The polar head groups of lipid molecules are directed to the water adjoining outer surface of the membrane. The nonpolar hydrocarbon tail of the lipid molecules are apposed to each other i.e. they are oriented into the middle core of the lipid bilayer.

TRILAMINAR MODEL OF DAVSON-DANIELLI :

In 1935, Davson and Danielli proposed a trilaminar model to explain the resistivity and low interfacial tension of the natural membrane. According to this model, plasma membrane is made up of a lipid bilayer bounded on either side by a layer of globular proteins. The protein molecules are electrostatically held by the polar head groups of the phospho lipids of the respective bilayers. Subsequently it was suggested that surface protein instead of globular conformation has α helical or β pleated form. It has been proposed also that the membrane has hydrophilic pores (0.7 mm diameter) being lined by polar side chains of proteins which help in the transport of polar solutes like K^+ and Cl^- ions.

A trilaminer structure of the plasma membrane and organelle membrane was observed under electron microscope in osmium/ permanganet fixed tissues. Hence, Robertson in 1959 proposed a trilaminar structure of the membrane and it was termed as unit membrane. Under electron microscope, a middle electron-lucent layer with two electron-dense layers one on either side of the electron-lucent layer was observed. Robertson interpreted the electron dense layer as the surface layer of proteins and the middle electron lucent layer as the nonpolar fatty acid tails of the lipid bilayer. The electron-dense appearance may also be due to polar head groups of the lipids. This is because Stoecknius demonstrated a similar trilaminar structure of osmium fixed artificial lipid bilayer free from proteins.

FLUID MOSAIC MODEL OF SINGER - NICOLSON :

Freeze- fracture replica of the plasma membrane was studied under electron microscope. The electron micrographs revealed that the fracture face that passes along the midline between the lipid monolayers are studed with many particles of 8.5 nm diameter. These particles remain embeded in the lipid bilayer and also extend along the fracture face. Similar studies with artificial membrane revealed that freeze fracture replica of artificial lipid bilayer do not show presence of such particles. However, such particles are seen on the fracture - face of the freeze - fracture replica of artificial lipid bilayer mixed with proteins. It has also been observed that protein chains penetrate into the lipid monolayer. Phospholipids and proteins were also found to redistribute themselves rapidly in the membrane which was possible only if it were fluid in nature.

Basing on these observations, S.J. Singer and G.L. Nicoloson proposed a fluid mosaic model of the membrane in 1972. According to this model, the membrane is composed of a continuous semifluid lipid bilayer. In this bilayer many globular proteins are embeded as a discontinuous mosaic of particles. The particles are so arranged that the continuity of the lipids is interrupted at different points. The structure of the lipid bilayer is basically same as seen in other models. It consists of two monalayers. In each monolayer,

the polar head groups of the phospholipids are directed towards the adjoining aqueous phase. On the other hand the nonpolar hydrocarbon tails of both the monolayers are directed towards each other in the nonaqueous middle core of the membrane. The nonpolar tail remain perpendicular to the membrane surface and remain closely side by side in the monolayer. They are held in position by hydrophobic interactions between the nonpolar tails.

Two types of proteins are present in the lipid bilayer. One type is integral/intrinsic or transmembrane protein that extends across the entire lipid bilayer and sometimes they spand the membrane several times, Hydrophobic interaction between nonpolar aminoacids and the acyl side chains of the membrane lipids firmly anchor the membrane proteins. The other type is peripheral/ extrinsic proteins. They do not penetrate the lipid bilayer. The remain associated with the polar part of the intrinsic protein and the polar head groups of the phospholipids by electrostatic and hydrogen bondings.

The proteins, lipids and carbohydrates present in the membrane have been studied in detail to reveal their role in the structure and function of the cell membranes. The carbohydrates present in the membrane are glycoproteins and glycolipids. The percentage of protein, lipid and carbohydrate in the erythrocyte membrane has been found to be as 52, 42 and 8 respectively. However, the protein : lipid ratio of the membrane show much variation and it is dependent on the functional variation of the cellular component. For example, the protein : lipid ratio is 0.2 :1 in myelin membrane; it is 2:1 in sarcoplasmic reticulum membrane; it is 3.2 :1 in mitochondrial inner membrane.

MEMBRANE LIPIDS :

To study the lipid composition, the lipids are extracted from tissued by lipid solvents. They are then separated by thin-layer and gas-liquid chromatography. Individual lipids are identified by their chromatographic behaviour, their susceptibility to specific enzyme action or by mass spectral determination of their molecular masses. The lipid content of the membrane varies. 45-55% of the mass of plasma membrane is lipid. Whereas it is about 80% in myelin sheath and about 30% in inner mitochondrial membrane. Lecithin, cephalim and sphingomyelin constitute about 50 - 65% of the membrane lipids: 10-20% of the lipids are phosphatidylserine, phosphatidyl, inositols, phosphatidyl glycerol, cardiolipin, cerebrosides, sulfatides and gangliosides. Cholesterol and cholester esters constitute about 25% of the total lipids of the plasma membrane. But they are much less in inner mitochondrial membrane. It has been found that compared to plasma membrane, the membranes of subcellular organelles are poor in cholestoral and sphingolipids.

Most of the membrane lipids are amphipathic in nature. They have both polar and nonpolar groups. The

ionizable phosphocholine, phosphoethanalamine and phosphoaminoacid residues of phospholipids, the suger residues of cerebrosides, the sulfated sugar residues of sulfatides and the OH group of free cholesterol are the polar head groups of the respective lipids. The nonpolar tail groups of these lipids are the hydrocarbon chains of fatty acid residues and sphingosine; the sterol ring and the side chain of cholesterol.

The lipids are arranged in a bilayer. The polar head groups are directed towards the outer side and the nonpolar tail part is apposed to each other. The hydrocarbon chain of the fatty acids are straight, closely packed and hydrophobically bonded to each other. The unsaturated fatty acids however, bend at the unsaturated bond (cis). Hence, these fatty acid tails are not closely packed and so their position is not rigid. For this reason, the mobility of the membrane lipids increase as the unsaturation of the lipid is unsaturattion of the lipid increased. Hydrophobic inter action also occur between the nonpolar amino acids of the membrane protein and the acyl side chains of the membrane lipids. The polar head groups of the lipids remain associated with the membrane mainly by hydrogen bonding and electrostatic interaction with the polar proteins of the intrinsic protein.

The lipids show asymmetric distribution. Lecithin and sphingomyelin are located mainly on the outer bilayer; cephalin and phosphacidyl serine are mostly present in the inner bilayer. Cholesteral is also generally present in large amount in the outer than in the inner bilayer.

The cholesterol molecule is so oriented in the membrane that the sterol ring remain below the polar head group of the phospholipids and the side chain is near the middle part of the lipid bilayer. It is held that both the starol ring and its side chain are hydrophobically bonded to the fatty acid tail of the nearby phospholipids. The polyunsaturated fatty acids wound round the distal part of the cholestoral side chain. The3-CH group of nonesterified cholesterol remain polar bonded with the charged polar head groupds of the phospholipid molecule. The membrane phospholipids are more compactly packed in presence of cholestrol. Hence, it decreases fluidity of the membrane. It has been shown that membrane fluidity increases as the phospholipid content increase and cholestrol content of the membrane decreases. The phospholids not only show a specific asymmetric distribution, they also exhibit movement. The phospholipid molecules are able to rotate about their axes. In addition, at physiological temperature (37°C), the hydrocarbon tails are mobile and they undergo rapid flexing movements. The rate of this movement is higher if the hydrocarbon tail is short and contains double bonds. The phospholipid molecudes also show lateral movement in the same layer (lateral diffusion). They also can move from one layer to the other layer by 'flip-flop' movement. Studies with synthetic membrane revealed that initially the flip-flop movement is slow but when a membrane protein e.g. glycophorin is introduced into the synthetic bilayer this movement increases 100 fold. Thus proteins can alter the rate of flip flop movement. However, in the intact membrane this flip-flop movement seems to

be slow to maintain asymmetric distribution of membrane lipids.

α - tocopherol molecules are also present in the lipid bilayer. The isoprenoid side chain of these molecules are hydrophobically bonded to the nonpolar tails of polyunsaturated fatty acids of neighouring phospholipids. Whereas the chromane ring ir oriented towards the aqueous surface of the lipid bilayer.

Mechanisims involved in maintaining the phospholipid asymmetry are not well understood. It has been postulated that translocases are present which transfer certain phosphilipids like lecithin from the inner layer to the outer layer. There are specific proteins that bind specific phosphalipids. Such proteins are specifically distributed in the two layers and this helps in accumulation of specific phospholipid in a particular layer. It is held that phospholipid exchange proteins may recognize specific phospholipid and transfer them from one membrane to another membrane.

FUNCTIONS OF MEMBRANE LIPIDS :

1. It is the structural component of membranes. They keep the proteins in position by proper bonding. It confers fludity to the membrane. Phospholipid; cholesterol ratio determines the fludity. If this ratio is high, the fludity is more and vice-versa. In alcholic cirrhosis of liver, cholesterol content of erythrocyte membrane becomes very high. This decreases the fluidity of the membrane considerably. As a result, the erytheocytes asume spiculated shape, their O_2 transporting capacity is decreased, their fragility is incrdased. They are prematurely destroyed leading to development of spur cell anaemia.
2. Membrane phospholipids are the source of polyenoic fatty acids like arachidonete, timnodonate used in the synthesis of prostaglandins, thromboxanes and leukotrienes. Phosphatidyl inosital is the substrate for the synthesis of second messenger in hormone action.
3. Phosphilipids are also required for some enzyme activation. Lecithin of inner mitochondrial membrane activates D(-) 3-hydroxybutyrate dehydrogenase.

MEMBRANE PROTEINS :

There are two types of membrane proteins - *extrinsic* and *intrinsic*. The extrinsic variety constitute about 30% of the membrane protein and they are found on the surface of the membrane. They do not penetrate lipid bilayer. They remain associated with the membrane mainly by electrostatic interaction and hydrogen bonding with the polar portions of the intrinsic protein as well as with the polar head groups of the phospholipids. These are weak bonding and so the extrinsic proteins can be easily extracted from membranes by simple procedures.

The intrinsic or integral membrane proteins constitute about 70% of the membrane protein. They penetrate

lipid bilayer partially or completely. Most of them span the bilayer completely, so that parts of the protein are exposed on both sides of the bilayer. Some integral proteins span the membrane several times. These proteins are usually amphipathic molecules. The hydrophilic regions of the molecule remain exposed to the aqueous medium outside the bilayer. The hydrophobic part of the molecule cluster in the interior or the lipid bilayer. The three dimensional structure of much of the hydrophobic part of the integral proteins is usually an α helix. Any polar amino acid side chains in this portion of the molecule are sequestered within the α -helix. Only the nonpolar side chains (hydrophobic) are exposed to the hydrophobic interior of the bilayer. In the part of the protein that is projected outside the lipid bilayer, the nonpolar side chains are barried in the interior of the protein structure, whereas the polar and ionic side chains are exposed to the hydrophilic environment.

Many of the intrinsic proteins also exhibit dynamic properties, like those of lipids. They show lateral diffusional movement as well as rotational movement about an axis perpendicular to the plane of the membrane. The fluidity of the lipid bilayer makes these movements possible. The flip-flop movement possibly does not occur. Such lateral mobility of these intrinsic proteins is checked or can be prevented by interaction with peripheral components of the membrane (such as other proteins) or with components of the cell cytoskleton (microtibules and microfilaments).

Hydrophobic interaction between the monpolar aminoacids and the acyl side chains of the membrane lipids firmly anchor the membrane proteins and thus provides a transmembrane pathway for protein translocation.

Asymmetry of membrane proteins :

Both extrinsic and intrinsic proteins are distributed unequally between the two halves of the bilayer. A specific protein may be associated with only one side of the bilayer. This asymmetry is related with the function of the cell. For example, the extrinsic protein, spectrin is present only on the inner side (cytoplasmic side) of the plasma membrane of rbc. It forms a part of the filamentous network inside the cell that is important in maintaining the biconcave shape of rbc. Moreover, spectrin is also flexible enough to allow the cell to change its shape as required during its passage through the capillaries. Many receptors for specific chemical signals including the hormones are located on the outer surface of the cell membrane and very often it is a part of an integral protein. When an intrinsic protein span the entire membranes, a mechanism exist for converting the extracellular signal to an intracellular response. For example, binding of a ligand (or hormone) to the receptor triggers a conformational change in the protein. This change may be opening of a hydrophilic channel, between the protein subunits. Consequently, a specific ion passes through the membrane from ECF to ICF.

FUNCTION OF MEMBRANE PROTEINS :

Proteins in the membrane carry out many functions :

1. They act as cell adhesion molecules that anchor cells to neighbouring cells or to basal lamina.
2. They act as pumps that actively transport ions across the membrane.
3. They function as carriers for transporting substances across the membrane via facilitated diffusion and active transport.
4. They function as receptors for neurotransmitters and hormones.
5. They function as enzymes, catalyzing reactions at teh surface of the membrane.
6. Some also function in antibody processing and distinguishing self from nonself.

MEMBRANE CARBOHYDRATES :

2-10% of the mass of membrane is carbohydrates. Although it is a minor component of the cell membrane, it has important functions in membrane physiology. Most of the carbohydrates of the membrane are bound either with intrinsic membrane proteing forming glycoprotein. Some are bound to lipids in the lipid bilayer forming glycolipids. The carbohydrates are heteroglycans being composed of sugars such as D-glucose, D-galactose, D-mannose, L-fucose, N-acctyl hexosamines and sialic acid. They are distributed unequally (asymmetric distribution) in the membrane. Glycoproteins are presesent in both inside and outside of the membrane. They are more abundunt than glycolipids. The glycolipids are present only in the external half of the bilayer. However, less than 10% of the lipid molecules in the external half of the bilayer have attached carbohydrates. The amount of carbohydrate present in individual glycoprotein may be large relative to the amount of protein. For example, glycophorin, an intrinsic glycoprotein in rbc membrane is composed of 131 amino acid residues and about 100 monosaccharide units. The monsaccharides together make up about 60% of the mass of glycoprotein molecule. Location of carbohydrates in the membrane is obviously related to function. Carbohydrates on the surface of the plasma membrane is probably an important factor in cell to cell recognition preocesses. The carbohydrates also probably serve as specific membrane receptors for ECF, messengers like hormones.

LIPOSOME

Liposomes are artificially made spherical or ellipsoidal vesicles surrounded by amphipathic molecules (lipid bilayer). The vesicles enclose a hollow central aqueous core. The vesicle may have a single bilayer (unilamellar liposome) or few concentric closed bilayers (multilamellar liposome) forming a multilayer

membrane. Liposomes have colloidal dimensions. The diameter ranges from 10^{-5} to 10^{-7} cm. Since the lipid bilayer is considerably impermeable to plar materials, the composition ofhte aqueous core will not be changed even if the composition of the surrounding medium is changed. Liposomes can fuse with other liposomes or with the plasma membrane of any other cell.

PREPARATION OF LIPOSOME :

Artificial membrane systems can be prepared using special techniques. These systems generally consist of mixtures of one or more phospholipids of natural or synthetic in origin. The mixtures are treated e.g. sonication, to form spherical vesicles made up of lipid bylayer. They form aqueous suspension from which they can be separated by dialysis, ultrafiltration and molecular sieve chromatography.

Erythrocyte ghosts may be fragmented by detergents. Such membrane fragmants may subsequently close up to form a new liposome.

IMPORTANCE :

1. The lipid composition of the liposome is changed to study the effect of lipid composition on the function of the cell. In this way the effect of the nature of phospholipid or fattyacid on cell-function has been studied.
2. Purified enzymes or proteins can be incorporated into the vesicle membrance to study the different factors which the protein require to reconstitute their functions. Using this procedure, it has been shown that only a single protein and a single lipid are required to reconstitute an ion pump.
3. The liposomes contain specific receptor protein. Hence, the liposomes can be exposed to known ligands to get the effect.
4. They can be used for delivery of drugs, to the target cells. When liposomes are formed they can be made to entrap drugs in the central core. Moreover, some proteins or ligands, that can interact with target cells, are incorporated into the lipid bilayer of the liposomes. Thus the liposome membrane will attach itself to the specific target cell membrane. The two membranes will fuse and the drug will be delivered to the specific cell.
5. Efforts are being made to deliver enzymes and isolated gense to the target cells with the help of liposome.

GLYCOCALYX

Most of the cells have an external carbohydrate coat called glycocalyx. (Gr. Glykys-sweet; Kalyx-cup of

a flowr). The existence of such carbohydrate rich layers outside the plasma mebrane has been demonstrated mainly by cytochemical techniques. The chemistry, morphology and the nature of association with plasma membrane show variation. Glycocalyses have been divided into two general categories, depending on their degree of connection to the cell surface. An attached glycocalyx (or surface coat) is an inherent part of the cell surface that cannot be removed without simultaneously removing part of the plasma membrane itself. These coatings often appear in electron micrographs as fuzzy layer of filamentous material covering the cell surface.

The other category of glycocalyx is the *unattached glycocalyx*. It consists of material located outside the plasma membrane that can be easily removed without affecting the viability of the cell or disrupting the plasma membrane. The membranous surroundings of most animal eggs, the outer coat of amoeba and the sarcolemma of muscle fibers belong to this category.

Glycocalyses are mainly glycoproteins and glycolipids. In other wards, the outermost glycoprotein and glycolipid covering of the plasma membrane is called the glycocalyx.

Most of the integral proteins are glycoproteins. Eight different sugars are mainly involved in the formation of the oligosaccharide side chain of glycoproteins. These are D-glucose, D-galactose, D-mannose, L-fucose, D-xylose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid.

Ten percent of lipids are glycolipids. The carbohydrates present in the glycolipids are D-glucose, D-galactose, N-acetylgalactosamine and sialic acid. Cerebrosides, sulfatides, gangliosides and globosides are the different glycolipids.

Proteoglycans also contribute to the glycocalyx. These are proteins covelently bound to glycosaminoglycans. The protein part of proteoglycan is called 'core protein'. 95% of proteoglycan may be carbohydrate. They are built up of repeating disaccharide units. The glyco-part is often bound to small core protein which are often loosely attached to the outer surface of the cell.

FUNCTIONS OF GLYCOCALYX :

1. It modulates physiochemical properties of cells like solubility, viscosity, charge, conformation, denaturation and binding sites for bacteria and viruses. It also affect insertion into membranes, intracellular migration, sorting and secretion.
2. Sialic acid present in the oligosaccharide side chain contribute to the negative charge on the surface. Such negative charge can repel other negative objects.

3. The glycocalyx of some cells attaches to the glycocalyx of other cells and thus help in the attachment of one cell with the other.

4. Many of the carbohydrates act as receptor substances for binding hormones like insulin and thus activates the attached integral protein which in turn also activates a casade of intracellular enzymes.

5. Glycocalyses are involved in immune reaction. The blood group antigens (ABO-system) are glycosphingolipids; some are glycoproteins. They are involved in the recognition and immune response related to organ transplantation. They take part in the onset of some infections diseases. It has been observed that the cholera toxin and influenza viruses enter their target cells by first binding with cell surface gangliosides.

6. Glycocalyx acts as a protective physical barrier on epithelial surface. it may contain or mask certain surface antigens.

7. The glycocalyz of intestinal microvilli contain enzymes involved in the terminal digestion of carbohydrates and proteins.

8. Glycocalyx confers unique surface specificity on different types of cells and thus play a key role in cell recognition during morphogenesis. It takes part in fertilization. The glycoprotein in zona pellucida in some species, help in the transport of sperm through it to bind with oocyte; in some other species, it helps in fusion of sperm membrane with oocyte membrane.

9. Glycocalyx present outside the sarcolemma can trap Na+ ions.

10. Glycolyx may affect the sites of metastasis selected by cancer cells.

FREEZE - FRACTURE ELECTRONE MISCROSCOPY

Freeze-fracture technique is a preparative process used to study the ultrastructure of cell membrane. The cell membrane is supercooled and then fractured by microtone or specially made knife. A metal-carbon replica of the fractured face is then proepared and it is observed under electron microscope. It is an extremely valuable technique used in the study of the membranes. But it is also coupled with "Freeze-etching" to make the technique more useful. In the "Freeze-etching" processes, the freeze-fractured surface is exposed in a vaccum to an elevated temperature for one to few minutes to evaperate a layer of ice from the surface (sub-limation). This sublimation of ice expose some intra or extracellular substance which were otherwise masked from view. This process has been termed etching, hence the name 'freeze-etching'.

The fracture is usually made in the middle of the lipid bilayer. Study of the fractured surface revealed that the surfact is studed with particles of about 8.5 mm diameter. These membrane particles remain embeded in the lipid bilayer and also extend along the fracture-face. Such study with artificial membrane synthesized from lipids only did not show membrane particles but when such synthetic lipid bilayer is mixed with proteins, the fracture-surface exhibit membrane particles. Freeze-fracture study provides the first direct evidence for the presence of integral proteins in many biological membranes. It is also a good supporting evidence in favour of 'fluid mosaic' theory of membrane structure.

METHOD :

It has been observed that majority of plant and animal cells (excepting bacteria, yest and spores) cannot withstand freezing process in respect viability of cell or in the preservation of ultrastructure. This happens becuase during freezing intracellular ice crystals are formed which greatly interfere with the assessment of normal ultrastructural information. This problem has been solved by treating the tissue with cryprotectants like glyerol, ethylene glycol, certain sugars, dimethyl sulfoxide (DMSO) & dextran etc. and this technique has been extensively used to sutdy the structure of the membranes. The method involves the following steps.

1. **Pretreatment with cryoprotectants :**

   Tissue or cells or membrane fragments are pretreated commonly with glycerol or other cryoprotectants. The time of exposure depends on the nature of the tissue (rbc of sheep and human require exposure for several hours and few minutes respectively).

2. **Freezing the specimen :**

   Small pieces of tissue in a suspending medium are placed on a metal disc or carrier and then ultrarapidly frozen. The specimen sample is usually deposited on a copper specimen carrier. The carrier is then plunged into liquid freon cooled close to its preezing point (-165°C) with liquid nitrogen.

   Other procedures like freezing the specimen is mall boats, tubes or rolled up electron microscope grid are used. The sample may be placed against a metal block that has been cooled by liquid helium. The frozen specimen may be kept stored in liquid nitrogen for several weeks if required.

FREEZE - FRACTURING :

The disc or carrier containing the frozen tissue is then placed in a special holder. The tissue block is struck by the edge of a cooled knife, so that the tissue is split into two pieces. A good quality stanless steel razor blade,

thoroughly cleaned with xylol or other organic solvents is commonly used to cause the fracture. The procedure is done in a cold chamber and different types of appartuses are available for this.

FREEZE - ETCHING :

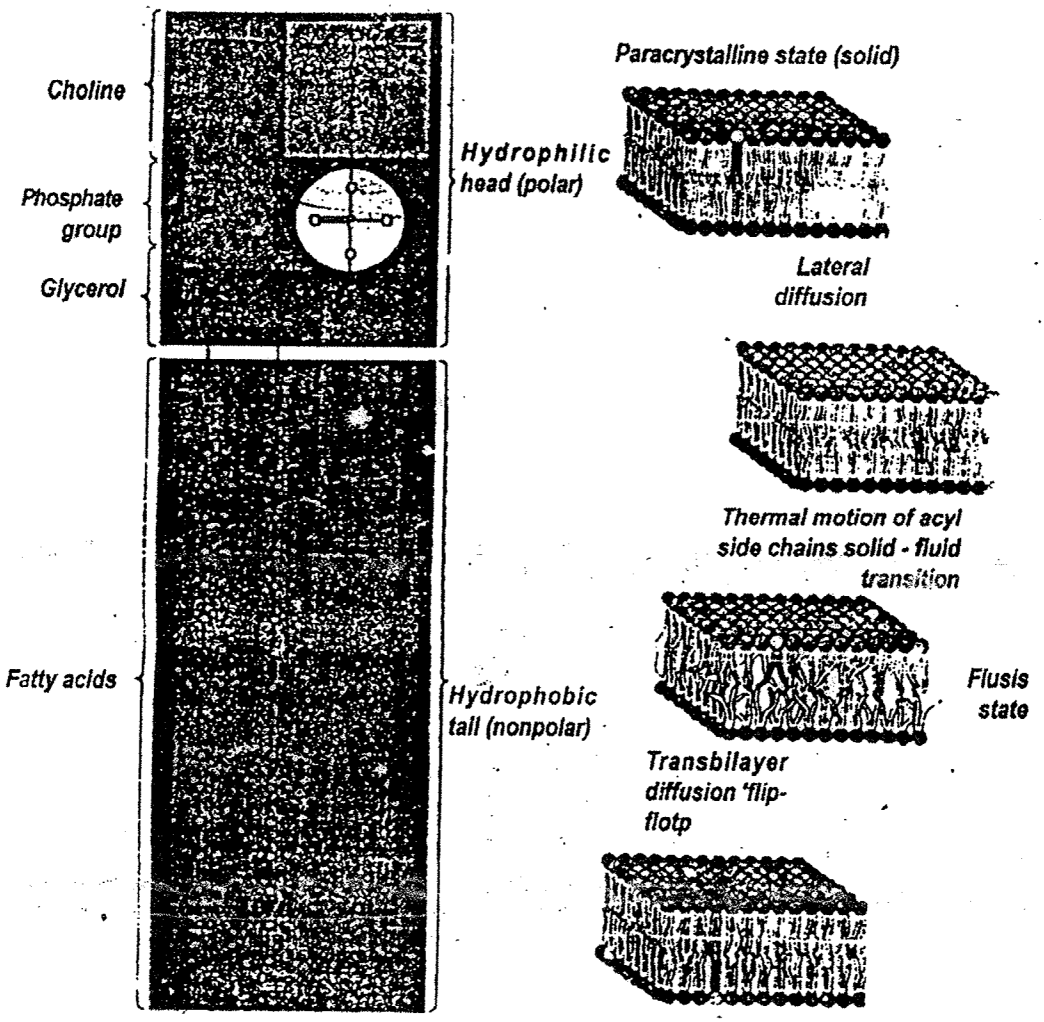
It is a step followed after freeze-fracture. In this step, the frozen, fractured specimen, while still in place within the cold chamber, is exposed to a vaccum at an elevated temperature (about 100°C), for one to few minutes. During this time a layer of ice evaperates from the surface. The etching rate followed is about 23 A depth per second.

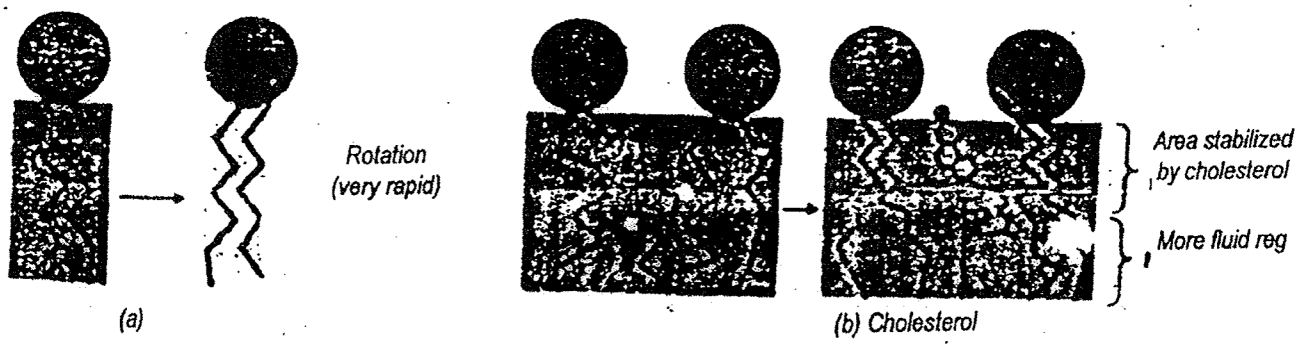
REPLOCATION :

After the surface is obtained it is replicated for future viewing in the electron microscope. A replication technique that takes a minimal time and imposes a negligible heat loade on the specimen is used. The fractured surface act as a template. Upon it a layer of heavy metal, usually platinium is deposited. The heavy metal is deposited on the newly exposed surface of the frozen tissue in the same chamber where fracturing was carried out. The metal is deposited at an angle to provide shadows that accentuate loca topography. A Carbon layer is then deposited on top of the metal layer from directly overhead rather than at an angle, so that a uniform layer of carbon is formed. This layer cements the patches of the metal into a solid layer. A replica is thus formed.

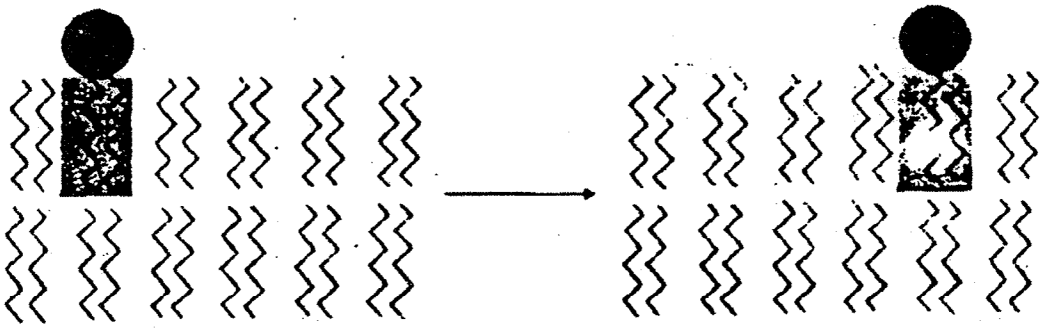
CIEANING THE REPLICA :

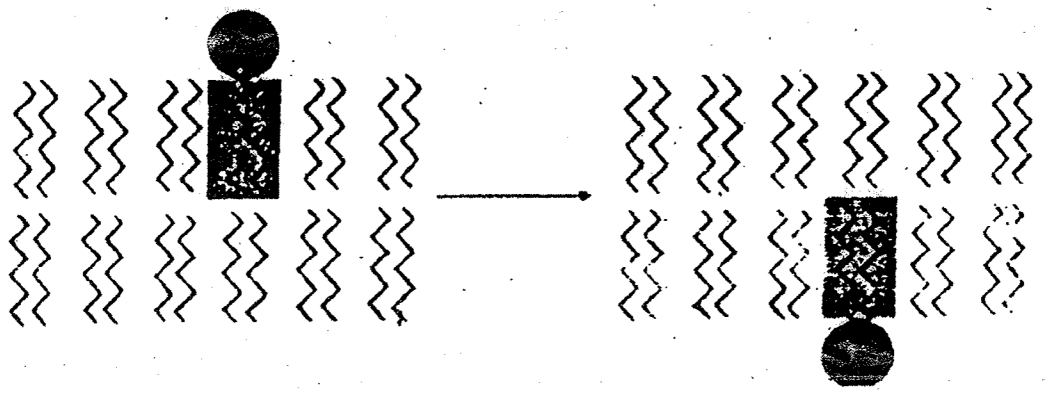
The tissue alongwith the replica is further treated. The tissue is thawed, removed and discarded. Initially it is floated in a medium in which the tissue was frozen. The replica is loosened. It is then cleaned by a cleaning solution (5% sodium hypochlorite is commonly used). Other cleaning solution are strong sodium hydroxides, sulfuric and hot nitric acid, dicromate solution as well as proteolytic enzymes. Subsequent to leaning, replicas are carefully rinsed in several changes of distilled water. Finally, the metal-carbon replica is picked up on a wire-loop and transferred to coated or uncoated grids and viewed under electron microscope.


Choline
Phosphate group
Glycerol
Hydrophilic head (polar)
Fatty acids
Hydrophobic tail (nonpolar)
Paracrystalline state (solid)
Lateral diffusion
Thermal motion of acyl side chains solid - fluid transition
Flusis state
Transbilayer diffusion 'flip-flotp



Phospholipid molecules (a) rotate on their axes and (b) undergo flexing motions. The rigid steroid ring structures of cholesterol stabilizes certain portions of the bilayer.





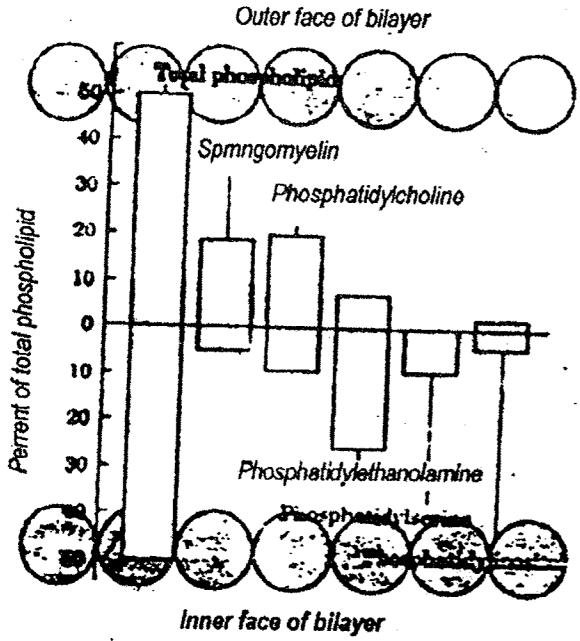
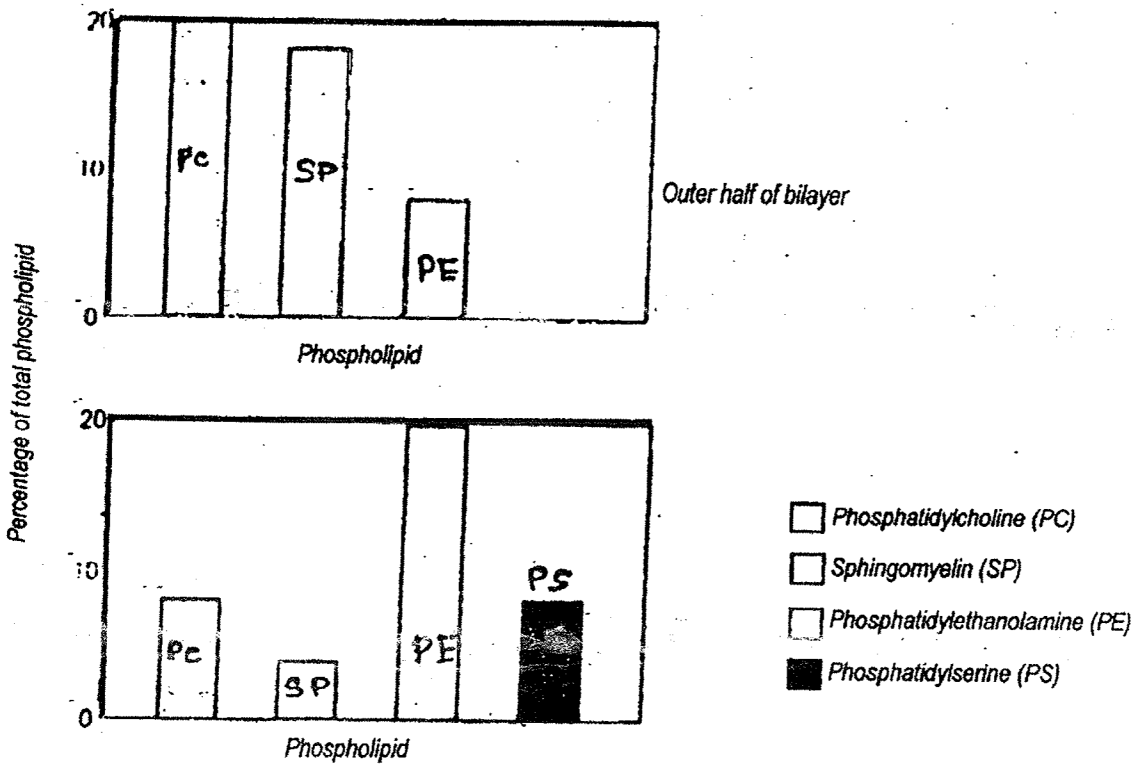
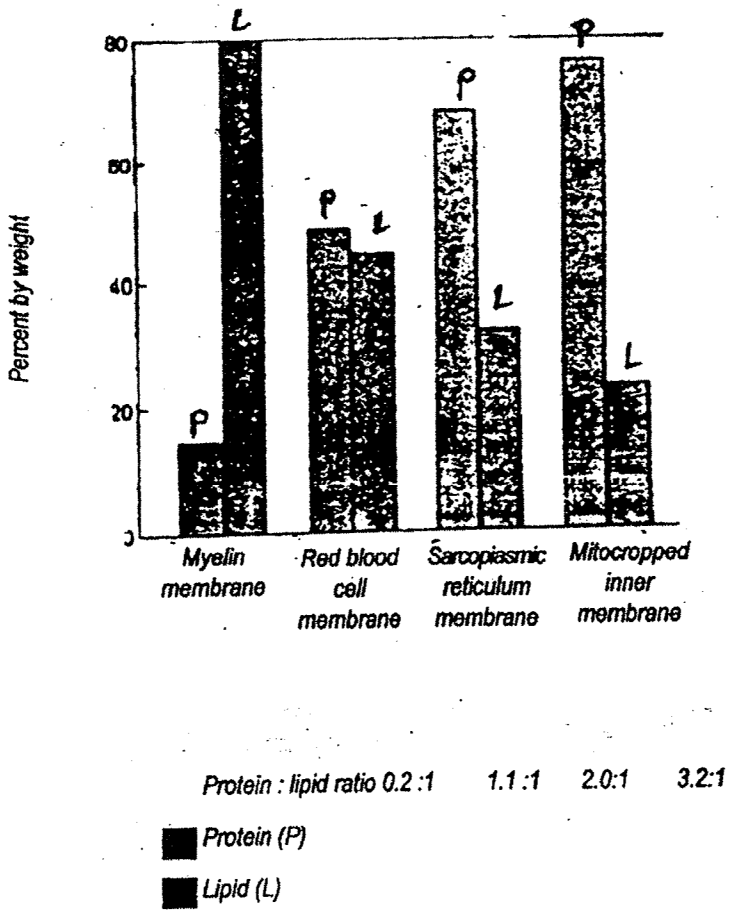


Figure 16-5 : The distribution of specific erythrocyte membrane lipids between the inner and outer face is asymmetric.




Percent by weight
80
60
40
20
0
P
L
Myelin membrane
Red blood cell membrane
Sarcoplasmic reticulum membrane
Mitocropped inner membrane
Protein : lipid ratio 0.2 :1
1.1 :1
2.0:1
3.2:1
Protein (P)
Lipid (L)

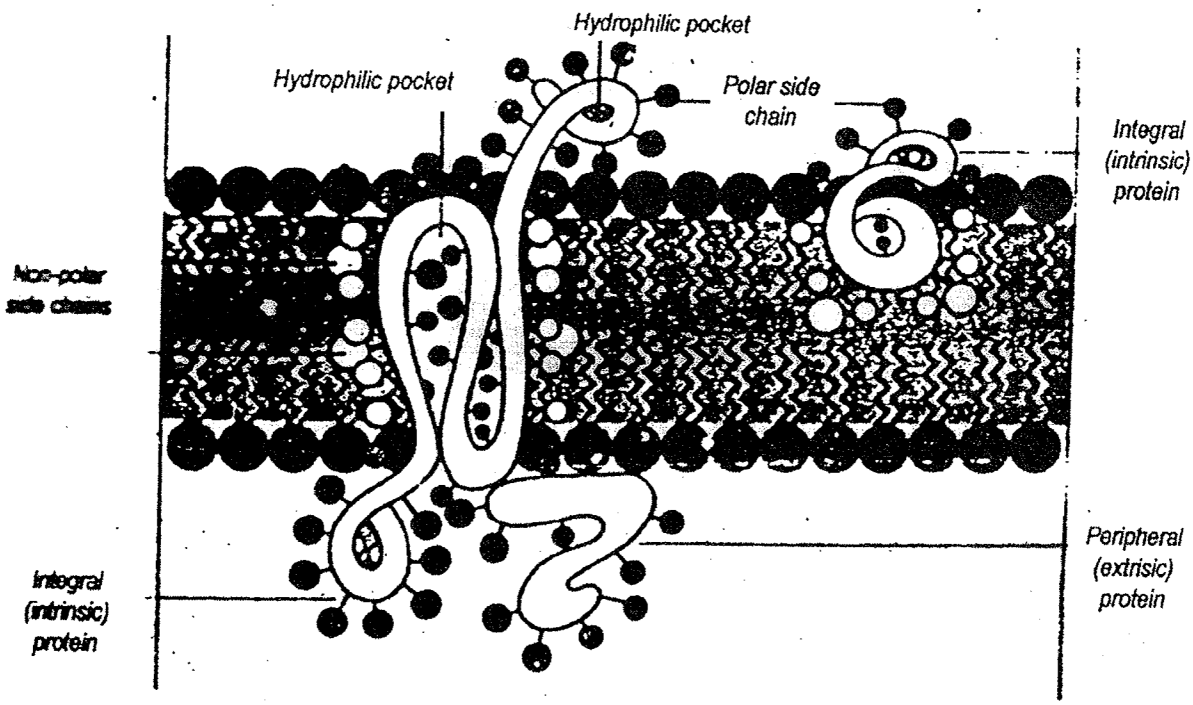
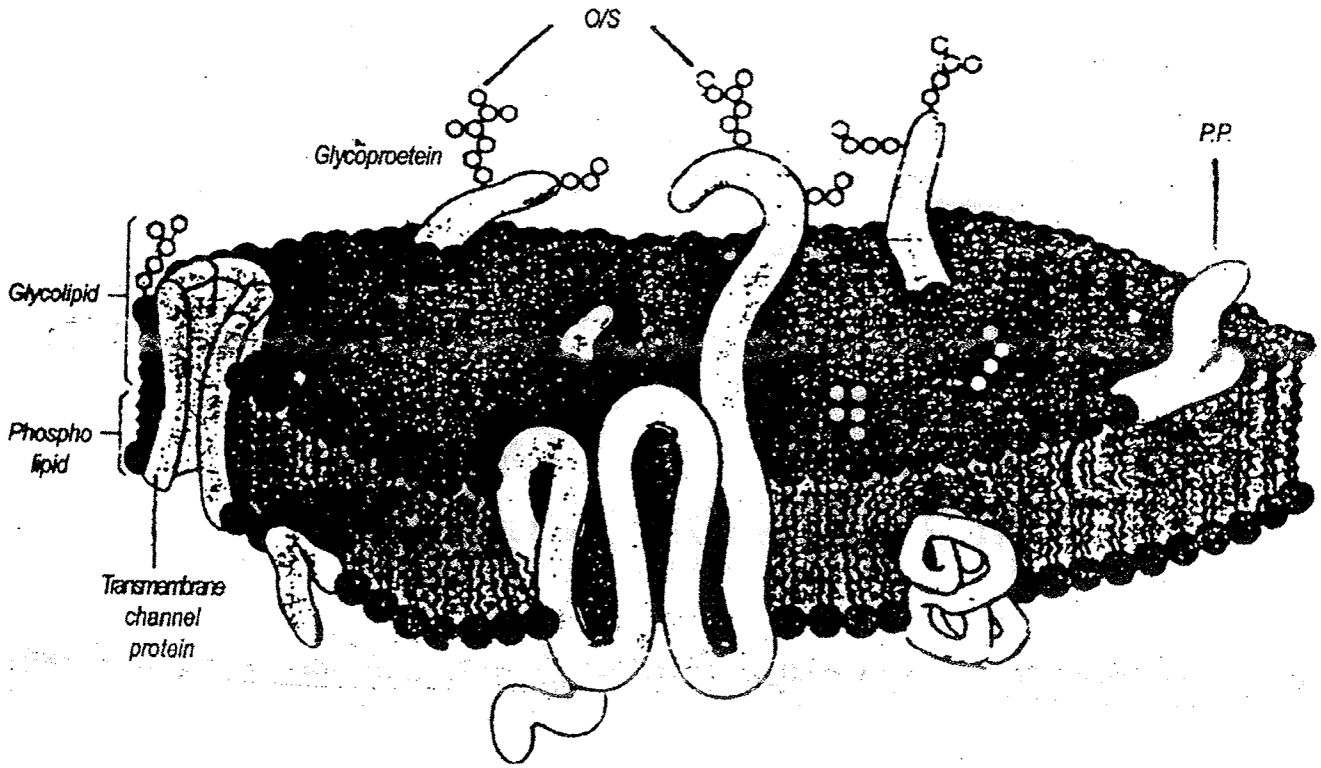
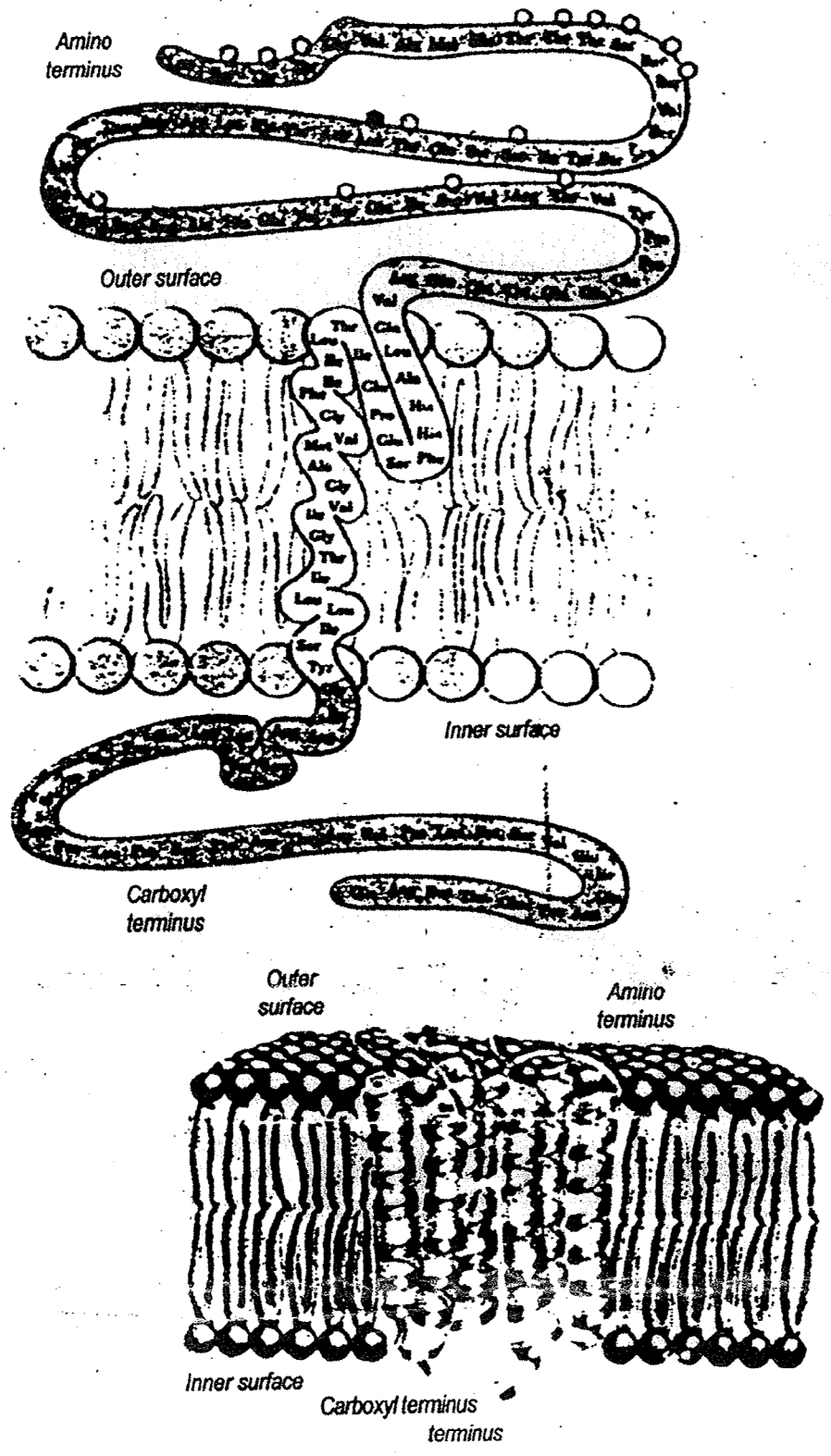
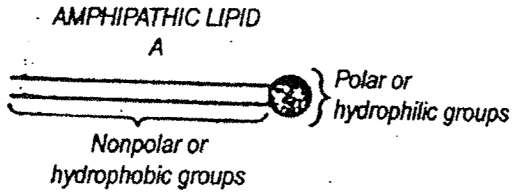


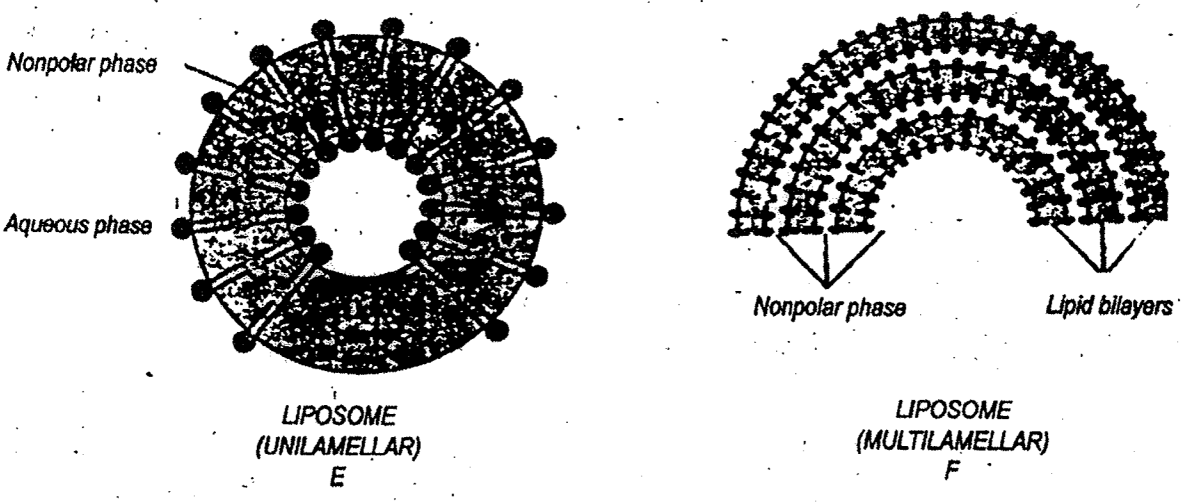
Figure 4 - 9 : The arrangement of integral and peripheral proteins in a biological membrane.



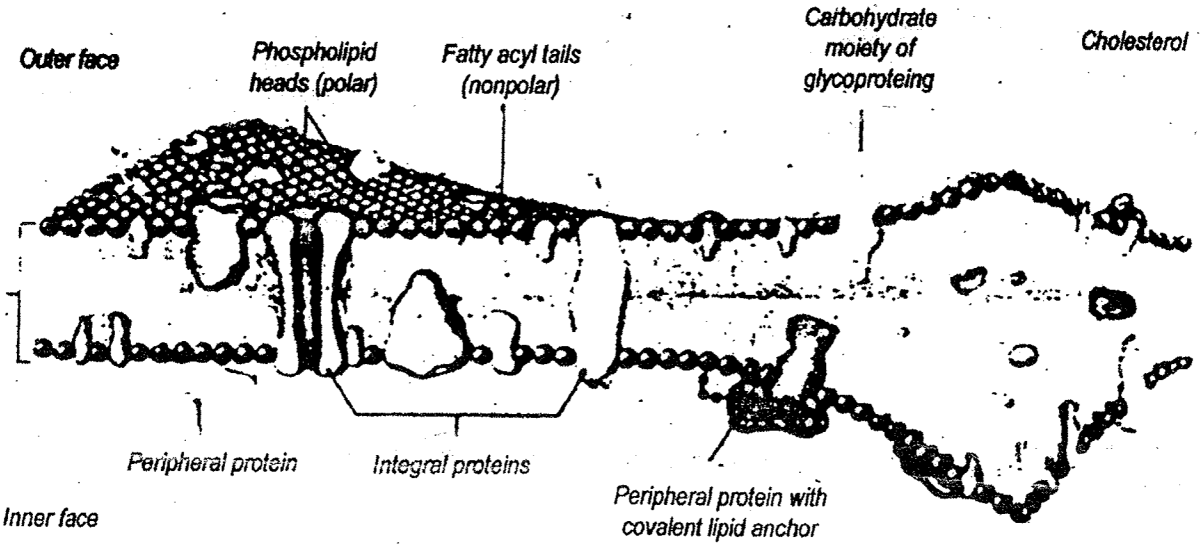
The current fluid mosaic model of the structure of plasma membrane.

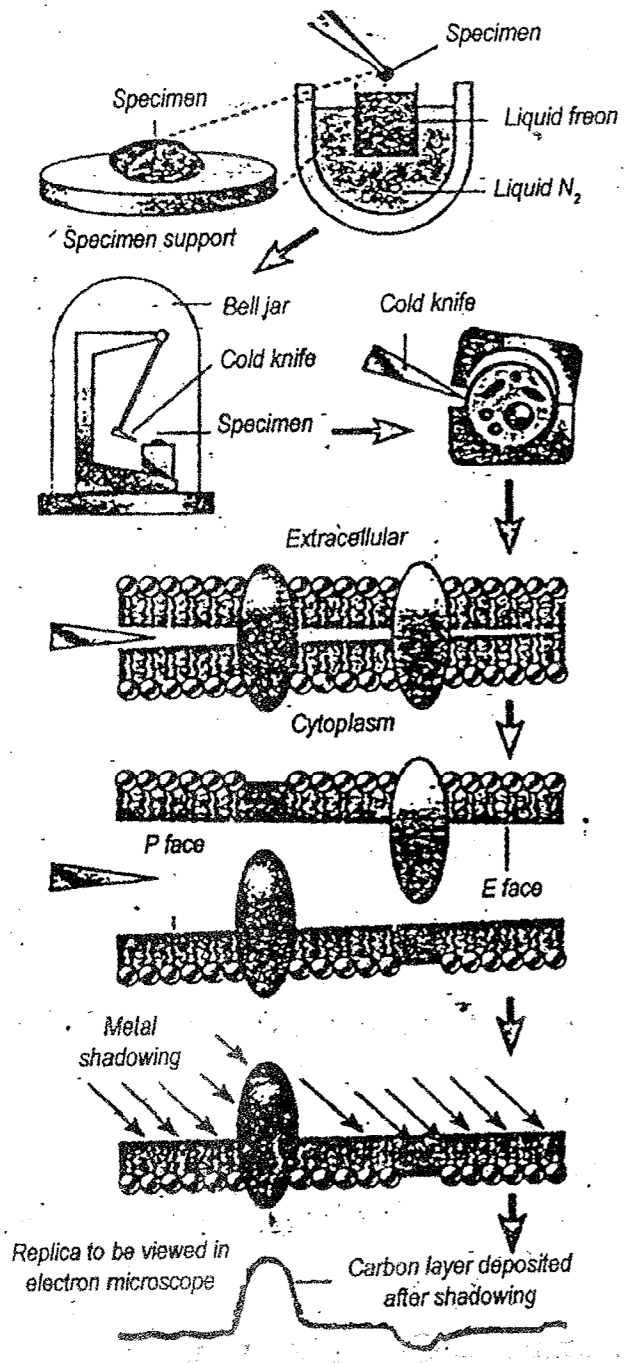

Amino terminus
Outer surface
Inner surface
Carboxyl terminus
Outer surface
Amino terminus
Inner surface
Carboxyl terminus
terminus

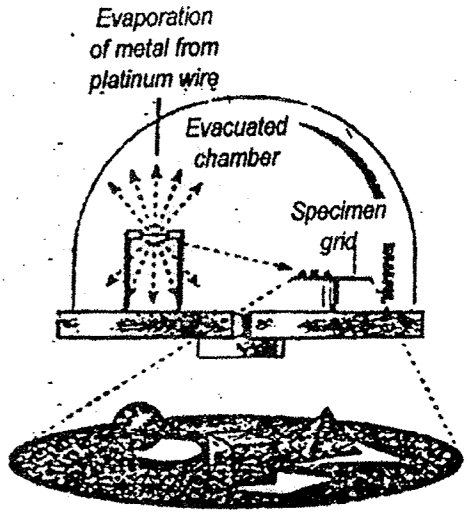




Formation of lipid membranes, micelles, emulsions and liposomes from amphipathic lipids, eg, phospho







The procedure used for shadow casting as a means to provdie contrast in the electron microscope. This procedure is often used to visualize small particles, such as the virus shown in the previous figure. DNA and RNA moleucles are often made visible by a modification of this procedure known as rotary shadowing in which the metal is evaporated at a very low angle while the specimen is rotated.

MEMBRANE PROTEINS

(1st para – Page No. 5)

Proteins are extracted from tissues by aqueous solvents like water, dilute salt solutions, dilute acids or alkalies, and occasionally with mixtures of water and organic solvents like alcohol or glycerol, Protease present in the crude extract are then subjected to various treatments and procedures to separate specific proteins. In general, the protein solution is kept at high concentration and at a nearly neutral pH to keep the proteins stable. Salting out proceduces, adsorption techniques, chromatography, electrophoresis, ultra-centrifugation, molecular sieving are very often used to separate the proteins. After a pure proteins sample is obtained, its physical, chemical and biological properties have been studied.

FLUIDITY OF THE MEMBRANE

(Before Liposome, Page No. 8)

The membrane lipids are in a gel phase below the transition temperature. Whereas, above this temperature they remain in sole phase. At physiological temperature they remain in sole phase. This happens because at higher temperature, the hydrocarbon tail of fatty acids remain in bent conformation, so they are less closely packed and hydrophobically bonded. The close packing of the fatty acid tail decreases membrane fluidity. Hence, any factor that reduces the close packing will increase fluidity. Bending at the unsaturated bond of fatty acids reduce close packing and so increases fluidity. Thus more the unsaturated fatty acid and lower fatty acids in the lipd molecule, more will be the fluidity. Hence, if the phospholipd concentration is high the membrane remains in the fluid state. It has been observed that in cold, polyunsaturated fatty acid in the phospholipid increase and this lower the transition temperature and fluidity is maintained even at low environmental temperature.

Cholesterol decreases fluidity. It rigid flat ring, decreases the bending of the fatty acid residues of the lipid. Consequently, their close packing and hydorphobic interaction increase. Hence, higher the cholesterol less is the mobility. It has been observed that phospholipid : Cholesterol ratio determines fluidity. If the ratio is increased, the fluidity is more and vice-versa.

Ca^{++} ions decrease fluidity. If favours formation of aggregates of lipid molecules. Moreover, Ca^{++} bind to the anionic polar head groups of membrane lipids and so decrease their mutual electrostatic repulsion. Hence, it enhances close packing and hydrophobic interaction between their non-polar tails.

Owing to membrane fluidity reorientation, reorganisation, incorporation and removal of membrane segments; flow of membrane molecules, cellular changes like evagination, invagination, pinching off and fusion of membranes are possible. Moreover, due to this fluidity, membrane lipids show movements like lateral and rotational diffusion, flexion of the nonpolar tail and flip-flop movement.

Movement of proteins in the membrane is also due to fluidity of the membrane lipids. Some membrane proteins show lateral diffusion due to membrane fluidity. Such movement has been found to be increased as the fluidity of the membrane is increased. Cell fusion studies using fluorescent dye clearly revealed lateral diffusion of proteins. However, a number of factors restrain such movement. These are (1) Aggregation of proteins into large oligomers, (2) their binding with oligosaccharides and peripheral portiens, (3) hydrophobic interaction and vander Waals forces between nonpolar side chain of amino acids and nonpolar tails of membrane lipids; (4) inability of membrane proteins to cross tight junctions.

FUNCTIONS OF MEMBRANES :

1. Plasma membrane separates intracellular compartment from extracellular environment. The organelle-membranes separate the organelle-compartment from the cytoplasm.

2. Owing to fluidity and mobility, many intramembrane molecules move from one part of the cell to another along the membrane, parts of the membranes, can undergo evagination, invagination, pinch-off or fusion with other cell. Such alteration of membrane conformation helps in cellular movement, formation of pseudopodia, renewal of membrane, exocytosis and endocytosis. Membrane bound secretory vesicles are pinched off from the membrane of Golgi cisternae and then fuses with the plasma membrane and the contents are extruded. By way of phagocytosis or pinocytosis extracellular materials are taken into the cell, membrane bound endocytotic vesicels are formed which fuses with lysosome or Golgi cisternae.

3. Membranes have selective permeability property. Lipid soluble substances and gases pass through the lipid core of the membrane, water soluble particles pass through transmembrane channels or water-filled pores. There are voltagegated and ligand-gated ion channels in the membrane that take part in the movement of ions across the membrane.

4. Some integral proteins act as membrane transporter and take part in the transport of solute. The transport may be uniport, cotransport or antiport. On the basis of energy requirement, the transport

may be passive or active. Exocytosis, endocytosis, selective diffusion, carrier-mediated transport help in the transport of material across the membrane.

5. Membranes have integral proteins which can act as hormone receptor, receiptor for neurotransmitters or drugs. Sometimes one part of the receptor directed to ECF is the ligand binding part, the other part of the receport help in transduction processes.

6. Many integral proteins function as enzymes, e.g. adenylate cyclase.

7. The membrane acts as a dielectric and separate two aqueous electrolytic solutions. A membrane potential with positivity outside and negativity inside is seen in the resting state. On stimulation, due to Na^+ influx, the membrane polarity is changed. The outside becomes negative and inside positive (action potential).

8. Plasma membrane of adjacent cells may undergo modification to form cell junction.

9. Polyunsaturated fatty acids of membrane phospholipids are used in the synthesis of most important biomolecules like Prostaglandins, thromboxanes and leukotriens.

10. Phosphatidylinositols of the membrane is used in the synthesis of phosphoinositols and diacylglycerol which act as second messenger.

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION
MIDNAPORE - 721 102

M.Sc. in Zoology

Part-I

Group-B Paper-II Unit-I

Module No. - 20

Animal hormones and mechanism of action

Hormones are informational molecules synthesized in the specialized endocrine cells. They are released from the cells by the process of exocytosis into the extracellular fluid and carried to the target cells. Most of the hormones act on target cells situated at a distance (telecrine secretion); some act locally (paracrine secretion); some hormones act on the very cells which secrete them (autocrine secretion); some neurones release hormones into the blood (neurohormones). They are of diverse chemical nature like peptide, protein, amine and steroid.

Classification and characteristics of hormones:

The hormones have been classified according to chemical nature, solubility, receptor location and nature of the signal used to mediate hormone action within the cell.

According to solubility they are of two groups – hydrophilic and lipophilic.

Hydrophilic:

The largest number of hormones are water soluble. They are polypeptides, proteins, glycoproteins and catecholamines in nature. They have no transport proteins. Their plasma half-life is very short and within minutes. Their receptors are located in the plasma membrane. The action of these hormones are mediated through different second messengers like cAMP, cGMP, Ca^{-}, metabolites of complex phosphoinositols, kinase cascades.

Lipophilic:

These hormones are not soluble in water. They are lipophilic. They are steroids, iodothyronines, calcitriol.

They are transported in the blood in combination with transport proteins. Their plasma half-life is long and ranges from hours todays. The receptor for these hormones are intracellular. Their action is mediated through formation of hormone-receptor complex. The complex binds with specific region of DNA, called hormone response element, through the receptor and activates or inhibits specific genes.

1. Hormones produce effects after binding with its receptor. Such binding generates a signal for initiating biological effects.
2. Hormones bind with the receptor by non covalent bonds. The binding is reversible. Hence, the hormone dissociates from the receptor when the hormone titer is low.
3. Receptors possess higher affinity for its own hormone. Hence, they can bind with the hormone even its concentration is very low. Usually, the receptor number is such that they can be saturated at physiological concentration of the hormone.
4. Hormones which on binding with the receptor produces biological response are called agonists. Hormone binds with its own receptor with highest affinity. There are hormones which can bind with receptors for other hormones but the potency will vary. The potency or sensitivity of an hormone is expressed as EC_{50}. The effective concentration of a full agonist for eliciting half maximal response is called its EC_{5}. Binding of hormone to receptors other than its own is termed as **specificity spillover**. This happens because of some structural similarity between the two hormones. When a full agonist binds with more than one receptor, its EC_{50} will vary according to the receptor with which it binds. EC_{50} will be minimum when it binds with its own receptor and it shows an increase when it binds with receptor for other hormones. However, in both the cases maximum response will be obtained if the concentration of the hormone is suitably increased. However, a partial agonist cannot fully activate the relevant receptor and consequently produces a partial response. For example, cortisol, corticosterone and aldosterone are all full agonist for glucocorticoid receptors. However, they show a descending order of potency and an ascending order of EC_{50}. Thus regarding EC_{50} cortisol < corticosterone < aldosterone and regarding potency certisol > corticosterone > aldosterone. In all these cases maximum response will be obtained if the concentration of the hormone is raised to required concentration. Progesterone is a partial agonist for glucocorticoid receptor. It produces a partial response. Fig. 1.

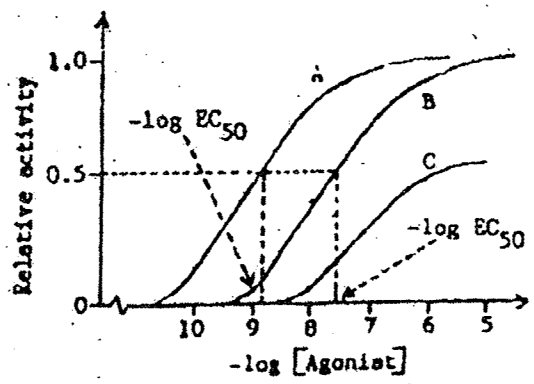


Fig. 1: Dose-response curves of (A) a full agonist of higher potency. (B) a full agonist of lower potency, and (C) a partial agonist.

5. In many cases a minimum fraction of the total receptors must get bound with the hormone to produce the response. This is called **threshold receptor occupancy**. In some cases like steroids and some peptide hormones, the full contingent of the receptors should combine with the hormone to produce maximal response. But catecholamines and some peptide hormones produce maximum response when many of their receptors remain unoccupied. Such unoccupied receptors are called **spare receptors** for the specific response. These spare receptors increase the sensitivity of the hormone. If spare receptors exist for a response the loss of some receptors increase the EC_{50} of the hormone, but maximum response will be obtained if the hormone concentration is suitably increased. However, in case of hormone action where spare receptors are not present loss of some receptors will reduce the maximal response but the sensitivity of the hormone is not changed as evidenced by no change in EC_{50}. Fig. 2.

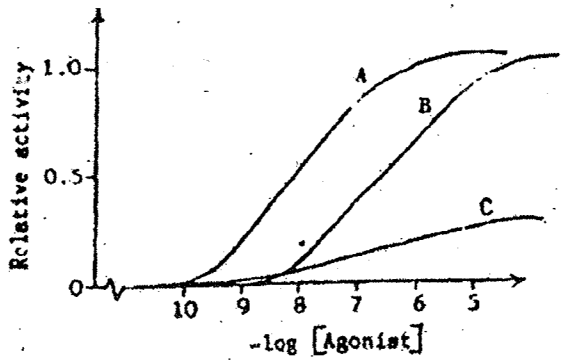


Fig.2 : Effects of loss of receptors on the dose-response curve of an against. (A) Normal receptor number. (B) Loss of some receptors when spare receptors exist. (C) Loss of some receptors in the absence of spare receptors.

An antagonist is an agent which binds with the receptor and blocks the action of the hormone. A competitive antagonist (propanolol, a β blocker for adrenaline) binds with the receptor and inhibits the hormone action by increasing EC_{50}, the maximum response is not affected. A noncompetitive antagonist does not alter EC_{50} but the maximum response is decreased. Fig. 3.

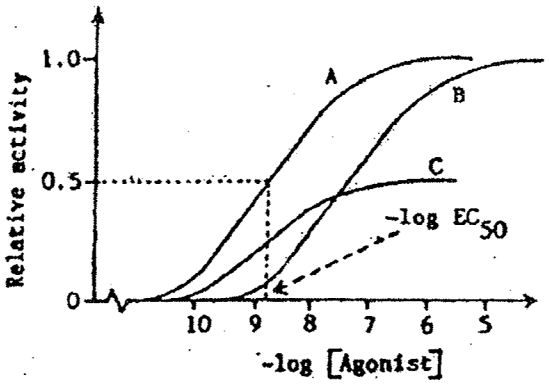


Fig. 3: Dose-response curve of (A) an agonist. (B) the agonist plus a competitive antagonist, and (C) the agonist plus a noncompetitive antagonist.

6. As a single receptor can bind with many hormones so also there are situations where one hormone can bind with more than one receptor. These receptors are called **isoreceptors**. The hormone will exhibit different response depending on the nature of the receptor with which it binds. For example, vasopressin binds to V_1 and V_2 isoreceptors on the plasma membrane of arteriolar smooth muscles and the cells of distal and collecting tubules of the nephron respectively. The effect of the hormone on arteriolar smooth muscle is contraction through Ca^{++} and increased reabsorption of water from the distal and collecting tubule via cAMP mechanism. α and β receptors are isoreceptors for catecholamines. The D_1 and D_2 are the isoreceptors for dopamine. In case of isoreceptors the action of the hormone depends on the nature of isoreceptor with which it binds and tissue distribution of the isoreceptors.

7. Cellular responsiveness to a hormone is regulated by changing the number and activity of the receptors.

   a) **Regulation of receptor concentration.** When the concentration of an agonist is increased, the cellular concentration of its receptor is rapidly reduced. This is called **down regulation**. If spare receptors exist, such down regulation will reduce EC_{50} but maximal response will remain unaffected. But if spare receptors does not exist, the down regulation will not change EC_{50} but maximal response will be decreased. The down regulation is brought about by a decreased synthesis or

increased degradation of the receptor. Rise in blood level of adrenaline or insulin will down regulate β adrenergic or insulin receptors respectively. Some hormones can also down regulate the receptors for other hormones. For example, progesterone down regulate or decrease the number of prolactin receptors in the plasma membranes of mammary glands.

A few hormones (agonists) can increase the number of their own receptors in the target cells. For example, prolactin increases the number of prolactin receptors in the plasma membrane of mammary gland and ovary. Such **up-regulation** of the receptors increase the cellular responsiveness to an agonist on chronic exposure. Some hormones can up-regulate the receptors for other hormones. For example, estrogen enhances the number of prolactin receptors in the liver and of progesterone receptors in the uterus and mammary gland.

b) **Regulation of receptor effector coupling.** The down regulation of the receptors is often accompanied with a functional uncoupling of the remaining receptors. For example, increased blood adrenaline level down regulates the β adrenergic receptors; moreover, it causes a cAMP mediated phosphorylation of the remaining receptors after down regulation. These phosphorylated β receptors become inactive, they cannot active adenylate cyclase after binding to adrenaline. The responsiveness is much decreased due to combined effect of down regulation and functional uncoupling. On the contrary, thyroxine not only up-regulate the β adrenergic receptors, it also enhances their functional coupling with adenylate cyclase.

A prolonged use of an antagonist, however, increase the number of its receptors and thus enhance the cellular responsiveness to the agonist.

MECHANISM OF ACTION OF PEPTIDE HORMONE

Hydrophilic peptides and protein hormones, catecholamines bind to specific receptor proteins on plasma membrane (fixed receptors). This causes change in concentration of certain chemicals which are termed as 'second messenger'. These second messengers, cause various changes in the composition of the cytosolic proteins leading to specific effect.

Various types of second messengers discovered till date. Some of the widely known and involved in hormone action are discussed below.

A. Hormone action mediated through cAMP.

Adrenal catecholamines and most of the peptide hormones increase intracellular cAMP level through activation of adenylate cyclase.

I. The first step involves **hormone-receptor binding.** The receptor proteins are serpentine in nature and spans the cell membrane 7 times. These receptors have two binding sites generally. One extracellular domain for bidning with the peptide or protein hormones. And other intracellular 3rd loop for interacting with G-Protein.

When the hormone binds to extracellular domain of the receptor, there is a conformational change in the receptor which causes activation of G-Proteins. Fig. 4.

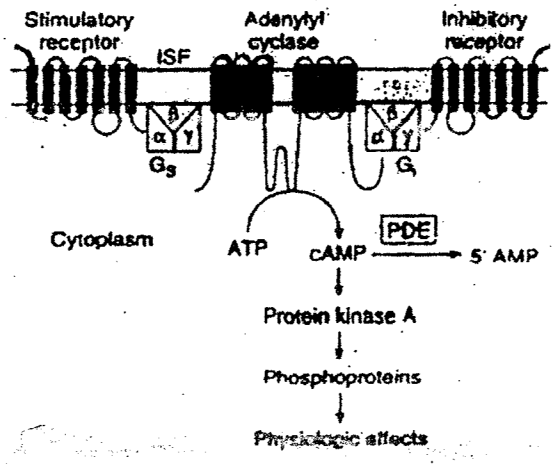


Fig. 4. The cAMP system. Activation of adenyly or adenylate cyclase catalyzes the conversion of ATP to cAMP. Cyclic AMP activates protein kinase A which phosphorylates proteins producing physiologic effects. Stimulatory ligants bind to stimulatory receptors and activate adenylyl cyclase via G_s inhibitory ligants inhibit adenylylcyclase via inhibitory receptors ISF, interstitial fluid.

II. The next step comprises of regulation of adenylate cyclase by **G-Proteins.** The G-Protein has three subunits namely β subunit (35 kdal), γ subunit (8.4 kdal), and an α subunit, which has two varieties. α-stimulatory or α_s- - subunit (45 kdal) and α inhibitory or α_i subunit (40 kdal). The G-Protein is termed as stimulatory or inhibitory on the nature of its α subunit.

At inactive state the α-subunit of G-Protein is bounded with GDP molecule. As the G-Protein is activated by serpentine receptor, the GDP goes out and a GTP molecule binds with the α-subunit of the G-Proteins and the α-subunit comes out of the G-complex.

The α-GTP complex binds to cytoplasmic surface adenylate cyclase and regulate it. If α_s binds to adenylate cyclase, ATP is converted to cyclic AMP, but if α_i binds to adenylate cyclase aforementioned conversion stops.

After a very short time, the α-GTP complex is converted to α-GDP due to GTPase activity of α-subunit. The α-GDP complex rebinds to βγ proteins of the G-Protein.

Cyclic AMP formed, is cleaved to 5 AMP by the action of phosphodiesterase (PDE). cAMP is very short lived molecule. Fig. 5 and 6.

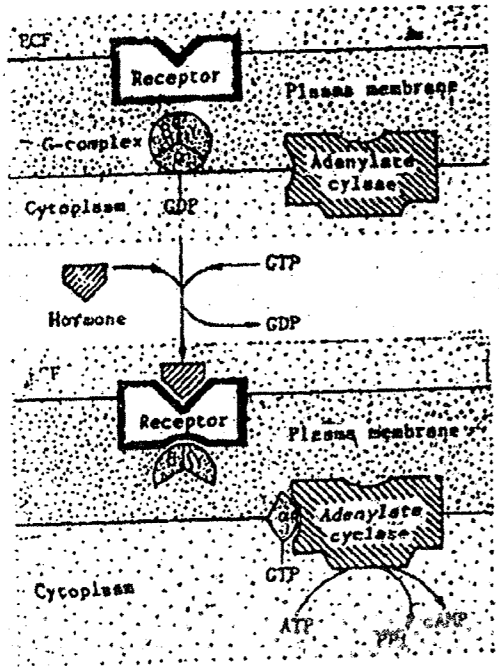


Fig. 5 : A model for the signal transduction by the G-complex in activating adenylate cyclase.

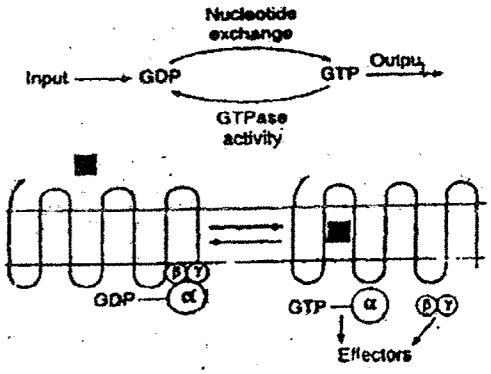


Fig. 6 : Heterotrimeric G proteins. **Top :** Summary of overall reaction. **Bottom :** When the ligand (square) binds to the serpentine receptor in the cell membrane GTP replaces GDP on the α subunit GTP-α separates from the βγ subunit and GTP-α and βγ both activate various effectors producing physiologic effects. The intrinsic GTPase activity of GTP-α then converts GTP to GDP and the α, β and γ subunits reassociate.

III. Next step is activation of protein kinase A by **cyclic AMP**. Protein kinase A remain in heterotetrameric form normally. In this form two catalytic subunits are bounded to two allosteric subunits and the enzyme is in inactive form because the substrate binding site remain covered. When 4 molecules of cAMP binds with two regulatory subunits, they dissociate and the catalytic subunits become free, the substrate binding site is exposed, and become active. Fig. 7.

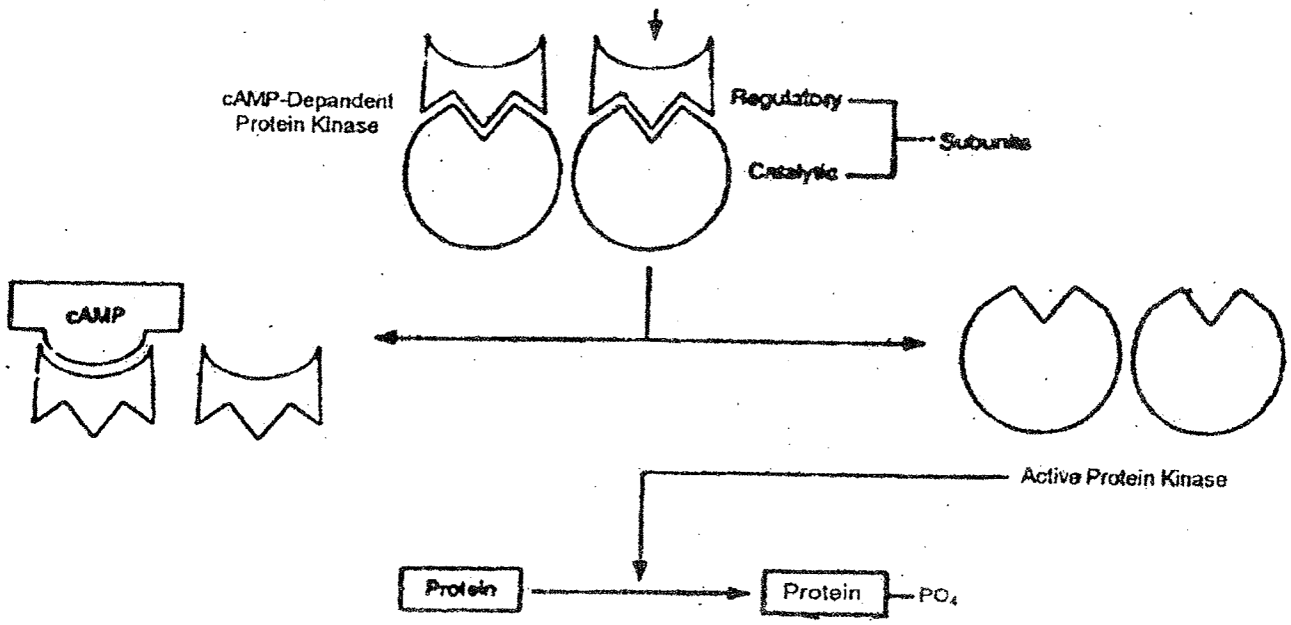


Fig. 7 : Protein kinase activation

IV. The last step is activity of protein kinase A. Protein kinase A activates many proteins and enzymes by phosphorylating the hydroxyl group of Ser or Thr. amino acids of proteins and the metabolic activity of the cell is altered.

Genetic regulation by the enzyme Protein kinase A:

Small amount of protein kinase A enters the nucleus through porous nuclear membrane and activates cAMP response element binding (CREB) protein by phosphorylating it. This CREB-protein binds with cAMP regulated enhancer (CRE) site of the DNA molecule and thus regulate DNA synthesis. Fig. 8.

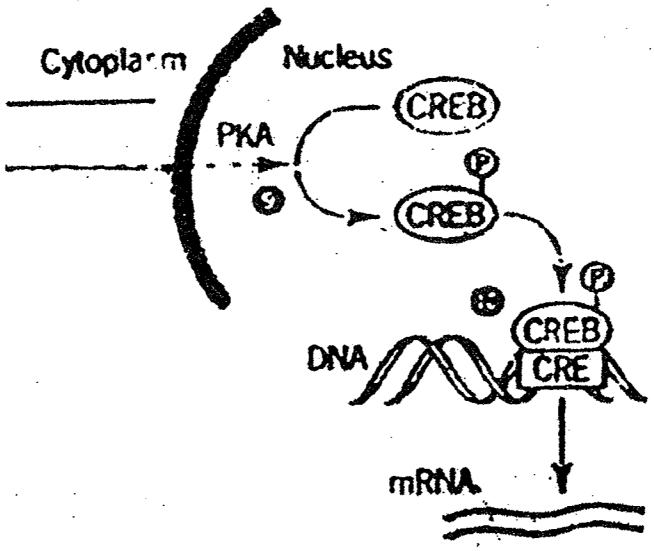


Fig. 8 : Genetic requlation by PKA

Thus, the cAMP mediated action is effected both by activating and deactivating the proteins as well as by altering the synthesis of the proteins.

B) Cyclic GMP:

Cyclic GMP or cGMP acts as a second messenger. Atrial natriuretic peptide (ANP) and Nitric Oxide (NO) acts via CGMP.

cGMP is made from GTP by the action of Guanylyl or Guanylate cyclase which exist in two forms (i)membrane bound and (ii) soluble form.

The membrane bound form has an extracellular aminoterminal receptor domain, a transmembrane domain and a cytoplasmic carboxyl terminal that has a tysosine kinase like and a guanylate cyclase catalytic domain. Three such receptors are known. Two are receptors for ANP and third binds with E. coli enterotoxin and guanylin.

The soluble form of the enzyme is completely intracellular and it contains heme. It has several isoforms. They are activated by nitric oxide (NO) and NO-containing compounds. Hormone receptor interaction leads to formation of cGMP. This allosterically activates protein kinase G which in turn phosphorylates a number of smooth muscle protein including myossin light chain leading to vasodilation, smooth muscular relaxatino and other effects.

C) Phosphoinosital and Diacylglycerol:

Hormones like vasopressin (smooth muscle action via V_1 receptor) TRH, GnRH and gastrin act through phospholipase C-Phosphoinositol system.

These hormones bind to its specific receptor protein on external surface of plasma membrane. This activates a G_s-like heterotrimeric and GTP binding membrane protein called G_p-protein.

The G_p protein activates phospholipase C of the cell membrane. This phospholipase C catalyses conversion of Phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and Diacylglycerol (DAG).

IP_3 is phosphorylated in the cytoplasm by IP_3-3 phosphokinase and Inositol 1,3,4,5 tetrakis phosphate (IP_4) is formed. This IP_4 enhances mobilization of Ca^{2+} from extracellular fluid. DAG activates one of the seven subspecies of protein Kinase-C. It then phosphorylates various proteins and modulate their activities. DAG is also converted to Monoacyl glycerol (MAG) & arachidonic acid. This arachidonic acid is converted to leukotriene and prostagladins which in turn modulates cellular activities. Fig. 9.

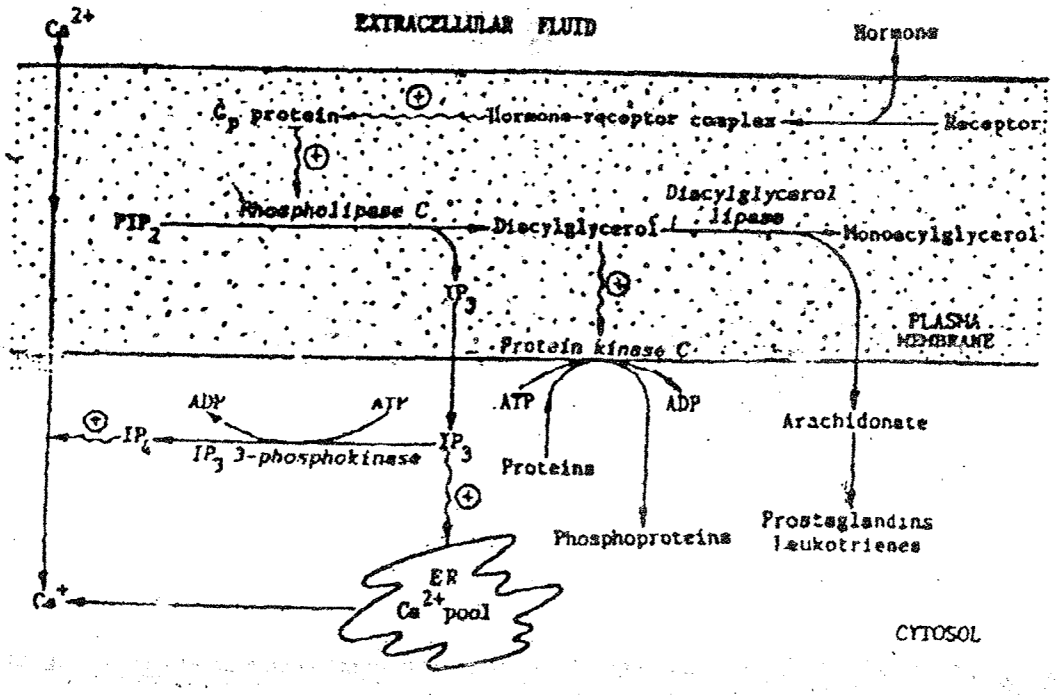


Fig. 9 : Action of the phospholipase C-phosphoinositol system in signal transduction.

The activation of protein KinaseC (PKC) has some relevance. Normally, PKC contain 3 polypeptide domain. A catalytic domain C, an allosteric domain A & a pseudosubstrate domain P. In inactive state, P fits in the catalytic unit of C and thus blocks any catalytic action. P is conformationally supported by A. But as conc. of DAG & Ca^{2+} with phosphatidyl serine increase, the P site comes out from catalytic unit of C and there is a conformational changes in C that makes the substrate binding more suitable.

Genetic regulation by Protein KinaseC:

Some of the protein KinaseC, diffuses into nucleus and causes production of C-Fos and C-Jun by immediate early genes, by phosphorylation. The C-Fos and C-Jun heterodimer binds to AP-I site having a sequence TGAGTCA and alter transcription. Fig. 10.

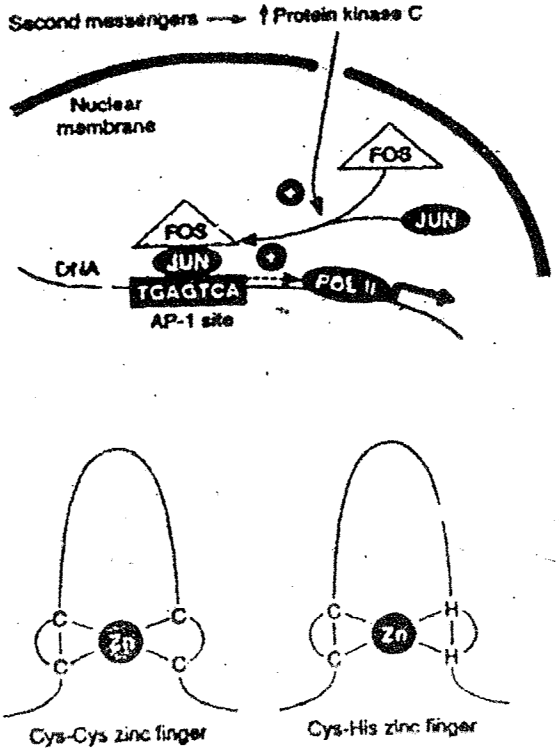


Fig. 10 : Top : Activation of genes by second messengers. Increased protein kinase C causes production of c-Fos and C-Jun by immediate-early genes. The c-Fos-c-Jun heterodimer binds to an AP-1 site, in this case activating RNA polymerase II (Pol II) and increasing transcription of other genes. **Bottom** : Zinc fingers. The curved lines represent polypeptide chains of proteins that bind to DNA, and the straight lines indicate coordiante binding of zinc to cysteines (C) or cysteines and histidines (H).

D) Hormone action mediated through calcium:

Gn RH, Vasopressin (action on smooth muscle), Oxytocin, Catecholamines (α_1 effect) when bind to their respective membrane receptors, intracellular Ca^{2+} concentration is increased. Ca^{2+} then acts as a second messenger and produces biological response. Ca^{2+} regulates the activity of many enzymes like phospholipases A_2 and C; PKC; adenylate and guanylate cyclases; glycogen synthase, cyclic nucleotide phosphodiesterase; myosin light chain Kinase and thus modulate cellular reactions. Ca^{2+} also affects cell motility, membrane permeability, exocytosis, endocytosis and actin-myosin interactions in different target cells.

Hormone-receptor interaction increases calcium ion concentration in different ways:

1. Phospholipase C-polyphosphoinositol system is activated and IP_3 and IP_4 are produced. They increase Ca^{2+} concentration.
2. Ca^{2+}-ATPase or Na^{-} - Ca^{2+} exchanger of plasma membrane is inhibited and the outflow of Ca^{2+} is decreased. Consequently, intracellular Ca^{2+} concentration is increased.
3. Voltage independent Ca^{2+} channels in the membrane are opened directly and entry of Ca^{2+} into the cell is increased. Fig. 11.

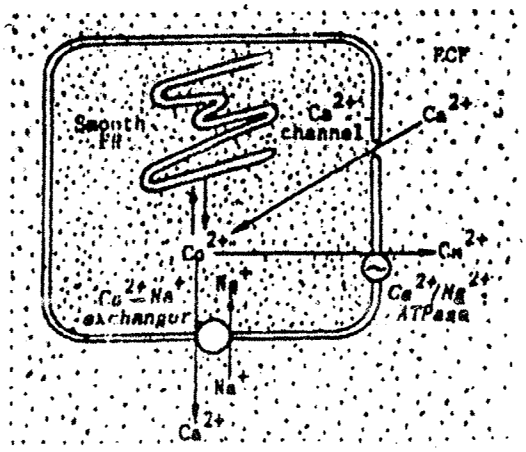


Fig. 11 : Ca^{2+} fluxes between the cell and the extracellular fluid.

Hormone action mediated through tysosine Kinase. There are two situations.

I) In one case the receptor of the hormone of the hormone has an intrinsic **tyrosine kinase activity**, i.e. tyrosine kinase catalytic domain is present in the cytosolic end to the receptor itself. Receptors for EGF, IGF-I & insulin have this type of activity.

As the hormone binds to the receptor, the receptor exhibit a conformational change resulting in activation of tyrosine kinase dmain.

The activated domain at first phosphorylates itself. Phosphorylated tyrosine kinase thus formed has more activity compared to the non-phosphorylated form.

This phosphorylated receptor phosphorylates Insulin receptor substrate (there are at least four of these molecules called IRS 1-4). SH_2 domain of a variety of proteins, that are directly involved in mediating the action of hormones, gets bound with phosphorylated IRS, get activated and exhibit sequential activation.

General regulation by tyrosine kinase:

One of the SH_2 domain containing protein is Growth factor receptor binding (Grb) protein 2. This Grb2 is associated with son of sevenless (SOS) protein. Combination of Grb2-SOS with IRS-IP causes the Ras protein to exchange bounded GDP for GTP, Ras-GTP complex is formed and it activates Raf kinase. After a while Ras-GTP is converted to Ras-GDP by GTPase action of Ras protein.

The activated Raf kinase formed, phospherylates and activates Mitogen activated protein kinase kinase (MAPKK) which in turn activates Mitogen activated protein kinase (MAPK) and convert it to phosphorylated MAPK (MAPK-P). This MAPK-P acts in two ways. It phosphorylates P90 ribosomal protein S-6 kinase (P90) rsk) and MAPKinase activated protein kinase-2 (MAPK APK-2). These two causes activation of various transcription factors, (TF) which enters the nucleus, binds with DNA and modify transcription. Fig. 12, 13.

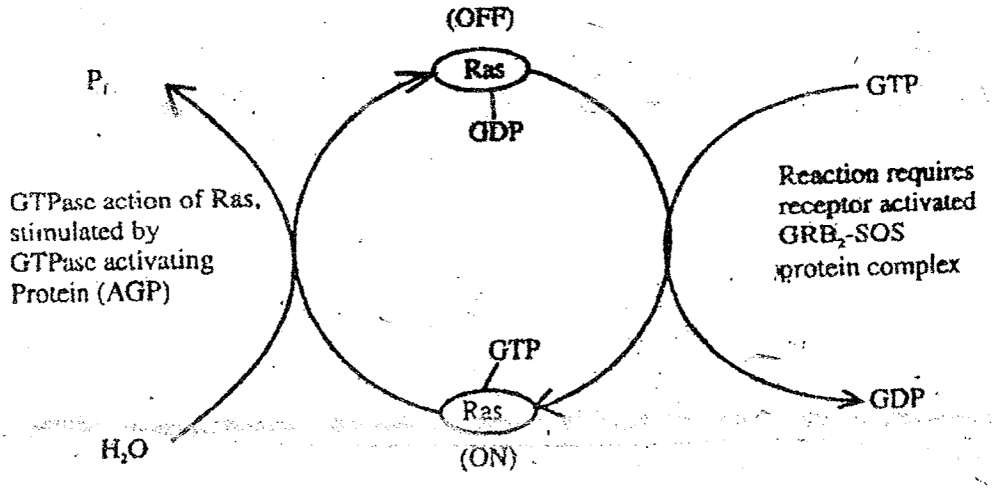


Fig. 12 : Activation deactivation of Ras

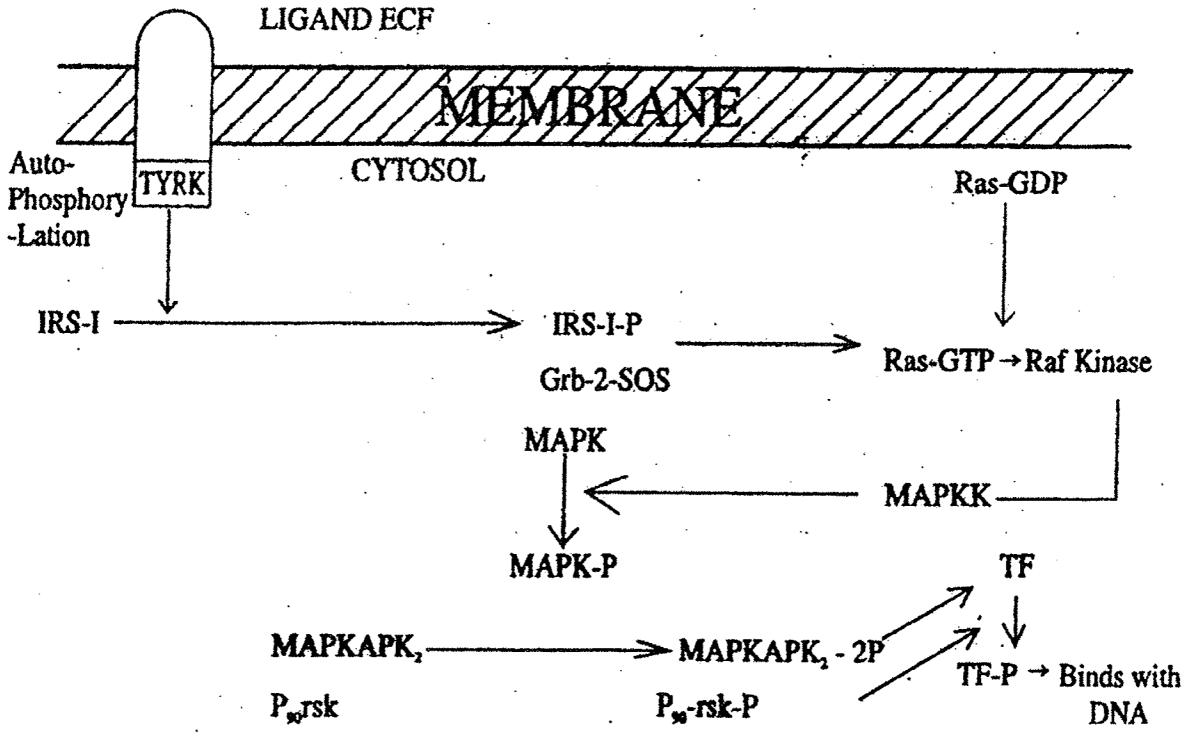


Fig. 13 : Ras Pathway

In the second situation, the hormone receptor itself has no tyrosine kinase activity. But when hormone binds with the receptor, the receptor dimerize and cycloplasmic protein tyrosine kinases (like Tyk-2, JAKI, or JAK2) associated with the receptor are activated. This results in phosphorylation of the receptor as well as tyrosine kinases on tyrosyl residues. The phosphotyrosine residues of the receptor bind several SH_2 domain containing proteins present in the cytoplasm and they are subsequently phosphorylated by JAK kinase. These events result in the activatino of a number of signaling pathways.

The STAT (Signal transduction and activators of transcription) proteins associate with phosphorylated receptor. They are then phosphorylated by AJK-P. STAT-P (phosphorylated STAT Protein) dimerises, translocates to the nucleus, binds to specific DNA elements and regulates transcription. Similar docking events result in (i) SHC/Grb2 associated activation of MAP kinase pathway; (ii) IRS phosphorylation with activation of PI3 kinase; (iii) G-protein mediated activation of PLC with production of diacylglycerol and activation of protein kinase C. Some hormones like growth hormone, prolactin, erythroposetin produce their effect through this pathway. Fig. 14.15.

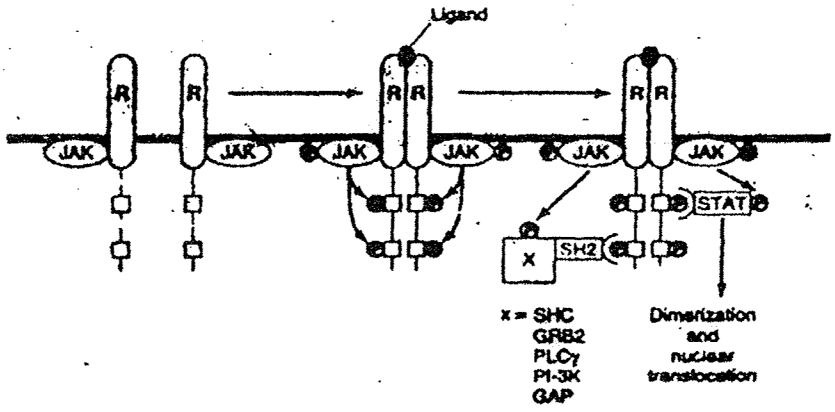


Fig. 14 : Initiation of signal transduction by receptors linked to JAK kinases. The receptors that bind prolactin, growth hormone, interferons, and cytokines lack endogenous tyrosine kinase. Upon the ligand binding, these receptors dimerize and an associated protein (JAK1, JAK2, or TYK) is phosphorylated. JAK-P, an active kinase, phosphorylates the receptor on tyrosine residues. The STAT proteins associate with the phosphorylated receptor and then are themselves phosphorylated by JAK-P. STAT. P dimerizes, translocates to the nucleus, binds to specific DNA elements, and regulates transcription. The phosphotyrosine resides of the receptor also to several SH2 domain-containing proteins. This results in activation of the MAP kinase pathway (through SHC or GRB2), PLCγ or PI-3 kinase.

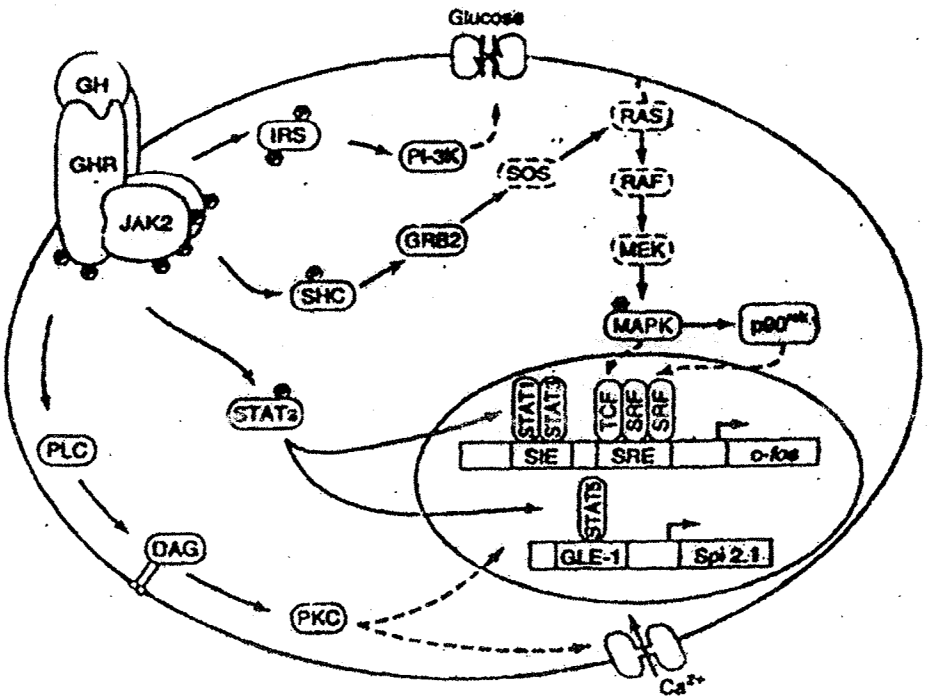


Fig. 15 : Signal transduction pathways activated by the interaction of growth hormone (GH) with its receptor (GHR). As described in the text. OH action may involve four different pathways, each of which is shown in this figure. The activation of JAK2, with subsequent activation of STATs 1 and 3, results in the binding of these transcription factors to specific genes, *c-fos* and Spi 2.1 in this case. The other pathways can be used by different hormones.

Recent evidence suggest that HRE associate with other elements to function optimally. Such assembly of DNA and other factors is called hormone response unit (HRU). An HRU consists of one or more HREs and one or more DNA elements with associated accessory factors. The communication between an HRU and the basal transcription apparatus is accomplished by one or more of a class of coregulator molecules. One such corregulator molecule in CREB binding protein called CBP. A similar related molecule, called p300 interact with other signaling molecules including CREB. It has been observed that CBP/p300 has intrinsic histone acetyltransferase activity (HAT). It has been suggested that this enzyme causes acetylation of histone and results in the remodeling of chromatin into a transcription-efficient environment and thus transcription is stimulated. The observation that histone deacetylation causes inactivation of transcription supports this concept. Fig. 16.

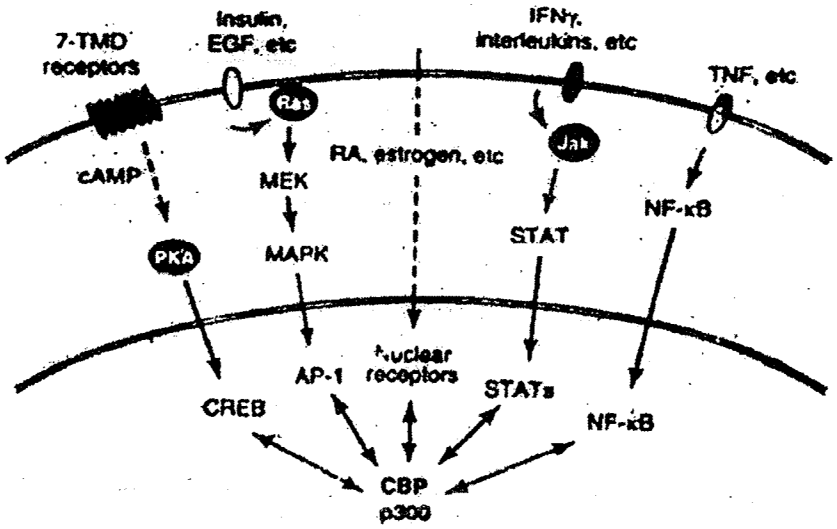


Fig. 16 : Several signal transduction pathways converge on CBP/p300. Ligands that associate with membrane or nuclear receptors eventually converge on CBP/p300. Several different signal transduction pathways are employed.

MECHANISM OF ACTION OF STEROID HORMONE

Steroid hormones act by binding with steroid hormone receptors located in the cytoplasm and mainly in the nucleus. Basically the receptor molecule has a ligand (Hormone) binding domain near the c-terminal end of the receptor. Towards the middle it has a DNA binding domain which is rich in cystein residue. Fig. 17.

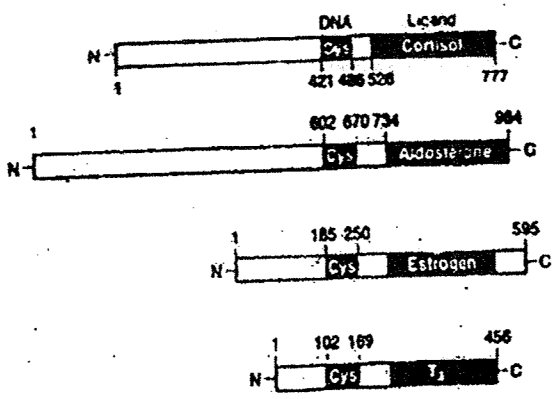


Fig. 17: Structure of human glucocorticoid, mineralocorticoid, α-estrogen, and βT_3 receptors. None that each receptor has a cysteine-rich DNA-binding domain and a ligand-binding domain at or near the crboxyl terminal, with considerable variability in the amino terminal part of the protein. The numbers identify amino acid residues.

When the hormones bind with the receptors the DNA binding domain of the receptor becomes capable of binding with the hormone response element (HRE) of DNA and thereby modulate the activity of DNA (Fig. 18 & 19).

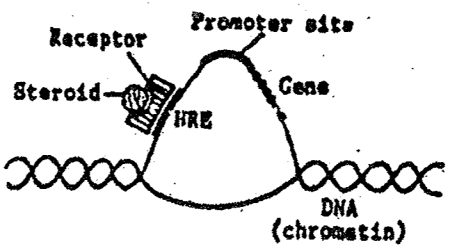


Fig. 18 : Interaction of steroid-receptor complex and hormone responsive element (HRE) on the upstream side of the promoter site of a gene.

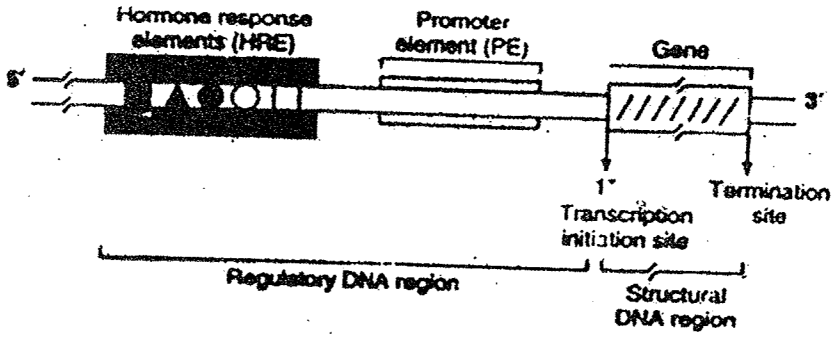


Fig. 19 : Structural requirements for hormonal regulation of gene transcription.

The specificity of receptor DNA interaction is modulated by the N-terminal domain of the receptor. Interaction of hormone receptor occurs with the DNA through DNA-motifs. There are different DNA-motifs; of them, cys-cys-zinc finger motif (Fig. 10) is involved in the mechanism of action of steroid hormones. The receptor binds with DNA with Zn-finger motif.

The expression of most genes is also controlled by enhancer and silencer elements of DNA. These elements may be located far away from the transcription initiation site. However, when transcription factors bind with enhancer or silencer elements, they increase or decrease respectively the rate of transcription.

The steroid hormone receptor has several domains one binding with hormone, one binding with DNA and one that activates transcription. In case of glucucorticoid receptor (777 amino acid residues), the molecule has been found to contain several functional domains:

I) A hormone binding region in the C-terminal portion.

II) An adjacent DNA binding region.

III) At least 2 regions that activate gene transcription.

IV) At least 2 regions responsible for translocation of the receptor from the cytoplasm to the nucleus.

V) A region that binds heat shock protein in absence of the ligand.

The DNA sequence to which the hormone receptor binds is a palindrome —

$$\xleftarrow{5'AGAACA}\ NNN\ \xrightarrow{TGTTCT3'}$$

$$\xleftarrow{3'TGTTCT}\ NNN\ \xrightarrow{AGAACA5'}$$

N means any one of the four can be used in that position. The arrows pointing in opposite directions illustrate the slightly imperfect inverted palindromes present in many HREs. DNA sequences of several hormone response elements (HREs) have been worked out (Table 1).

Table 1: The DNA sequences of several hormone response elements (HREs).[1]

Hormone of Effector	HRE	DNA Sequence
Glucocorticoids	GRE	
Progestins	PRE	
Mineralocarticoids	MRE	GGTACA NNN TGTTCT
Androgens	ARE	AGGTCA TGA/TCCT
Estrogens	ERE	AGGTCA N3,4,5 AGGTCA
Thyroid hormone	TRE	
Retinoic acid	RARE	
Vitamin D	VDRE	
cAMP	CRE	TGACGTCA

[1]Letters indicate nucleotide; N means any one of the four can be used in that position. The arrows pointing in opposite directions illustrate the slightly imperfect inverted palindromes present in many HREs; in some cases these are called "half binding sites" because each binds one monomer of the receptor. The GRE, PRE and ARE consist of the same DNA sequence. Specificity may be conferred by the intracellular concentration of the ligand or hormone receptor, by flanking DNA sequences not include in the consensus, or by other accessory elements. A second group of HREs includes those for thyroid hormones, estrogens, retinoic acid and vitamin D. These HREs are similar except for the orientation and spacing between the half palindromes. Spacing determines the hormone specificity. VDRE (N=3), TRE (N=4) and RARE (N=5) bind to direct repeats rather than to inverted repeats. Another member of the steroid receptor superfamily, the retinoid X receptor (RXR), forms heterodimers with VDR, TR and RARE, and these constitute the *trans*-acting factors. cAMP affects gene transcription through the CRE.

In case of glucocorticoid receptor it has been found that the DNA binding domain of each subunit of the dimer receptor bind with one half of the DNA sequence. This is actually an enhancer element GRE (Glucorticoid response element). Hence, such binding enhances the rate of transcription. If this sequence is deleted, the level of transcription is much decreased, when such enhancer element (DNA sequence) is introduced into the up stream region of a gene that normally does not respond to glucocorticoids the transcription of this gene is increased by glucocorticoid. The dNA motif has two α-helical loops (Zn-fingers). The two zn-fingers have

different roles. One recognizes and binds with DNA sequence, the other one binds with other subunit of the receptor dimer and thus helps in dimerization. DNA-protein interaction involves the forces like — (i) van der walls (hydrophobic) forces; (ii) ionic bonds; (iii) hydrogen bonds between amino acid residues and various parts of DNA including the backbone.

The hormone receptor in its free form cannot bind with DNA but binding occurs when hormone binds with receptor. Different possibilities have been suggested for such action.

1. i) The steroid hormone-receptor molecule normally when not bound with hormone, remain phosphorylated and aggregated probably with some inhibitory proteins into oligomers.

   ii) These inactive receptor heteromers have poor affinity for the nuclear chromatin (DNA).

   iii) As the specific steroid hormone binds to the ligand binding site of the receptor molecule, the receptor heteromers dissaggregates and release the inhibitor proteins.

   iv) This results in the formation of steroid-bound-receptor monomers, exposing the DNA-binding site in the central domain of the latter.

   v) The consequent rise in affinity of that site for receptor accepting region of DNA enables the active steroid-receptor complex to bind to the HRE in the DNA strand.

   vi) This changes the transcription rate of the relevant gene by altering the efficiency of the promoter in binding to the RNA polymerase. Fig. 20.

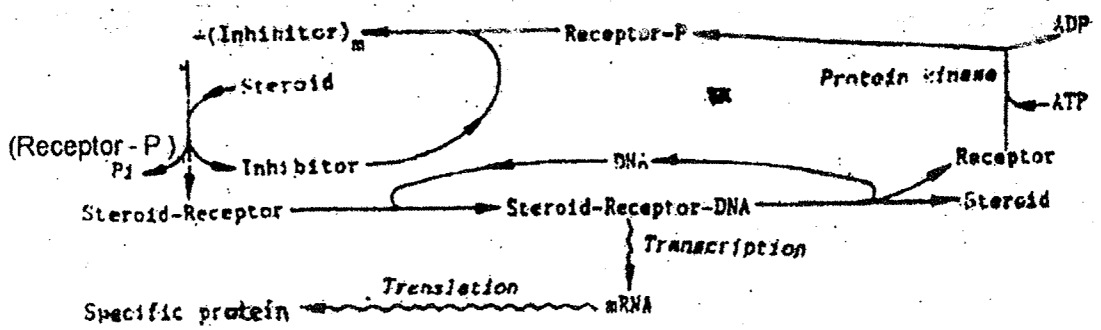


Fig. 20 : Mechanism of action of steroid hormones through interaction with nuclear reactors.

2. Heat shock proteins have been shown to be involved in the activation of DNA. It is held that at least in case of glucocorticoids, estrogen and progesterone the receptors remain bound with heat shock protein (Hsp 90) and other proteins when it is not bound with steroid hormone. It is believed that the Hsps cover the DNA binding domain. When steroid binds with the receptor the heat shock protein is released and thus the DNA binding domain is exposed.

3. It has also been suggested and observed specially in case of glucocorticoid receptor that in absence of steroid the steroid binding domain prevents the receptor from recognising the glucocorticoid response element of DNA and prevent activation of transcription. This prevention may be due to an interaction of steroid binding domain and the DNA binding domain either directly or by interacting proteins. Binding of the steroid disrupts this interaction and thus facilitates interaction between DNA binding domain and the HRE.

RAPID ACTION OF STEROID HORMONES

Some of the action of steroids are much more rapid than those mediated via binding to DNA. For example, rapid increase in Ca^{++} conc. in sperm heads that is produced by progesterone; prompt steroid induced alteration in the functions of various neurons. This has led to the hypothesis that steroids can also bind to $GABA_A$ membrane receptors and thus the rapid actions are produced. Steroids have been found to bind to $GABA_A$ receptors, facilitating their action.

It has been found that metabolites of steroid hormones like progesterone and dexycorticosterone bind to $GABA_A$ receptors and increase Cl^- conductance. It has been known for many years that progesterone and deoxycorticosterone are sleep inducing and annesthetics in large doses. It has been observed that aldosterone binds with membrane receptor and increases the activity of membrane $Na^+ - K^\bullet$ exchangers. This produces an increase in intracellular Na^+ and the second messenger involved is probably IP_3.

Questions:

1. Write briefly on:

   (i) Threshold receptor occupancy and specificity spillover.

   (ii) EC_{50}

(iii) Spare receptor

(iv) Isoreceptor 5x4=20

2. Give a short account of regulation of hormone receptor. 10

3. Describe the mechanism of hormone action through adenylate cyclase. 10

4. How protein kinase A is activated Add a note on genetic regulation by PKA. 5+5

5. Describe the mechanism of hormone action through phosphoinositol and diacylglycesol. 10

6. What is Zn-finger mohf? How hormone action is mediated via calcium? 4+6

7. Describe the mechanism of hormone action mediated through Ras pathway. 10

8. Write briefly on the mechanism of hormone action through JAK-STAT pathway. 10

9. Describe the mechanism of a action of steroid hormones. 10

10. Comment on the rapid action of steroid hormones. Write briefly on genetic regulation by Protein kinaseC. 5+5

Further reading:

1. Review of Medical Physiology - by W.F. Ganong.

2. Text book of Medical Physiology - byGuyton/Hall.

3. Best and Taylor's Physiological basis of medical Practice. Edited by J.B. West.

4. Principles of Biochemistry — by Lehninger/Nelson/Cox.

5. Harper's Biochemistry — by Murray / Granner / Mayes / Rodwell.

6. Biochemistry — by D. Das.

VIDYASAGAR UNIVERSITY

DIRECTORATE OF DISTANCE EDUCATION
MIDNAPORE - 721 102

M.Sc. in Zoology

Part - I
Paper - II, Unit - II, Group - B

Module No. 22

OSMOSIS

The moment of solvent from solvent side to the solution side or from dilute solution to a concentrated solution when the two are separated by a seminpermeable membrane is called osmosis. Semipermeable membrane is that membrane which allows only the solvent molecules to pass through and not the solute molecules.

Osmotic pressure (OP) is the pressure which has to be applied on the solution side to stop the osmotic inflow into it from pure solvent.

Van't Hoff laws of osmotic pressure :

1. It is directly proportional to the molar concentration of the solute so long as the temperature remains constant.

   $\pi = k_1 c$

   π = op k_1 = constant; C = molar concentration.

2. OP of a solution is directly proportional to the absolute temperature (T) so long as its concentration remains constant,

   $\pi = K_2 T$ K_2 = constant.

3. Van't Hoff–Avogardo Law :

   Identical members of moles of different solutes produce an identical osmotic pressure when dissolved in the same volume of the solvent at the same temperature.

OP is expressed as atmosphere, mm of Hg or dynes per sq. cm. (dynes cm^{-2})

1 mole of a nonionized solute is equivalent to 1 osm. (osmol).

Osmolarity of a solution is its solute conc. in osmols per litre of the solution.

A solution of one m0sm of any solute in a litre possess an osmotic pressure of 19.3 mm HG at 38°C.

Osmolality of a solution is its solute conc. in 0sm. per KG of solvent.

Osmotic pressure can be determined by –

1. Osmometer.
2. Barkley – Hartley method.
3. Freezing point method. This method depends on the direct proportionality between the osmotic pressure and the depression of freezing point of a solution. (Depression of Freezing point is measured by Backmann Thermometer).

$\Delta t = KfM$ M = Molal conc. of solute.

$\therefore M = \frac{\Delta t}{Kf}$ Δt = Depression of freezing point of the solution.

Kf = Cryoscopic constant.

The freezing point of one Molal soln. is called cryoscopic constant or Kf / Molal freezing point.

It varies with the solvent. The Kf for water is –1.858°C.

$\Delta = CRT$ C = Molal conc.

R = Molar gas constant (0.082)

T = Temp. in Abs. degree.

π = Osmotic pressure in atmosphere.

A sample of urine freezes at –0.56°C calculation of its OP at 37°C.

Molal conc. $C = \frac{-0.56}{-1.858} = 0.3$

The sample will have OP at 37°C. ($\pi = CRT$).

0.3 x 0.082 x 310 = 7.6 atmos. or

7.6 x 760 mm. Hg.

Osmotic work :

When substances are transferred from a lower concentration to higher concentration osmotic work must be performed upon them. But when substances pass from higher to lower concentration. Osmotic work is done by them. Relation between osmotic work and conc. change is given by the equation:

$$W_{min} = NRT \ln \frac{C_2}{C_1} \text{ or } 2.NRT \log \frac{C_2}{C_1}$$

W_{min} = Minimum osmotic work in small calories involved in the transfer of N mols of substances from a molal conc. of C_1 to a molol conc. C_2.

R = Gas constant 1.987 cal / mol / degree.

T = Absolute temp. In = Natural log. 2.3 is the factor for converting natural log to log to the base 10.

Calculation of osmotic work to be done to transfer or secrete 3.54g of Cl^- from plasma to urine at 37°C when the Cl^- concentrations in plasma and urine are 0.1 and 0.2 Mol respectively.

Cl^- conc. in Plasma – 0.1 Molal C_1

Cl^- conc. in urine – 0.2 Molal C_2

Osmotic work to be done to transfer / secrete 3.54g of Cl^- at 37°C in urine.

N – 0.1 T = 310 R = 1.987

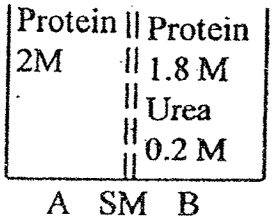
$$W \text{ min} = NRT\ 2.3 \log \frac{C_2}{C_1}$$

$$0.1 \times 1.987 \times 0x\ 0310 \times 2.3 \log \frac{0.2}{0.1}$$

141.7 log 2 = 1.417 x 0.301 = + 42.65 cal.

Osmoticity and tonicity :

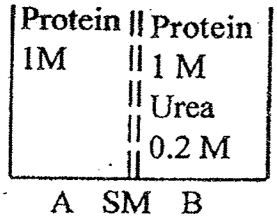
The osmoticity of a solution depends on the total solute concentration, both diffusible and non diffusible. Whereas, tonicity of a solution depends on the concentration of non diffusible solute only. Hence, two solutions may be isosmotic but may not be isotonic.



Solution A is isosmotic with solution B.

But solution A is hypertonic to solution B.

Similarly, two solutions may be isotonic but may not be isosmotic.



Solution A and B are isosmotic but the solution B is hyperosmotic to solution A.

SM = Semipermeable membrane.

Solvent always flows from hypotonic solution. In biological system, tonicity is considered because biological membranes are not strictly semipermeable. They allow some solute to pass.

Biological application of Osmosis :

1. **Hemolysis, crenation, plasmolysis :**

   RBC is hemolysed or animal cell ruptures if placed in hypotonic solution. This happens because solvent flows from hypotonic solution to RBC or cell. Consequently, they swell and burst at a certain degree of swelling. Similarly these cells or RBC will shrink (creanated) if placed in hypertonic solution. It is due to osmotic outflow of water to the hypertonic solution.

   Plant cells lose water when placed in hypertonic solution. So the cell membrane collapses and withdraws from the cell wall. This is called plasmolysis.

   Plant cells swell if placed in hypotonic solution, but they do not burst due to rigidity of cell wall.

2. **Osmotic distension of RBC :**

   Osmotic pressure of RBC fluid is 1.5atm. higher than plasma due to higher electrolyte concentration in RBC then plasma. So RBC remain slightly distended, but they do not rupture. However, due to genetic disorder, vit. E deficiency or selenium deficiency and other defects RBC may not be able to withsand such distension and rupture.

RBCs of camel are more resistant to osmotic distension. Camel can drink more than 100 liters of H_2O in 10 minutes. Blood becomes temporarily highly hypotonic but RBCs do not rupture. It has been observed that RBC may be distanded upto two times its volume but hemolysis does not occur.

3. Osmotic pressure of plasma is higher than tissue fluid. It is due to plasma proteins. Total osmotic pressure of plasma is about 5453 mm. Hg and that of ECF is 5430 mm. Hg. The difference of 23 mm. Hg is due to plasma proteins. It is called colloidal osmotic pressure of plasma. This osmotic pressure, hydrostatic pressure i.e. capillary pressure and pressure of the tissue fluid play an important role in the exchange of body fluid across the blood capillaries. Owing to pressure differences, fluid passes out from arterial end of blood capillaries to the tissue fluid and body fluid enters at the venous end. These forces also govern the flow of fluid between any two compartments.

   In Kwashiorkor, hepatic cerrhosis and nephrosis, plasma protein concentration is decreased. So colloidal osmotic pressure is decreased. This decreases water retention by the plasma and so edema develops.

4. Osmotic pressure plays a vital role in the absorption of water from intestine and kidney tubules.
5. Water absorption by plant roots is also governed by osmotic pressure. Root hair cells have higher osmotic pressure than surrounding soil-fluid. So water enters into root hair cells. Water moves from one cell to the next cell by cell to cell osmosis and thus other cells are also distended. Hence, the cells become turgid and rigid and they stand erect on watering.

DIFFUSION :

Particles, molecules or ions have a tendency to spread uniformly in the entire available space by their incessant random movements. This tendency to spread is called *diffusion*. Though they are moving at random in all directions, a greater number of particles move from a region of higher concentration to the area of lower concentration, than in the reverse direction. Hence, there occur a *net diffusion* from higher to lower concentration and the two concentrations become equal. In this state equal number of particles move in all directions and the net diffusion in zero.

Fick's first law of diffusion states that the rate of diffusion (flux) of a solute particle is directly proportional to the magnitude of concentration gradient and it occurs down the concentration gradient.

Graham's law states that the diffusional flux (J) of a gas varies inversely with the square root of its density (ρ) and and molecular weight (M).

Diffusion coeficient (D) denotes the diffusibility of the particle or gas. In case of solid, the 'D' is the mass of solute diffusing across 1 sq. cm. area in 1 sec. down a concentration gradient of unity. Diffusion coefficient of a gas is the volume of gas that diffuses across 1 sq. cm. area per sec. down a ΔP (partial pressure difference) of unity (i.e. ΔP of 1mm Hg per cm.).

Diffusion is directly proportional to :

i) Concentration gradient / pressure gradient / electrical gradient.

ii) Solubility in the medium.

iii) Temperature of the medium.

iv) Cross sectional area through which diffusion is taking place.

Diffusion is inversely proportional to :

i) Distance to be travelled / thickness of the membrane.

ii) Diameter of the diffusing particle.

iii) Viscosity of the medium.

iv) Shape of the particle –

Spherical particle diffuse easily than elongated particle.

v) Charge of the particle and charge of the pore. If the charges are same, diffusion will be less, if the charges are opposite, diffusion will be more.

Biological application :

1. Absorption of certain substances like pentoses, some minerals, some water soluble vitamins from intestine is carried out by diffusion; same is the case of renal reabsorption of Urea.

2. Water, water soluble substances pass largely by diffusion through water filled pores of the cell membrane. This is dependent on the pore size and size of the diffusing particle. Pore size is about 0.8 nm. Hydrated K^+ ion is 0.4 nm. diameter; hydrated Na^+ ion has 0.5 nm. diameter. Hence, movement of K^+ is two times faster than the movement of Na^+. Glucose and galactose molecules have a diameter of 0.85 nm. Hence, they cannot pass via pore. They pass via membranes after combining with some carrier.

3. There are voltage gated ion channels in the membrane. When these channels open, ions move by diffusion along electro-chemical gradient.
4. Exchange of respiratory gases occur by way of diffusion. The partial pressure of O_2 is higher in alveolar air and lower in the deoxygenated blood. So O_2 enters blood from the lungs. Alternatively the partial pressure of CO_2 is higher in deoxygenated blood and lower in the alveolar air. So, CO_2 diffuses from blood to alveolar air. In the same way, due to difference of partial pressure O_2 flows from the oxygenated blood to the tissue cell and CO_2 diffuses from the tissue cells to the blood.
5. The alveolar surface area per unit body weight is larger in children than adult humans. So the resting O_2 uptake from the alveoli is higher in children than in adults.
6. There is a difference of partial pressur of O_2 and CO_2 between alveolar air and inspired air. So, O_2 and CO_2 diffuse down their respective pressure gradients between the terminal bronchioles and alveoli. This is how the alveolar air has its O_2 renewed and CO_2 partially removed.
7. When the cell size is increased, the surface volume ratio is decreased. This decreases diffusion of gases and solute across the plasma membrane per unit volume of the cell. Enhanced cell-size also decreases rate of diffusion.
8. Replacement of cutaneous respiration across the general body surface by gill respiration and pulmonary respiration enhances the respiratory surface. So respiratory exchange increases with the evolution of gills and lungs.

Membrane transport :

Substances are transported across the membrane. It may occur without the help of a carrier, (non mediated transport) or with the help of a carrier (mediated transport). Depending on energy requirement, the transport is of two types. A passive transport where energy is not required and an active transport which requires energy. Active transport is carrier mediated. However, carrier mediated transport may also be passive, then it is called facilitated diffusion.

Mediated transports are of three types –.

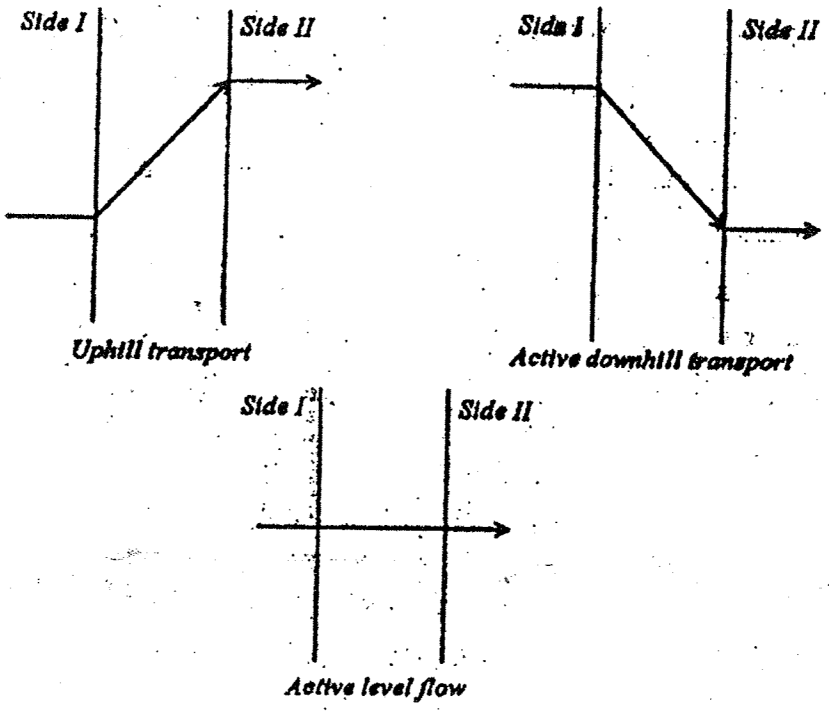
i) Transmembrane transport of a single substrate (uniport);

ii) simultaneous transport of two substrates in the same direction across the membrane (symport);

iii) transport of two substances simultaneously across the membrane but in opposite direction (antiport).

ACTIVE TRANSPORT :

Substances are transported across the cell membrane. When the transport occurs by expenditure of energy, it is called active transport. Transport of substances against electro-chemical gradient (up hill transport) is always active transport. But this can also operate in case of transport in favour of electro-chemical gradient (downhill transport) to increase the rate of transport (active "down-hill" transport). Active transport also operates in absence of electro-chemical gradient (active level flow).



Substances transported by this process :

Among the different substances that are actively transported are ions of Na, K, Ca, Fe, H, Cl, I, Urea etc. as well as several different sugars and amino acids.

Site of active transport :

Active transport is known to occur across cell membranes, membranes of mitochondria and endoplasmic reticulum. It is also possibly operative in other membranes of the Cell. It also occurs through cellular sheets. Substances are transported by changing the contour of cell-membrane (e.g. phagocytosis and pinocytosis), and these are caused by expenditure of metabolic energy. According to Stein, the enzyme carries in the reduced state act as carriers and when oxidised release the ions. The source of energy is the potential difference between two redox systems.

Mechanism of active transport :

It is a carrier mediated transport. The carrier substances are intramembrane molecules. They are proteins or lipo-proteins. It is held that the protein moiety provides the sepcific site to which the specific substance is attached and the lipid moiety provides the solubility in the lipid phase of the cell membrane. The carriers are called porters, translocase or transport system.s They bind reversibly with the substance to be transported and thus carry the substnace from one side to the other side of the membrane. It is held that the carriers carry their ligands across the membrane by diffusion, or they may rotate within the membrane or they may undergo conformational changes to create a 'hole' in the membrane for the transport of specific substances.

Heinz and Walls proposed the following mechanism for the transport :

A. The substance binds with the carrier. (1)

B. Substance – Carrier complex moves to cytoplasmic side (2).

C. The complex dissociates and the substance is discharged into the cytoplasm. (3)

It is postulated that the carrier is enzymatically (ε) degraded into an inactive form, i.e. a form which shows low affinity for the substance. Due to such low affinity the transported substance dissociates from the carrier. The released substance being insoluble in the membrane cannot diffuse back to the lipd matrix of the membrane. Hence, it moves to the cytoplasm. (4)

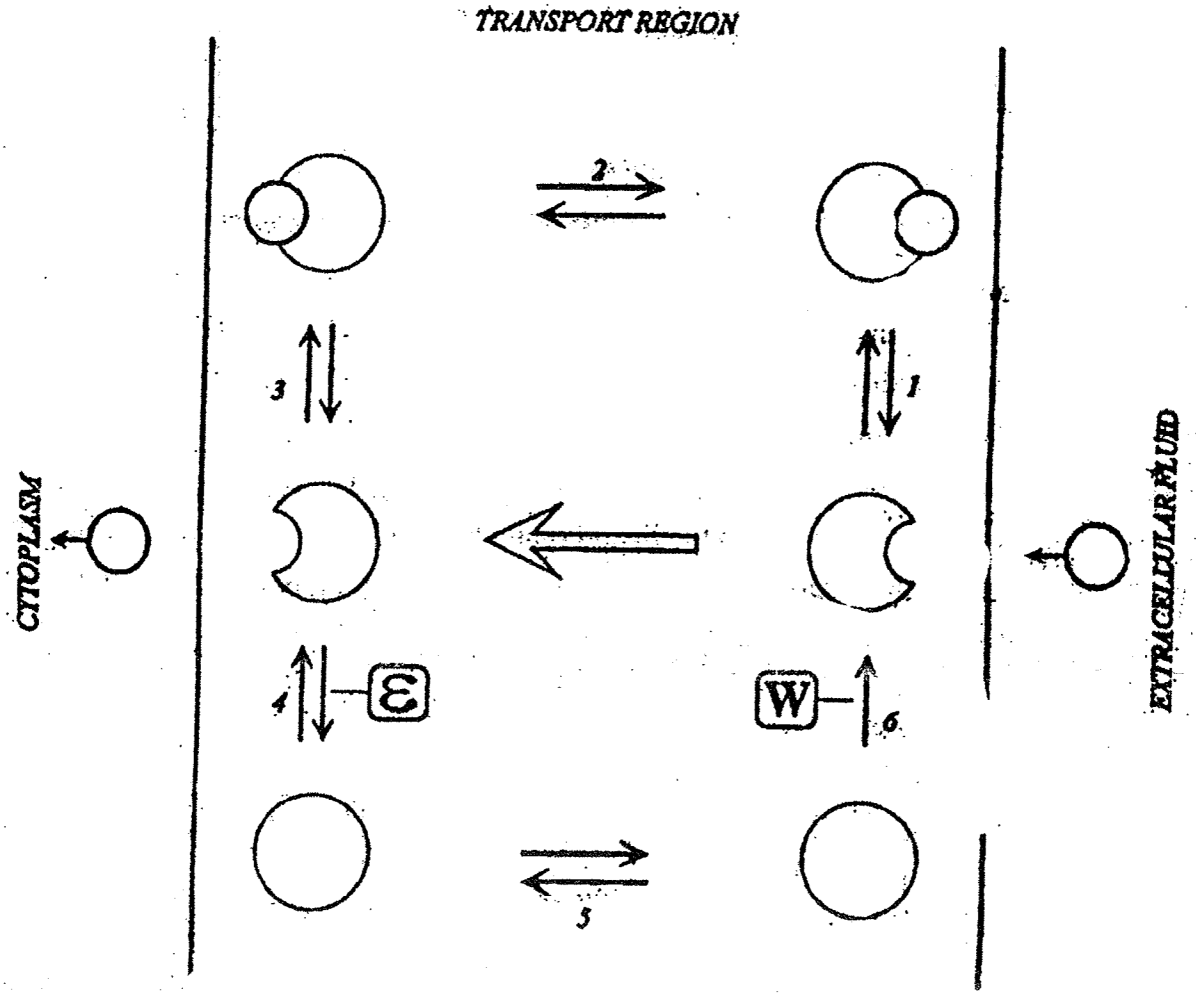
D. The inactive carrier passes to the other side of the membrane. (In this case extracellular side) (5).

E. Here, the carrier is activated by an energy requiring process (ω), (6)

F. After activation it again combines with the substance and transport it.

Movement of substances from cytoplasm to extracellular fluid also operates in the same way.

S.J. Singers proposed a mechanism for the transport of substances with the help of peripheral and integral proteins:



Model for active transport after Heinz and Walsh.

Transport via integral proteins :

The ligand binds with the active site of the integral protein (I). After binding, some energy - yielding enzyme reaction occurs. This causes conformational changes in (I). As a result, the substance is sequeezed through the membrane.

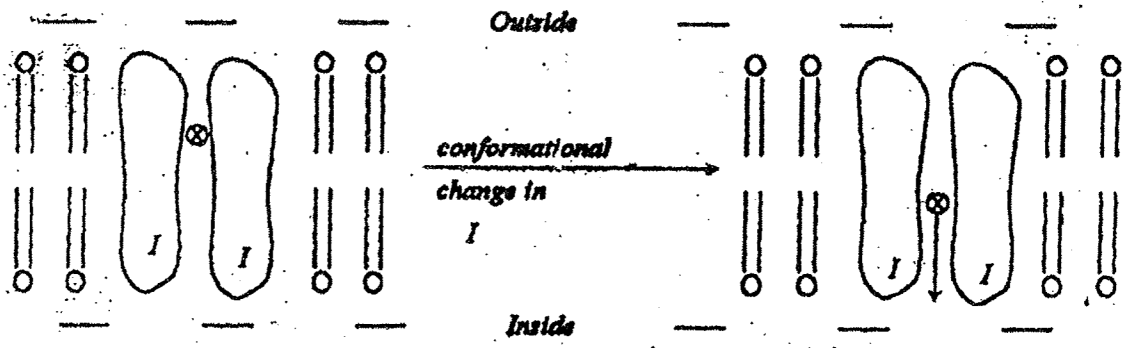


Fig. 1 (A) Model for active transport through internal protein (I)

Transport via peripheral and integral proteins :

Initially, the integral protein molecule is so arranged that there is water filled pore which allows water molecules to pass through, but not other solute molecules.

The peripheral protein (P) is attached to the integral protein (I). The former (P) has a binding site for a specific substance (ligand). When the ligand is attached to 'P' some energy yielding steps occur. This results in quarternary rearrangement of this structure and the pore is opened. Now the ligand is released to the other side of the membrane.

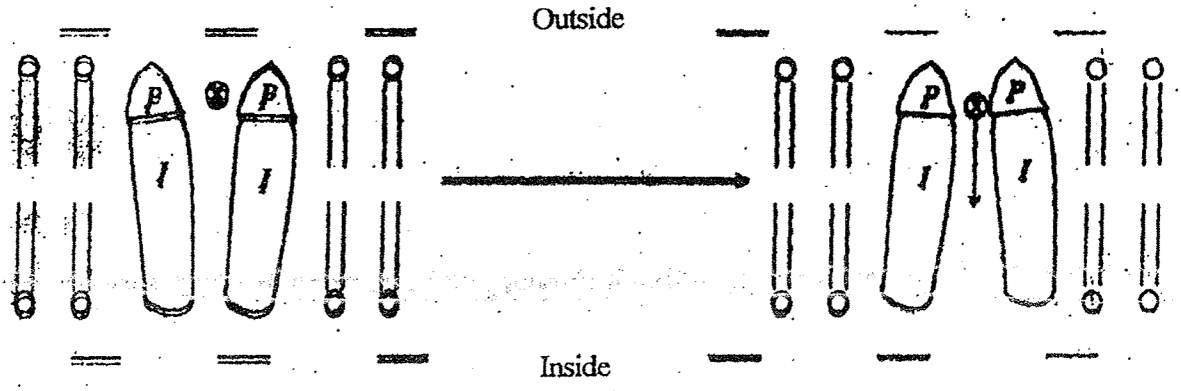


Fig. 1 (B) Model for active transport through peripheral protein (P)

Characteristics of active transport :

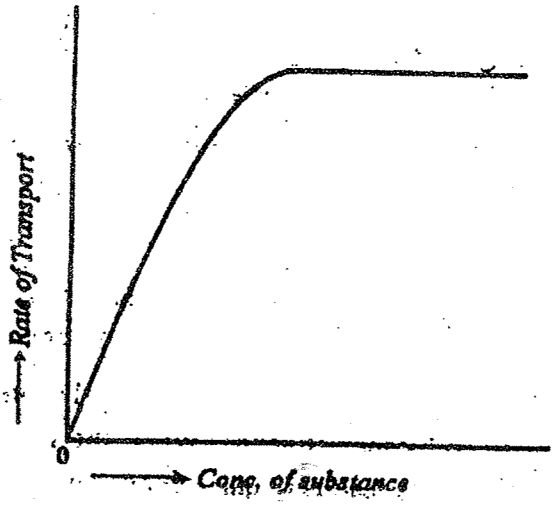
It has different characteristics.

i) Specificity :

The carrier transports specific substances. The specificity may be of very high order in some cases, e.g. glucose transport, while in others it may be group specific, e.g. arginine, histidine and lysine are carried by the same carrier.

ii) Saturation :

The rate of transport in proportionately increased as the concentration of the substance to be transferred is increased. This happens upto a certain level of concentration. Beyond that, the rate is not increased any further because all the carriers are statured.



iii) Competition :

When two substance X and Y are transported by the same carrier, concentration of X will decrease the rate of transport of Y and vice versa because X molecules will occupy some carrier molecules that transport Y.

iv) Inhibition :

The inhibitor will combine with the carrier and so the transport of the substance will be inhibited.

v) Succeptibility to metabolic inhibitors :

Since active transport requires metabolic energy. The transport is blocked by metabolic inhibitors.

vi) Energy dependent :

It requires a continuous supply of energy. The metabolic energy may be directly linked to the transport process (primary active transport). Alternatively, active transport of one substance provides the energy for the transport of another substance (secondary active transport).

Energetics of active transport :

Active transport requires high amount of energy. The free energy change (ΔG) is determined by

i) Concentration of the substance in the two compartments;

ii) Amoutn of substance transported from lower to higher concentration.

$$\Delta G = RTI_n \frac{C_2}{C_1}$$

R = Molar gas constant.

T = Temp. in absolute degrees.

C_1 = Conc. of the substances from which it is transported.

C_2 = Conc. of the substances in the compartment to which the substance is transported.

ΔG = Free energy change when 1 mol. of uncharged substance is transported.

To transport 1 mol. of glucose against a 10 fold concentration gradient, 1.34 Kcal is necessary.

Functions of active transport :

i) It takes part in the transport of fuels and essential nutrients from the surrounding medium.

ii) It keeps the optimum internal concentration of organic nutrients, metabolites, inorganic electrolytes, specially K^+ and Ca^{++} which are essential for normal intracellular activities.

iii) It maintains the electrochemical gradient in biologic system which is very important. For this, about 30-40% of the total energy input is consumed in a resting human.

iv) It maintains the osmotic relationship between internal and external medium and these maintain the volume of the cell.

v) It is involved in the transmission of information in the nervous system and in the excitation and relaxation cycle in animal tissues.

vi) It takes part in the absorptive activity of intestinal epithelia and secretory function of the kidney.

vii) It plays an important role in the formation of ATP during oxidative and photosynthetic phosphorylation since in these processes pumping of H^+ respectively across mitrochondiral and chloroplast membranes may be an essential intermediate step.

Energy is necessary for phagocytosis and pinocytosis. Hence, these are also active transport.

Major Transport Systems in Mammalian Cells : Table - 1

Substances transported	Mechanism of transport	Tissues
Sugars		
Glucose	Passive	Most tissues
	Active symport with Na^+	Small intestines and renal tubular cells
Fructose	Passive	Intestine and liver
Amino acids		
Amino acid specific transporters	Active symport with Na^+	Intestines, kidney, and liver
All amino acids except proline	Active group translocation	Liver
Specific amino acids	Passive	Small intestine
Other organic molucules		
Cholic acid deoxycholic acid, and taurocholic acid	Active symport with Na^+	Intestines
Organic anions, e.g., malate, α-ketoglutarate, glutamate	Antiport with counterorganic anion	Mitochondria

ATP-ADP	Antiport transport of nucleotides; can be active transport	Mitochondria
Inorganic ions		
H^+	Active	Mitochondria
Na^+	Passive	Distal renal tubular cells
Na^+, H^+	Active antiport	Proximal renal tubular cells small intestine
Na^+, K^+	Active ATP driven	Plasma membrane of all cells
Ca^{2+}	Active ATP driven	Plasma membrane and endoplasmic (sacroplasmic) reticulum
$Ca^{2+}, Na^+,$	Active antiport	Most tissues
H^{2+}, K^+	Active antiport	Partiel cells of gastic mucosa secreting H+
Cl^-/HCO_3^-	Passive transport	Erythrocytes and many other cells

Ion Channel :

In natural membranes there are transmembrane aquous channels that transport ions. These are called ion channels. Some of them are continously open. Whereas, others are gated i.e. they have gates that either open or close. Most of the ion channels are voltage gated or ligand gated, some are

also mechanically gated. These are widely distributed. They are found in the plasma membrane of neurous, muscle cells and many other cells, both prokaryotic and eukaryotic.

Voltage gated channels remain closed when the membrane is in the resting polarized state. They open briefly when the membrane is depolarized. Ligand gated channels open briefly when ligand binds with it. The ligand is often external. e.g. a neurotransmitter or a hormone. However, it can also be internal like intracellular Ca^{2+}, cAMP, or one of the G–Proteins produced in the cells can bind directly to the channels and activate them. Mechanically gated channels open on mechanical stretching. Most cells have a variety of Na^+, K^+, Ca^{2+} and Cl^- gated channel.

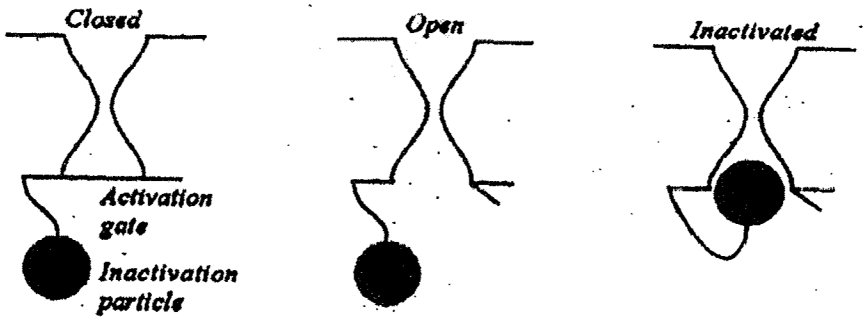


Fig. 5 : Fast inactivation of voltage-gated K^+ channels. Depolarization opens an activation gate, and then the amino terminal of the subunits swings into the channel, stopping conductance despite continuing depolarization. After repolarization of the membrane, the resting conformation of the protein is restored.

Forty different K^+ channels and more than thirty different Na^+ and Ca^{2+} channel have been described. For example, voltage gated Na^+ and K^+ channels are present in the axolemma. At teh tip of the axonal ending, there are voltage gated Ca^{2+} channels in the axolemma. Acetylcholine receptor has ligand gaged Na^+ channel. GABBA & glycine receptor has ligand gated Cl^- channel.

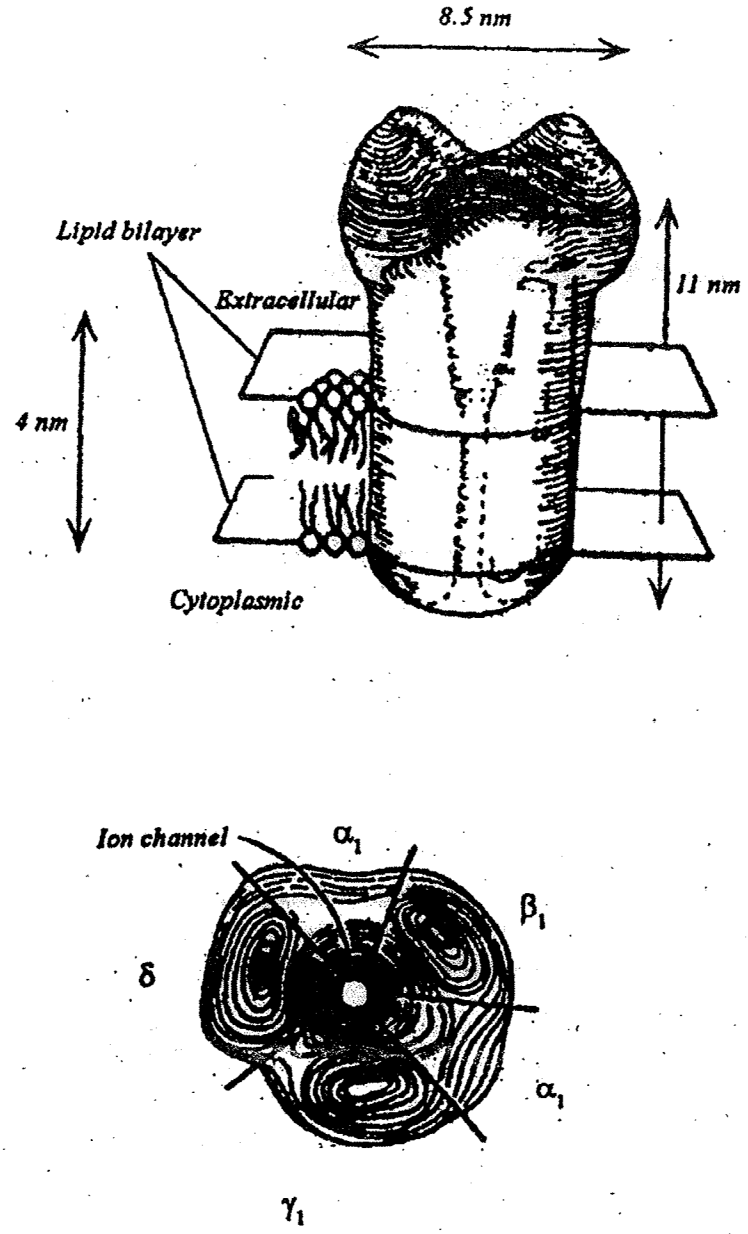


Fig. 6 : Diagram of fetal nicotinic acetylcholine receptor as viewed from the side (above) and from the top (below) $\alpha_1, \beta_1, \gamma_1, \delta$ ***:*** *receptor subunits.*

Chemical nature and important characteristics :

These channels are composed of transmembrane protein subunits. The subunits span the membrane several times. These subunits come together to form a central aquous pore through which the ions can diffuse, moving down their electrical and chemical concentration gradients. Most of the channels are made up of four subunits. However, there are variation also. For example, the epithelial sodium channel (E Na C) is made up of three sumits; the acetylcholine receptor, a cationic channel, is made up of five submits.

Each ion channel exist in multiple forms with diverse properties. Most of them are highly selective and pass one ion only and few are non selective. They allow impermeable ions to cross the membrane at rates approaching diffusion limits. They can permit ions at the rate of 10^6–10^7 per second. They are highly conserved across species. Their activities are regulated. One ion can regulate the activity of the channel for another ion. For example, a decrease of Ca^+ concentration in extracellular fluid increase the membrane permeability and increases the diffusion of Na^+. This depolarizes the membrane and triggers nerve discharge. This may explain the numbers, and muscle cramps which are the symptoms of a low level of serum Ca++. The activity of the channels is effected by drugs. Neuromuscular blocking agents like tubocurarine, Cobrotoxin, Bungarotoxin in block acetylcholine receptor or prevent opening of its channel and block neuromuscular transmission of nerve impulse, resulting in paralysis and death. Tetrodotoxin, saxitoxin are also deadly poison because they blcok neurotransmission by preventing opening of Na+ channels. The activity of GABBA receptor (Cl. channels) is potentiated by benzodiazepines which have marked antianxiety activity and are also effective muscle relaxant, antocanvulsants and seditives.

Mutations in genes encoding the channels can cause specific diseases. Mutation of the genes concerned with the synthesis of polypeptides makes the channels defective leading to varius disorders. For example, mutation of the genes related to Cl^- channels in skletal muscle cause "myotonia congenita" a disease characterized by muscle hyperexcitability.

Significance :

These are signal transducers. They have important role in the conduction of nerve impulse along

nerve fibres as well as synapses. They are also involved in the regulation of cell-volume, in the transepithelial ion transport; in the regulation of muscle and kidney functions.

Ionophore :

Ionophores are small organic molecules capable of transporting specific alkali metal ions across the membrane through a carrier mediated facilitated diffusion.

They are highly polarizable, neutral cyclic compounds. Many of them are antibiotics.

There are two types of ionophores.

(i) Carrier ionophore and (ii) Channel forming ionophore.

Carrier ionophore :

They are mobile in the lipid bilayer of the membrane and shuttle across the membrane for the movement of ions. These ionophores contain hydrophilic centre that bind specific ions and are surrounded by peripheral hydrophobic regions. This arrangement allows the molecule to dissolve effectively in the membrane and diffune transversely. In most of the cases it looks like a doughnut. The metal ion precisely fits into the core through coordination bonds with oxygen and the periphery of the molecule consists of hydrophobic groups. The number of oxygen atoms that bind the metal ions is typically six or eight. In an aquous medium, the metal ion binds several water molecules through their oxygen atoms. The ionophore carrier competes with water to bind the ion by chelating it to several appropriately arranged oxygen atoms in the central cavity. The specificity of the ionophore is due in part to the size of the pore into which the ion fits and to the alteration of the ionophore for the ion in competition with water molecules.

The hydrophobic periphery makes the ionophore-ion complex soluble in the lipid medium and thus it freely diffuses across the biomembrane.

Some carrier ionophores remain restricted within the membrane and shuttle across its lipid bilayer. They form ion-ionophore complex at one of its surfaces and release the free cation from the other surface. However, some other ionophores may form the ion, ionophore complex in one aquous phase, enter and cross the lipid bilayer in that form, then pass into the aquous phase on the other side of the membrane and finally release the free ion there. Mobile ionophores cannot induce ionic fluxes

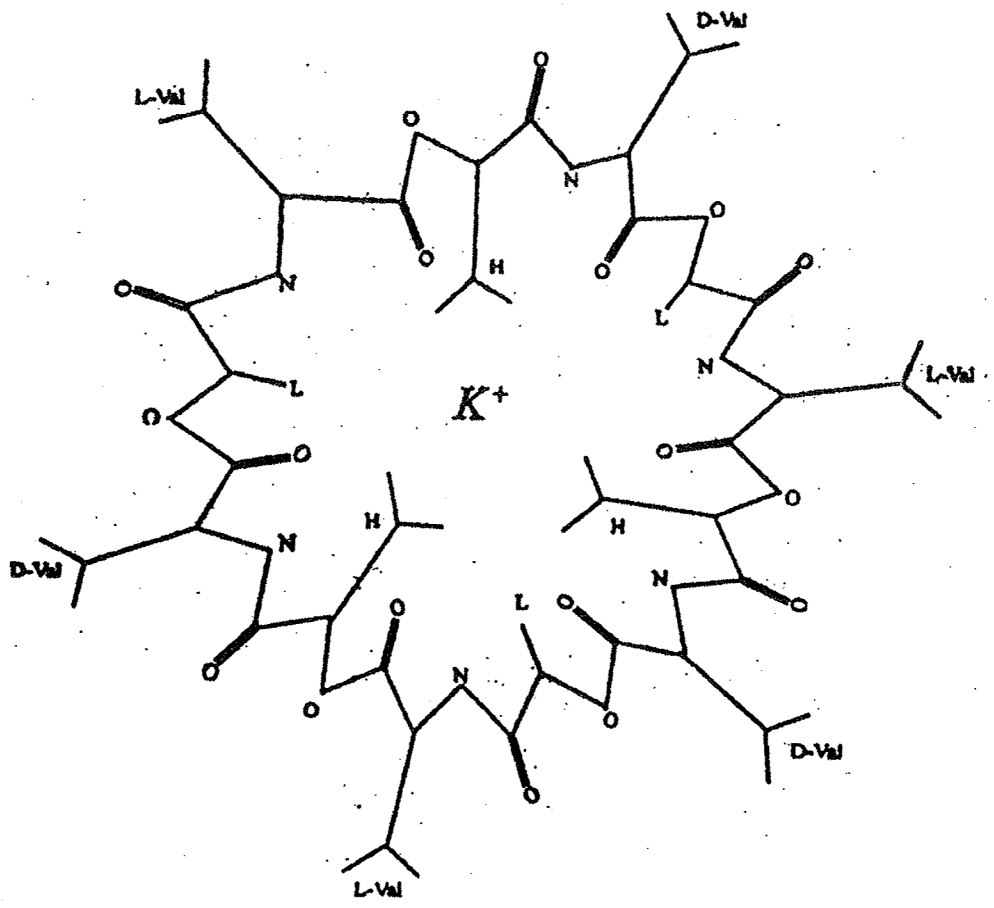


Figure 7 : The structure of the valinomycin – K^+ complex

Abbreviations : D -Val = D - Valine; L -Val = L - Valine;

L = L - Lactate ; and H = D - hydroxyisovalerate;

below the transition temperature of the membrane because the membrane is now in the gel phase and the ionophores cannot diffuse through this phase.

Channel forming ionophore :

These ionophores remain fixed in the one membrane. More than one molecule of such ionophore (e.g. Gramicidin A) may be sequentially arranged in the lipid bilayer into a relatively fixed helical structure. Such structures acts as a transmembrane channel. Of the ionophore molecules, the polar

backbone groups line the channel and the hydrophobic groups are on the periphery of the channel which interact with the lipid membrane. Specific ions diffuse through these channels to cross the membrane. This type of ionophore, however, has a lower degree of specificity towards the ions.

Major types of ionophore

Compound	Major cations transported	Nature
Valinomycin	K^+ or Rb^+	Cerrier ionophore (Uniport)
Nanoctin	NH_4^+, K^+	Cerrier ionophore (Uniport)
A 23187	$Ca^{2+}/2H^+$	Cerrier ionophore (Uniport)
Nigericin	K^+/H^+	Cerrier ionophore (Uniport)
Monensin	Na^+/H^+	Cerrier ionophore (Uniport)
Gramicidin	H^+, Na^+, K^+, Rb^+	Channel forming ionophore
Almethicin	K^+, Rb^+	Channel forming ionophore

Significance :

1. Ionophores are used as important model to study transport. The ionophores enable cations to be transported across synthetic phospholipid bilayers. Study of such artificial transport systems has given important information on the basic physical principles involved in biological transport system.
2. Many antibiotics are ionophores. The antibiotic effect is that the ionophores nullify vital transmembrane gradients of specific ions by increasing their facililated diffusion across the bacterial membrane. This can slow down ATP synthesis. Valinomycin, has been shown to uncouple oxidative phosphorylation by dissipating ion gradients across the inner mitochondrial membrane, due to K^+ transport, eliminating the contribution of transmembrane electrical potential ($\Delta\psi$) to the proton motive force. The antobiotic Nigericin also acts as an ionophore for K^+ but in exchange for H^+. Hence, it abolishes pH gradient across the membrane.

   The antibiotic valinomycin acts as an ionophore for K^+. It binds K^+ about thousand time as strongly as Na^+. This happens because it is energitically more costly to pull Na^+ away from water. The energy of activation for binding and release of K^+ with valinomycin is samll. Hence, valinomycin picks up and unloads K^+ many time a second. For this reason, the

transport of K^+ by valinomycin is very fast.

3. There have been reports that proteolipids, prostagladins and perhaps other lipids present in mammalian tissues may function as ionophores. The classic unconuplers such a sdinotrophenol are, in fact, proton ionophores.

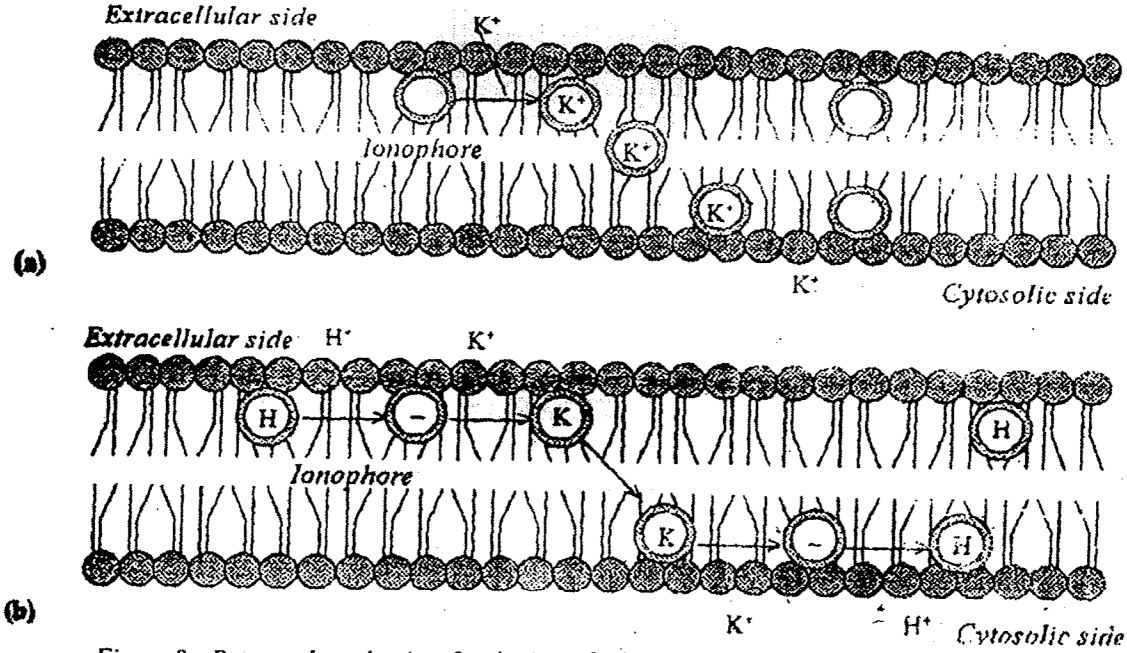
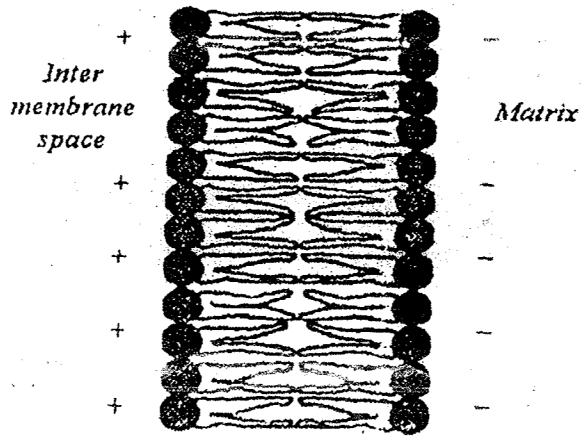
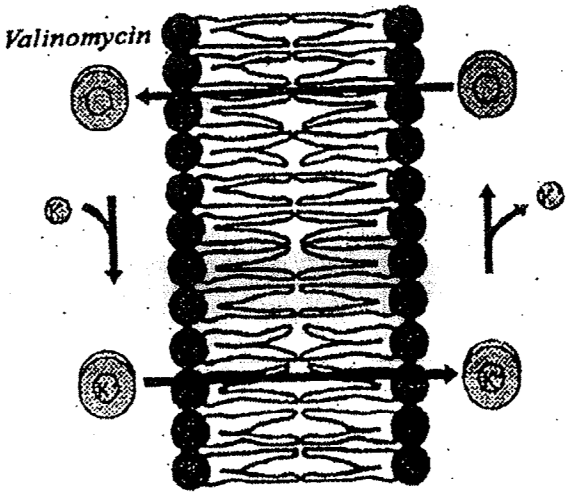


Figure 8 : Proposed mechanism for the ionophoretic activities of valinomycin and nigericin. (a) Transport by valinomycin. (b) Transport by nigericin. I represents the ionophore The y valinomycin –K complex is positivel charged and translocation of K^+ is electrogenic leading to the creation of a charge separation across the membrane. Nigericin translocates K^+ in exchange for a H^+ across the membrane and the mechanism is electrically neutral.



$\Delta\Psi > 0$

Electron transfer coupled to ATP synthesis.



Electron transfer coupled to ATP synthesis

Membrane :

1. Describe the fluid mosaic model of membrane with diagram. 10
2. Describe the functions of the membranes. 10
3. Write briefly on the fluidity of the membrane. 8
4. Give a brief account of asymmetry and functions of membrane lipids. 8
5. Write briefly on asymmetry and functions of membrane proteins. 8
6. Give a brief account of liposome. 8
7. Describe the functions of glycocalyx.

Diffusion :

1. Mention the factors that modify diffusion. 5
2. Discuss the role of diffusion in the transport of respiratory gases. 5
3. Write briefly on the biological application of diffusion. 8

Osmosis :

1. State the laws of osmotic pressure. 8
2. Mention the differences between
   i) osmolarity and osmolality.

ii) tonicity and osmoticity.

3. What is cryoscopic constant? Mention its use. 5

4. A sample of urine freezes at –0.56°C. Calculate its osmotic pressure at 37°C (R=0.082 litre-atmosphere). 5

5. Calculate the osmotic work to be done to transfer or secrete 3.54g of Cl^- concentration in plasma and urine are 0.1 and 0.2 Mol respectively. R =.987 cal / mol / degreee 5

6. Show with diagram that two solutions may be (i) isotonic but not isosmotic and (ii) isosmotic but not isotinic. 5

Membrane transport :

1. Describe the mechanism of active transport. 8
2. Write briefly on the characteristics of active transport. 5
3. Describe the functions of active transport. 8

Ion Channel and Inophore :

1. What are ion channels? Describe the characteristics and significance of ion channels. 4+6+2
2. What are ionphore? Describe the significance of ionophore. 4+8
3. Why valinomycin binds K^+ more strongly than Na^+ and transports K^+ vary fast. 4

Further reading :

1. Test book of Biochemistry
   by West / Todd / Mason / Van Bruggen.
2. Principles of Biochemistry
   by Lehninger / Nelson / Cox.
3. Harper's Biochemistry
   by Murrary / Granner / Mayes / Rodwell.
4. Biochemistry
   by L. Stryer.
5. Biochemistry
   by D. Das.
6. Review of Medical physiology
   by W.F. Ganong.

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M.Sc.
in
Zoology

Part - I
Paper - II, Unit - II, Group - B

Module No. 22/A

Module Structure

Topic : I. PROPERTIES OF MATTER :

1. Dalton's law of partial pressure of gases.

1.1 Solubility of gases in liquid.

(a) Absorption coefficient.

(b) Henry's law.

(c) Biological application.

2 DIFFUSION :

2.1 Definition.

2.2 Fick's First Law.

2.3 The diffusion coefficient.

2.4 Graham's Law of diffusion.

2.5 Facilitated diffusion.

2.6 Biological application.

3 VISCOSITY of liquid :

3.1 Definition.

3.2 Poisseuille's law.

3.3 Flow - pressure relationship.

3.4 Oswald viscometer.

3.5 Factors affecting viscosity.

4. SURFACE TENSION :

4.1 The Nature of Surface Tension.

4.2 Definition.
4.3 Capillary action.
4.4 Factors affecting Surface Tension.
4.5 Determination of Surface Tension.
4.6 Biological application.

5. CENTRIFUGATION :

5.1 Ultracentrifugation.
5.2 Detail of ultracentrifugation.
5.3 Moving Boundary ultracentrifugation.
5.4 Sedimentation coefficient (s)
5.5 Measurement of s
5.6 Measurement of MW
5.7 Density - gradient ultracentrifugation.

TOPIC : II. PROPERTIES OF SOLUTION :

OSMOSIS :

6.01 Semipermeable membrane.
6.02 Osmosis.
6.03 Abbe' Nollet's Experiment.
6.04 Definition of Osmotic pressure.
6.05 Van't Hoff Laws of osmotic pressure.
6.06 The Van't Hoff equation for ionised solution.
6.07 Units - Osmolarity.
6.08 Determination of Osmotic Pressure.
6.09 (a) Tonicity and isotonic solutions.
(b) Hypertonic and hypotonic solution.
6.10 Biological application.

TOPIC : III. pH AND BUFFERS :

7.1 The Ion product of water.
7.2 pH.
7.3 Acids and Bases.
7.4 Henderson - Hasselbach Equation.
7.5 Acid Base Buffer.

7.6 Acid Base Indicator.

7.7 Physiological buffers in pH regulation.

7.8 pH determination by pH meter.

TOPIC : IV COLLOIDS :

8.01 Colloids and crystallobids.

8.02 Definition.

8.03 Classification.

8.04 Solvation.

8.05 Tyndall effect.

8.06 Electrical double layer.

8.07 Salting in.

8.08 Salting out.

8.09 Dialysis.

8.10 Biological applications of dialysis.

8.11 Adsorption.

8.12 Biological applications of adsorption.

8.13 Donnan Membrane Equilibrium.

(a) Donnan effect.

(b) Factor affecting Donnan effect.

(c) Biological application.

TOPIC I. PROPERITIES OF MATTER :

1. Dalton's Law of Partial Pressure of Gases : A simple rule was put forward by Dalton in 1802 to express the pressure relationship of a gaseous mixture. The rule, which is known as Dalton's law of Partial Pressure, states that, " the pressure exerted by a gaseous mixture is equal to the sum of the pressures which the constituents would exert if each occupied separately the volume of the mixutre or in otherwords, the total pressure in a mixture of gases is equal to the sum of the partial pressures. Expressed algebrically ; at constant temperature and volume : $P = P_1 + P_2$ ..... (1 .1) where

P is the total pressure $P_1 P_2$ ... etc.

are partial pressures of various component gases.

Example : 1 : Calculate the total pressure in a mixture of 4 gms of oxygen and 2gms of hydrogen confined in a total volume of one litre at 0°C [R = 0.082 lit atm deg^{-1}]

Answer : Consider first oxygen alone is present in the total volume of 1 litre and calculate the pressure which is its partial pressure in the mixture.

Then, $P_1V = \left(\frac{g}{m}\right) RT$ where P_1 is the pressure of oxygen at 0°C

$$P_1 = \frac{4gm}{32gm} \times 0.082 \text{ lit atmdeg}^{-1} \times 273 \text{deg} \times 1lit^{-1} = 2.8 \text{ atm}$$

Similarly, P_2 is pressure of hydrogen, $P_2 = \frac{2gm}{2gm} \times 0.082$ lit atmdeg^{-1} $\times 273$deg$\times$ lit^{-1} = 22.5 atm

Total Pressure **P = (2.8 + 22.5) aton = 25.3 atm**

Dalton's law can be illustrated by the following example. Suppose we have three vessels having equal volume, maintained the same temperature. Each Vessel is attached to a closed manometer. Supose the first vessel contains x moles of hydrogen at a pressure P_1 and the second vessel contains y mole of helium at a pressure P_2. Suppose both gases are transferred to the third vessel at the same temperature. The partial pressures of these two gases in the third vessel will be P_1 and P_2. The total pressure will be $P = P_1+P_2$

Example : 2 : At 27°C, cylinder of 20 lit capacity contains three gases He, O_2 and N_2. Their masses are 0.002 gm., 0250 gm and 1.0 gm respectively (ideal gas). Calculate partial pressure of each gas and total pressure.

Answer : Moles of $He = \frac{0.0502}{4} = 0.125$, $O_2 = \frac{0.250}{32} = 7.81 \times 10^{-3}$

$$N_2 = \frac{1}{28} = 3.67 \times 10^{-2}$$

Volume = 20 Lit, T = 27 + 273 = 300° K

$$P_{He} = \frac{0.125ml. \times 0.082Litatm^{k-1} \times 300k}{20} = 0.154 \text{ atm}$$

$$P_{O_2} = \frac{7.81\times10^{-3}\times0.082\times300k}{20} = 9.62\times10^{-3}\,atm$$

$$P_{N_2} = \frac{3.67\times10^{-2}\times0.082\times300}{20} = 4.4\times10^{-2}\,atm$$

$$P_{total} = 0.154 + 0.00962 + 0.044 = 0.208 \text{ atm.}$$

1.1 Solubility of Gases in Liquid :

(a) Absorption coefficient : The amount of gas dissolved by a liquid is expressed in terms of solubility which is defined as the volume of gas dissolved by one cm^3 of the solvent either at the pressure and temperature of the experiment or calculated at STP (Standard Temparature and Pressure). The latter is called **Absorption Coefficient.**

The gases widely differ from one another in respect to their solubilities which depends on the nature of the gas and the solvent, and also on the temparature and pressure of the experiment. Oxygen is more soluble than nitrogen in water and so though air contains 21%. Oxygen, dissolved air contains as high as 34% of oxygen, and this is helpful for respiration of fish.

(b) Effect of Pressure on solubility. Henry's law :

Henry, a co - worker of Dalton, established the relationship between the solubility and the pressure of a gas. Henry's law states that **the amount of gas dissolved by a given volume of any liquid at a constant temperature is proportional to the pressure of the gas.** Thus, Henry's Law : g/p = constant (K) (2.1). Where g is the mass of the gas dissolved in a given volume of liquid and P is the pressure of gas.

Henry's law is obeyed fairly accurately by gases which are soluble to a small extent (like O_2) as evident from following table 1.1.

Table - 1.1 Solubility of Oxygen

Pressure (c.m.)	Mass of Gas Dissolved per litre	$g / p \times 10^5 = K$
76.0	0.0408	53.59
61.0	0.0325	52.28
41.0	0.0220	53.14
30.0	0.0160	53.33
17.5	0.0095	54.22

Common Phenomena in the light of Henry's Law : The ordinary soda water bottle contain CO_2 gas under pressure. As soon as the pressure is released, the liquid cannot now hold all the CO_2 in solution. Since the solubility of the gas is now less and therefore, a large part of the gas gets all at once expelled out in bubbles, imparting to it the characteristic sparkling freshness.

(c) Biological applications : (i) In accordance with the Henry - Dalton law, volume of O_2 and CO_2 dissolved in the plasma are linear functions of their respective partial pressure in blood. For example, following table 1.2 shows, for mm Hg rise in Po_2 increases the volume of dissolved O_2 by 3 mm^3 (0.003 ml).

Table - 1.2

Po_2 (mm Hg)	Volume of O_2 (ml) Per dl of Plasma(g)	$\frac{g}{p} = K$
10	0.03	0.003
20	0.06	0.003
40(Capilliary)	0.12	0.003
50	0.15	0.003
70	0.21	0.003
95(Art. blood)	0.285	0.003
100	0.30	0.003

In conformity with the Henry - Dalton law, arterial blood at normal pressure of 95 mm Hg carries $(95 \times 3) = 285 mm^3$ or 0.285 ml. of oxygen in solution per dl of plasma ; the same volume of plasma in capillaries at Po_2 of 40 mm. Hg can carry $(40 \times 3) = 120 mm^3$ or 0.12 ml. of oxygen in solution per dl of plasma. So each dl of plasma should liberate (285 - 120) = $165 mm^3$ (or 0.17 ml.) of the dissolved O_2 due to fall in Po_2 during the blood flow through tissue.

Suggested questions on Chapter 1 and 2 :

Q. 1. State Dalton's law of Partial pressure.

Q. 2. Example 1 in the text.

Q. 3 Example 2 in the text.

Q. 4. How does the solubility of gas in liquid depend on pressure? State Henry's law.

Keywords :

Partial pressure, total pressure, gas constant, absorption coefficient, solubility, Dalton's law, Henry's Law, arterial and capillary blood.

Suggested readings : At the end of the module.

2. DIFFUSION :

2.1 Definition : Diffusion is the tendency of particle, molecules and ions to spread uniformly in the entire available space by their incessant random movements. Though moving at random in all directions, a greater number of particles move from a *region of higher concentration to one of lower concentration.* Thus a net diffusion of particle down their concentration (or pressure) gradient occurs until a uniform concentration (or pressure) prevails throughout the available space. Random movements continue even afterwards but equal number of particles then moves in all directions, bringing the net diffusion to zero. The spontancons process must ultimately bring about uniformity of concentration everywhere within the system (diffusion equilibrium).

2.2 Fick's First Law :

$$dQ = -DA\frac{dc}{dx}dt \quad \text{..... (2.01)}$$

Where Q = quantity of net material diffuning from x to x + dx.

c = Concentration.

t = time.

x = distance.

D = diffusion coefficient.

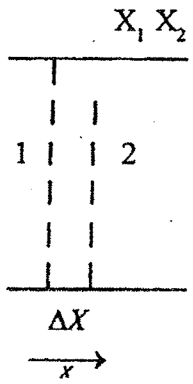
A = Area.

$$J_D = \frac{dQ}{dt} = -DA\frac{dc}{dx} \quad \text{.....(2.02)}$$

Steady state case (concentrations at any given place do not change with time) across a plane :

$J_D^{1\to 2} = \frac{\Delta Q}{\Delta t} = -DA\frac{\Delta C}{\Delta X}$ ..... (2.03) Where $J_D^{1\to 2}$ = rate of transport by diffusion from $1 \to 2$

$\Delta X = X_2 - X_1$ (Fig. 2.02)



(Fig. 2)

Fick's First Law of diffusion states that ***diffusion rate of a solute is directly proportional to its concentration gradient and occur down the latter, i.e. from an area of higher concentration to one of lower concentration***. Thus the greater the difference in solute concentration between two areas of solution, the higher is the diffusion rate of the solute down the concentration gradient. J is the rate of diffusion or flux of the solute across a unit area per second, the negative sign indicate the diffusion from a higher to a lower concentration, and D is the ***diffusion coefficient*** which is the mass of solute diffusing across 1 cm^2 area in 1 Sec down a concentration gradient of unity.

s 2.3 The diffusion coefficient (Cm^2Sec^{-1}) : Einstein derived the following expression for the diffusion coefficient for the case of spherical particle in a homogeneous medium, with the particle much larger than the molecules of the medium.

$$D = \frac{RT}{6\Pi r\eta N} \quad \text{..... (2.04)}$$

When, R = gas constant

T = absolute Temp.

r = radius of particle

η = Viscosity of the medium.

N = Avagadro's no.

For delute aquous solution at ordinary temperature glucose diffusing in water has D of

$0.6\times\left(10^{-5}\right)Cm^2Sec^{-1}$

Example - 1 :

Find out the unit of diffusion coefficent (D) when units of the following are as follows

R = ml atm deg $^{-1}$ mole^{-1} = (Cm^{3}gmSec^{-2} Cm^{-1} deg^{-1} mole^{-1})

T = deg

r = Cm

η = gm cm^{-1} Sec $^{-1}$

N = mole $^{-1}$

Answer : $D = \dfrac{[Cm^3 \times gm\ Sec^{-2}\ Cm^{-1}\ deg^{-1}\ mole^{-1}] \times \text{deg}}{Cm \times gm\ Cm^{-1}\ Sec^{-1} \times mole^{-1}}$

= Cm^2 Sec^{-1}

2.4 Graham's Law of diffusion : At constant temperature and pressure, the rate of diffusion of any gas is inversely proportional to the square root of its density.

$$\text{Rate of diffusion} \propto \frac{1}{\sqrt{density}} \quad \text{.....(2.05)}$$

A lighter gas like hydrogen, therefore, diffuse faster than a heavier gas such as nitrogen or carbon dioxide. Thus, the diffusional flux J of a gas varies inversely with the square roots of its density (ρ).

$$J \propto \frac{1}{\sqrt{\rho}} \quad \text{.....(2.06)}$$

If, J_1, J_2 are the rates of diffusion of two gases at same temperature and pressure, ρ_1, ρ_2 are their density then Graham's Law equation gives :

$$\frac{J_1}{J_2} = \sqrt{\frac{\rho_2}{\rho_1}} \quad \text{.....(2.07)}$$

Since molecular mass of a gas is twice its vapour density $M = 2\rho$, we have

$$\frac{J_1}{J_2} = \sqrt{\frac{\rho_2}{\rho_1}} = \sqrt{\frac{M_2}{M_1}} \quad \text{.....(2.08)}$$

The rate of diffusion of gas is defined as the ratio of the volume of the gas diffused to the time taken in diffusion i.e. $J = \frac{V}{t}$, V is the volume of gas, t is the time taken for diffusion.

If V_1 and V_2 are the volume of two gases which undergoes diffusion in time t_1 and t_2 respectively,

$$J_1 = \frac{V_1}{t_1}, J_2 = \frac{V_2}{t_2}$$

$$\text{Or, } \frac{J_1}{J_2} = \frac{V_1/t_1}{V_2/t_2} = \sqrt{\frac{\rho_2}{\rho_1}} = \sqrt{\frac{M_2}{M_1}} \quad \text{..... (2.09)}$$

$$\text{If, } V_1 = V_2, \quad \frac{t_2}{t_1} = \sqrt{\frac{\rho_2}{\rho_1}} = \sqrt{\frac{M_2}{M_1}} \quad \text{..... (2.10)}$$

$$\text{Or, if } t_1 = t_2, \quad \frac{V_1}{V_2} = \sqrt{\frac{\rho_2}{\rho_1}} = \sqrt{\frac{M_2}{M_1}} \quad \text{..... (2.11)}$$

Thus, comparing the rate of diffusion of unknown gas with that of a gas of known density or known molecular mass the density or molecular mass of unknown gas can be determined.

Example - 2 : The volume of gas X and chlorine during the same time are 35 ml. and 29 ml. If molecular weight of chlorine is 71, calculate mw of X.

Answer : $\frac{V_1}{V_2} = \sqrt{\frac{M_2}{M_1}}, \quad \frac{35}{29} = \sqrt{\frac{71}{M_1}}, M_1 = 48.74$

2.5 Facilitated diffusion : Solute and gases can pass across an intact membrane by two major processes:

(a) Non mediated transport or *diffusion*: It is a simple physical process, requires no carrier protein nor any energy expenditure, takes place down the electrochemical gradient of relevent substance.

(b) Carrier-mediated transports : They are far more rapid than diffusion and require specific *carrier molecules* for the transfer of specific solutes across a membrane. Mediated transports may be either *active transport* or *facililated* diffusion according to whether or not the process involves any cellular work.

Facilitated diffusion : This is a *passive mediated transport* of specific substrate e.g. transport of glucose across the erythrocyte membrane. Its characters are as follows :

(i) Specific carriers are required for the facililated diffusion of specific substrate across the membrane. Each carrier shows *Substrate - Specificity* and carries only one or few specific substrate across the membrane.

(ii) The process is far more rapid than simple diffusion.

(iii) It requires no energy expenditure and is unaffected by metabolic inhibitor.

(iv) The rate of facilitated diffusion depends on concentration gradients of substrate across the membrane. The carrier binds to a specific site of the substrate on one surface of the membrane, places the substrate on the other surface either by a conformational change in the carrier molecule or by diffusing across the membrane as the carrier - substrate complex, and finally releases the substrate into the adjoining aqueous medium. The same carrierr can transfer its substrate in either direction depending on the downward direction of the electrochemical gradient of the substance (Fig - 2.1A)

(v) Unlike simple diffusion in which the flux J is a *linear* function of the molar concentration [S] of the substrate, facilitated diffusion shows a *hyperbolic* saturation kinetics (Fig. - 2.1 B).

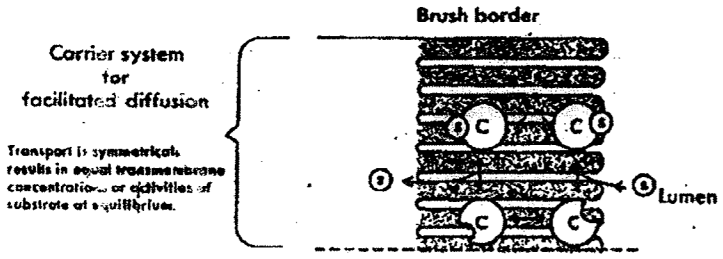
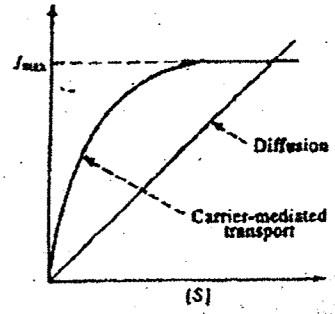


Fig 2.1 (A) The Carrier system for facilitated diffusion



(B) Hyperbolic saturation kinetics in facilitated diffusion

S = Substrate

C = Carrier

The rate of rise in the flux J of facilitated diffusion gradually declines with the rise in [S] ; ultimately, J reaches a maximum Jmax which cannot be surpassed by increasing [S] any further. This follows the Michaelis Menton equation for hyperbolic kinetics :

$$J = \frac{J\max[S]}{Km + [S]}$$

2.6 Biological application :

(a) Diffusion accounts for respiratory gas exchanges across the pulmonary alveolar membrane and the tissue capillary wall. This depends on the difference between the partial pressure of the relevant gas on two sides of the membrane. O_2 diffuses from alveolar air (Po_2 46 mm Hg) to alveolar air (Pco_2 40 mm Hg) until blood Pco_2 fall to 40 mm Hg.

(b) Diffusion accounts for the intestinal absorptions of pentoses, some minerals and some water soluble vitamins.

(c) The transport of some sugar is uniquely affected by the amount of Na^+ in the intestinal lumen ; a high concentration of Na^+ on the mucosal surface of the cells facilitates sugar influx into the epithelial cells and a low Na^+ concentration inhibits sugar influx.The glucose transporters responsible for facilitated diffusion in that it crosses the cell along the concentration gradient. Five different glucose transporter have been characterised. (Fig. 2.1 A)

Suggested questions :

Q. 1. State Fick's first law of diffusion.

Q. 2. State Einsteins equation of diffusion coefficient.

Q. 3. State Graham's Law of diffusion.

Q. 4. Discuss facilitated diffusion with an example. How is it related to enzyme substrate reaction?

Keywords : Random movement, Spontaneous process, Equilibrium, Diffusion coefficient, Avagadros number, Density, Molecular mass, Carrier-mediated transport, Passive transport, Substrate specificity, Hyperfolic saturation, Confarmational change.

Suggested readings : At the end of the module.

3 VISCOSITY of liquid :

3.1 Definition : Viscosity is the internal resistance against the free flow of a liquid due to frictional force between the fluid layers moving over each other at different velocities.

Let mn is a tube through which water is flowing. When such liquid moves, the 'Velocity profile' takes a parafolic shape (P) (Fig. 3.1). The lamina adjacent to the wall of the tube does not move at all, whereas the velocity increases more and more as the lamina in question is near the central longitudinal axis. That is, the molecules in the central longitudinal axis have the maximum velocity and it may be visualized that these molecules are 'dragging' the other laminae. This dragging force, that is,

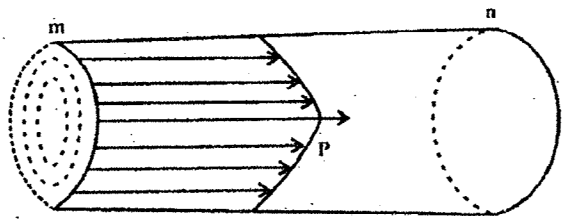


Fig 3.1 : Longitudinal View of the Velocity Profile

the force which causes the velocity gradient in the laminae is called the *shearing force.* So, in a moving column of water through a tube, it may be imagined, that a lamina is slipping out from its immediately outer lamina. As it slips out, there develops a *friction* and this frictional resistance opposes 'the sliping out.' If this frictional resistance is high, there will be little or no slipping out.

Viscosity, according to Newton, is the lack of slipperiness in between the adjacent laminae or in otherwords, the driving pressure remaining same, a viscid fluid will much less than a non viscid fluid. Liquids such as ether or gasoline have little viscosity and are quite mobile, whereas others such as honey and tar have high viscosity and more move slowly. The resistance experienced by one part of liquid in moving over another part is called viscosity.

3.2 Poisseuille's law : The idea can be quantitatively formulated from the simple picture of a liquid following in contact with a plane surface (PL). The plane surface would experience a *Tangential force* parallel to the direction of flow and would tend to move along the line of flow. Secondly there is a *gradient of velocity* of flow established at right angle to the direction of flow. The velocity of flow is zero on the plane surface (PL) and increases continuously as we move up into the liquid at right angle to the direction of flow(Fig. - 3.2) This Tangential force depends on the area of the surface and on the Velocity gradient.

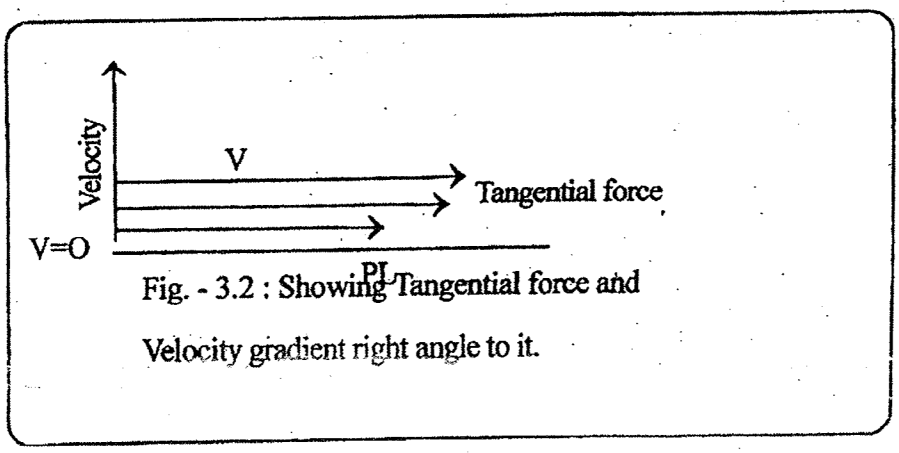


Fig. - 3.2 : Showing Tangential force and Velocity gradient right angle to it.

This Tangential force per unit area per unit Velocity gradient is called the coefficient of viscosity of the liquid or simply *Viscosity* and is denoted by η .

Evidently, Viscosity governs the flow property of a liquid, the higher the Viscosity the lower is its tendency to flow. Viscosity is generally formulated as follows :

$$\eta = \frac{Tangential\ force(F)}{Area \times Velocity\ gradient} \quad \text{..... (3.1)}$$

The *Unit of Viscosity* is *poise* named after Poiseuille who did pioneering work in this field.

$$\therefore \eta = \frac{gmCm\sec^{-2}}{Cm^2 \times (Cm\sec^{-1})Cm^{-1}} = gmCm^{-1}\sec^{-1}$$

Poiseuille formulated the law governing the rate of flow of liquid through Capillary tube. When a liquid flows through a Capillary tube of length *l* and radius r for a time *t*, under constant pressure head P and the volume flowing out of the tube is V, then the coefficient of viscosity η is given by (Fig 3.3)

$$\eta = \frac{\Pi Pr^4}{8Vl} t \text{.....(3.2)}$$

3.3 Flow - pressure relationship : If the flow rate (Volume per time) of the liquid amounts to Q mlsec^{-1} in the tube, then $Q = \frac{V}{t} = \frac{\Pi r^4 P}{8\eta l}$.....(4.3)$Since V = \frac{\Pi r^4 P}{8\eta l}$

When, the Poiseuille's formula is used in hemodynamics, the pressure head is written as $P_1 - P_2$, denoting the difference between the pressures at the two ends of the system.

$$Q = (P_1 - P_2)\frac{\Pi}{8} \times \frac{1}{\eta} \times \frac{r^4}{l} \text{.....(3.4)}$$

Since, Flow is equal to pressure difference divided by resistance $R[F = \frac{P}{R}]$

$$\therefore Q = (P_1 - P_2) \times \frac{1}{R} and R = \frac{8\eta l}{\Pi r^4} \text{ .....(3.5)}$$

There Flow is regulated by small change in r, the radius of the vessel.

Ppiseuille's law is valid only for stream line flow or laminar flow, or for Newtoman fluid i.e. they have a viscosity that is constant with respect to flow.

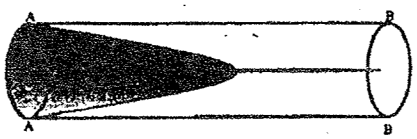


Fig. 3.3 : The velocity profile in a tube AB in steady laminar flow. This profile forms a parabola. The average velocity (V) is half the axial velocity (V_{axial})

3.4 Oswald Viscometer : The most frequently used method of determining liquid viscosity is based on the determination of time of flow of a given volume of a liquid through a length of Capillary tubing and to compare this time of flow with the time of flow of the same volume of another liquid of known viscosity. An apparatus in common use for this purpose is the Oswald Viscometer.

Oswald Viscometer consists essentially of a glass bulb A, with two mark x and y – one above and the other below - connected to a Capillary tube C and another bulb B called the storage bulb (Fig. 3.4)

Fig 3.4. Oswald Viscometer

A definite volume of liquid 1 of viscosity η_1 and density ρ_1 is poured into B, sucked into A and its time of flow between the marks $x \rightarrow y$ is observed. Let it be t_1. With a second liquid 2 of viscosity η_2 and density P_2, the experiment is repeated. Let t_2 be its time of flow between the mark $x \rightarrow y$. Now if the time of the flow of equal volume of two liquid through the same Capillary are measured under same pressure head of liquid (P) (P = h *pg*), then the pressure can be taken as proportional to density (ρ) of the liquid. As the same Capillary is used, radius and length are constant.

$$\therefore \frac{\eta_1}{\eta_2} = \frac{\Pi P_1 r^4 / 8Vl(t_1)}{\Pi P_2 r^4 / 8Vl(t_2)} = \frac{P_1 t_1}{P_2 t_2}$$

$$\text{Or,} \quad \frac{\eta_1}{\eta_2} = \frac{\rho_1 t_1}{\rho_2 t_2} [\because P = h\rho g]$$

Thus, the viscosity is proportional to the time of flow multiplied by the density.

Thus we could compare the viscosity of experimental lequid with known liquid at te same temperature.

Example : 1 : The density of liquid A is 1.102 $gmcm^{-3}$ and 5 ml. of it flows through a Capillary in 60 Sec. 5 ml. of water (density 0.9982 $gmcm^{-3}$) flows through the same capillary in 40 Sec. Calculate the *relative viscosity* of liquid A.

Answer: $d_1 = 1.02\ gmcm^{-3}$ $t_1 = 60$ Sec.

$d_2 = 0.9982\ gmcm^{-3}$ $t_2 = 40$ Sec.

Relative Viscosity $\frac{\eta_1}{\eta_\infty} = \frac{1.102 \times 60}{0.998 \times 40} = 1.66$

Example : 2 : In a certain experiment on the flow of a liquid through a Capillary tube, following data was obtained.

Length of tube = 25 Cm(*l*), radius = 1mm(r)

Volume of liquid issuing per min *(t)* = 15 ml (V)

head of liquid = 30 cm.

Calculate the viscosity if the density of liquid is 2.3 gm per c.c. (p)

Answer : Poiseulle's formula $\eta = \frac{\Pi\ r\ 4\ P}{8\ Vl} t$

$$(\frac{V}{t}) = \frac{15ml}{60Sec} = 25, r = 0.1cm., l = 25cm.$$

$$P = h\ \rho\ g = 30\ cm \times 2.3\ gm\ cm^{-3} \times 981\ cm\ Sec^{-2}$$

$$\eta = \frac{3.14 \times (2.3 \times 981 \times 30) \times (0.1)^4}{8 \times 0.25 \times 25} = [\frac{CmSec^{-2} \times gmCm^{-3} \times Cm^4}{Cm^3 Sec^{-1} \times Cm}]$$

$$[\frac{212543.46}{50} \times 10^{-4}] = 0.425 Poise(gmcm^{-1}Sec^{-1}) Ans.$$

3.5 Factors affecting viscosity :

(a) **Density :** Viscosity of a liquid varies directly with its density. For two liquids having densities ρ_1 and ρ_2 and viscosities η_1 and η_2 respectively,

$\frac{\eta_1}{\eta_2} = \frac{\rho_1}{\rho_2}$ The ratio of viscosity and density of a liquid is called its *kinematic* Viscosity (K): $K = \frac{\eta}{\rho}$

(b) **Temperature :** Viscosity of a liquid falls with the rise intemperature, because heat raises the kinetic energy of molecules for overcoming the resistance due to intermolecular attraction.

(c) **Solute Concentration :** Viscosity of a solution or suspension exceeds that of the pure solvent and rises in direct proportion to the solute concentration.

(d) **Size and Shape of Solute particle :** A larger or elongated solute molecules imparts higher viscosity to its solution than a smaller or spherical molecule, because a nonspherical or large particle presents a larger surface towards the direction of flow and consequently has a higher frictional coefficient.

3.6 Biological application :

(a) **Viscosity of protein solution :** Viscosity of a protein solution results from restrictions in the free movement of water in that solution owing to (i) solvation layer around protein particles, (ii) large protein particle, (iii) their non-spherical, elongated forms and (iv) their surface ionic group. Viscosity is the lowest at the isoelectric pH because protein zwitterions at this pH bear minimum net charge.

(b) **Viscosity of blood plasma and serum :** The plasma viscosity normally ranges from 15 - 20 mpoise at 20°C. Large fibrous fibrinogen molecule contribute more to plasma viscosity than smaller globuler molecules of other plasma proteins. Serum has a lower viscosity than plasma due to the lack of fibrinogen. But serum viscosity is raised even ten fold due to enhanced concentration and polymerization of immunoglobulin in multiple myeloma.

(c) **Viscosity of whole blood :** This amounts to about 40 mpoise at 20°C and depends partly on protein concentration, but more on the number of erythrocytes. The blood cells have like suspended particles to increase the viscosity. So the higher the hematocrit value, the greater are the viscosity. Thus, viscosity rises in polycythemia with an abnormally high RBC count and falls in chronic anemia with low hematocrit value.

(d) **Vascular resistance :** The resistance (R) against blood flow is proportional directly to the blood viscosity (η) and the vessel length (l) and inversely to the fourth power of the versel radous (r) $\left[R = 8\eta l / \Pi r^4\right]$. So, a rise in blood viscosity increases the resistance and consequently *enhance* the work of the heart in maintaining a steady blood flow.

(e) **Streamline blood flow :** The critical velocity Vc at and above which blood flow is turbulent is proportional directly to blood viscosity η and inversely proportional to its density ρ and radius r of the vessel : $Vc = \frac{\eta}{r\rho}Re$ Where Re is a constant (Reynold's number) amounting to 10^3 for blood. Normally blood flow is stream line at velocities upto the critical velocity.

Suggested Questions on Ch - 4 (Viscosity) :

Q. 1. Define coefficient of viscosity.

Q. 2. Give Poiseuelle's formula for viscosity.

Q. 3. Poiseuelle's law relates the flow and pressure analogous to Ohm's law. Give flow - pressure relationship in Poiseuelle's law. What are the resistance factors in Poiseuillis equation?

Q. 4. Write on the principle of Oswald viscometer.

The density of liquid A is 1.102 $gmcm^{-3}$ and 5mlofit flows through a capillary in 60 Sec. 5ml. of it flows through a capillary in 60 Sec. 5 ml of water (density 0.9982 $gmcm^{-3}$) flows through the same capillary in 40 Sec. Calculate the relative viscosity of liquid A.

Q. 5. Discuss **three** factors that affect viscosity.

Q. 6. Example 1 and 2.

Keywords : Velocity profile, Stream line flow, Shearing force, Friction, Tangential flow, flow - pressure relation, Viscometer, density, frictional coefficient.

Suggested Readings : At the end of the module.

4. SURFACE TENSION :

4.1 The Nature of Surface Tension : The molecules of a liquid attract each other. The force of attraction falls of rapidly with distance, and so it may be regarded that there is a *Sphere of attraction* for each molecule which will attract other molecules if they fall within this sphere of attraction.

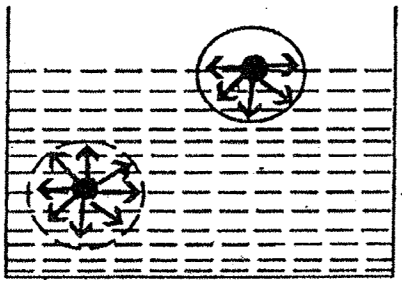


Fig. 4.1 - Molecular Attraction inside a Liquid.

A molecule which lies entirely inside the liquid is attracted equally from all sides. Since its sphere of attraction lies completely inside the liquid as a result of which there is no unbalanced force remaining on it. On the otherhand, a molecule on the surface layer has got the liquid only on one side of it and so will be attracted by all the molecules in its sphere of influence towards the interior of the liquid (Fig. 4.1). It is therefore acted upon by a force which tends to drag it in the interior of the liquid. This unsymmetrical attraction gradually diminishes with the depth. So, it appears that a thin layer on the surface is under a state of tension and work is to be done in bringing molecules from the interior to the surface.

4.2 Definition of Surface Tension : It is thus seen that every liquid behaves as if it were enclosed by a membrane, which always tends to contract so as to keep the liquid under tension.

Surface Tension (γ) is the force acting perpendicularly inward on the surface layer of a liquid to pull its surface molecules towards the interior of the liquid mass. It equals the energy required for bringing molecules from inside the liquid mass to surface against inward molecular attraction in order to expand the liquid surface by a unit area. Surface tension contracts the liquid surface to the minimum area and keeps it in a state of tension like a stretched membrane.

Surface Tension (γ) is expressed as the force in dynes acting perpendicularly to a 1cm. line on the liquid surface (dyne cm^{-1}). At 20^0C, γ amounts to 465, 72.8, 65.2 and 21.7 dyne cm^{-1} for mercury, water, glycerol and ether respectively on their liquid - vapour interface.

4.3 Capillary action : If a Capillary tube is immersed in a liquid that strongly attracts and consequently wets the tube material, a thin liquid film spreads upto some distance on the inner surface of the tube. This stretches the liquid surface into an upward concavity. To minimize the free surface energy, surface tension of the liquid then

tends to decrease the stretched surface by pulling it upwards into the tube. This raises the liquid in a short column with a concave upper meniscus inside the tube and above the liquid surface outside.

If the liquid does not a attract or wet the tube material, surface tension produces a convex upper meniscus and depresses the liquid surface in a Capillary tube.

4.4 Factors affecting Surface Tension :

(a) Density : Macleod's equation relates γ to the density (ρ) of the liquid and the ρ' of its vapour :

$$\gamma = K\ (\rho - \rho')^4$$

Where K a constant called MacLeod Constant which is characteristic of liquid.

(b) Temperature : The surface tension of a liquid in contact with its vapour *decreases* with rise of temperature (Table 4.1) and necessarily becomes zero at the critical temperature. Ramsay and shields (1898) formulated the following equation : $\gamma\left(\frac{M}{\rho}\right)^{2/3} = K(Te - T - 6)$

Table

Tem^{0}C	γ Water dyne cm^{-1}
0^0	75.64
20^0	72.75
40^0	69.56
80^0	62.61
100^0	58.85

Where, K is a constant called Eotvos constant

Te is critical temperature

T-temperature

M is molecular weight

ρ is the density.

γ is the surface tension.

(c) Solutes : Solutes, dispersing in a liquid instead of concentrating on its surface, raise its surface tension while those concentrating on the liquid surface lower its surface tension.

4.5 Determination of Surface Tension :

Drop weight method : Surface tension of a liquid is directly proportional to the weight of its drops from a vertical Capillary tube.

A *Stalagmometer* is a pipette - like glass tube with a smooth capillary end C and two calibrated portions of the stem G with an intervening bulb B (Fig. 4.2).A liquid of unknown surface tension (γ) is sucked up into the tube and it is placed vertically in a thermostat bath, using the calibrations on the stem, a known volume V of the liquid is dropped out in slow regular drops from the Capillary end and the number (n) of drops is counted.An identical volume V of a standard liquid of *known* surface tension (γ') is similiarly dropped out from the tube and the number (n') of its drops is also counted, where ρ and ρ' are the known densities of the liquids respectively, $\frac{\gamma}{\gamma'} = \frac{n'\rho}{n\rho'}$

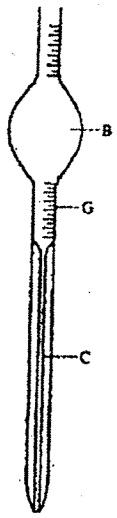


Fig. 42 Stalagmometer.

4.6 Biological application :

(a) *Emulsifying action of bile salt* : Bile Salts lower the surface tension of fat droplets in the duodenum.

(b) *Plasma - surface tension* : Due to the presence of surfactants like lecithins in the plasma, the latter has a surface tension, $\gamma = 50 dynes\ cm^{-1}$ at 4°C much less than water $\left(r = 69.56\ dyne\ cm^{-1}\right)$

(c) *Lung compliance*: Comphance or stretchability of lungs depends on the surface tension of the fluid lining the inner surfaces of pulmonary alveoli. The alveolar epithelium secretes *Lung Surfactants* containing amphipathic phospholipids. They form a monolayer on the inner surface of alveoli and reduce the surface tension.

(d) An identical alveolar surface tension (γ) develops a higher pressure (inwardly directed collapsing pressure) P in smaller alveoli than in larger ones [Lapaces law $\rightarrow P = 4\gamma / r$ where r is the radius of alveoli]. This would tend to blow up larger alveoli and collapse smaller alveoli. But lung surfactants normally prevent such incidents by lowering the alveolar surface tension.

Suggested Questions :

Q. 1. (a) Define Surface Tension (b) How does it expressed?

Q. 2. Discuss two factors that affect Surface Tension.

Q. 3. (a) Write on the determination of Surface Tension by Stalagmometer.

(b) Explain the Equation $\gamma / \gamma' = n'\rho / n\rho'$

Q. 4. Discuss how Surface Tension can be reduced by surfactant molecules present in Pulmonary alveoli.

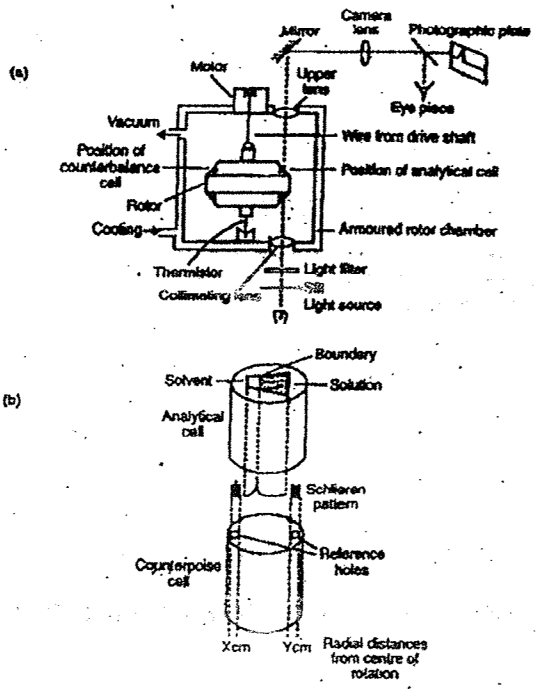
Keywords : Surface Tenion, Sphere of attraction, liquid - vapour interface, Capillary action, Drop weight method, Stalagnometer, Lung compliance, Surfactants.

Suggested Readings : At the end of the module.

5. CENTRIFUGATION : Moving boundary Ultracentrifugation and density gradient Ultracentrifugation.

5.1 Ultracentrifugation : An Ultracentrifuge developed by T. Svedberg may be used for speparating and isolating macromolecules such as protein, lipoproteins and nucleic acids and subcellular particles like microsomes and ribosomes from solutions and suspensions. It is also used for verifying the homogeneity and estimating *Sedimentation coefficients*, molucular weights of marcromolecules.

5.2 Detail of Ultracentrifuge : The instruments are capable of operating at speeds approaching 70,000 rev. per minute (*r* pm), generating centrifugal field of 3×10^5 to 6×10^5 g, and consist of a motor, a rotor contained in a protective chamber which is refrigerated and evacuated (to minimize the thermal effects of air friction), and an optical system which enables the sedimenting material to be observed so as to determine concentration distributions within the sample at any time during centrifugation (Fig. 5.1)



5.1 Fig. : Diagrammatic representation of (a) an analytical ultracentrifuge system and (b) an analytical and counterpoise cell.

The rotor is suspended on a wire coming from the drive shaft of a high speed motor which permits the rotor to find its own axis of rotation. Housed in the rotor are two cells, the *analytical cell* and the *counter balance* cell which counter balance the analytical cell.

The rotor chamber contains an upper and lower lens, the former, together with a camera lens, focuses light onto a *photographic plate* whilst the latter collimates the light so that the sample cell is illuminated by parallel light. The blackening of the film is inversely proportional to the concentration of particles in a layer. Alternately, monitoring of the sedimenting materials may be achieved by difference in *refractive index* utilising a *Schlieren optical system*. The Schlieren optical system makes use of the fact that, if light passes through a solution of uniform concentration it does not deviate but on passing through a solution having different density zones, it is refracted at the boundary between these zones. The optical system records the change in refractive index of the solution which will vary as the concentration changes.

5.3 Moving Boundary Ultracentrifugation : In the case of sedimenting materials in an analytical cell, a boundary is formed between the solvent, which has been cleared of particle, and the remainder of the solution containing the sedimenting material. The concentration can be determined from the area of the peak. As sedimentation proceeds the boundary, and hence the peak, shifts, the rate at which the peak moves giving a measure of the rate at which the material is sedimenting.

The rate of sedimentationof the particles being spun in a centrifuge tube under the influence of a strong gravitational force can be determined. The centrifugal force acting on the solute particle of mass m is mw^2r, where w is the angular velocity of the rotor in radians per second, r the distance from the center of rotation to the particle (Fig. 5.2)

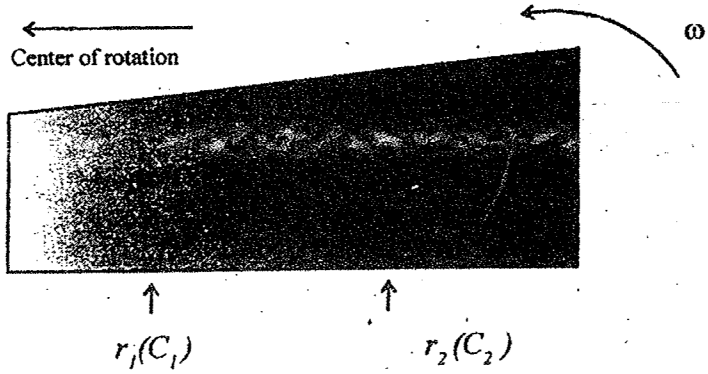


Fig. 5.2 : Concentration gradient established in the sample cell.

5.4 Sedimentation coefficient (s) : The sedimentation velocity v is determined from the outward migration (Δr) of a boundary during an interval Δt. It is expressed in term of *sedimentation coefficient(s)* which is the sedimentation velocity in $cmsec^{-1}$ per unit field of centrifugal force.

$$s = \frac{\Delta r / \Delta t}{r w^2}; \quad v = \frac{\Delta r}{dt} = srw^2 \quad \text{.....Eq.5.01}$$

A sedimentation coefficient of $1 \times 10^{-13} cm \sec^{-1}$ is expressed as one *Svedberg unit (S)*.

Human IgG and IgM have sedimentation coefficients of 7 S and 19 S respectively.

5.5 Measurement of *s* :

$$s = \frac{dr / dt}{\omega^2 r} \quad \text{..... Eq. 5.02}$$

$$\text{Or, } sdt = \frac{1}{\omega^2} \frac{dr}{r} \quad \text{..... Eq. 5.03}$$

Integration over distance travelled by the particle from $r = r_o$ $(t = o)$ *to* $r = r$ $(t = t)$ *give*

$$\int_o^t sdt = \frac{1}{w^2} \int_{r_o}^{r} \frac{dr}{r}$$

$$\boxed{s = \frac{1}{t\omega^2} \ln \frac{r}{r_o}} \quad \text{..... Eq. 5.04}$$

So, s can be determined from a plot of lnr *vs* time.

5.6 Measurement of Molecular Weight (MW) :

In addition to centrifugal force $\left(m \omega^2 r\right)$, we must consider the particle's *buoyancy* as a result of the displacement of the solvent molecules by the particle. This *buoyancy* reduce the force on the particle by $\omega^2 r$ times the mass of displaced solvent m_s, and v and ρ are the volume of particle and the density of solvent respectively.

net force = Centrifugal force - buoyant force

$$\text{net force} = \omega^2 r m - \omega^2 r m_s \quad \text{..... Eq. 5.05}$$

$$\text{net force} = \omega^2 r m - \omega^2 r v \rho \quad \text{..... Eq. 5.06}$$

A net force acting on a particle would cause it to accelerate. The initial acceleration lasts for a very short time $\left(10^{-9} Second\right)$, after which the particle moves at a constant velocity. This is because the medium exerts a

frictional force on the particle, which is proportional to the sedimentation velocity dr/dt. The frictional force is equal to the product of the frictional coefficient f and the sedimentation velocity. It acts in the opposite direction to the net force. *At Steady State*, then, the frictional force is equal to the net force :

$$f\frac{dr}{dt} = \omega^2 rm - \omega^2 r\,\bar{v}\,\rho \quad \text{..... Eq. 5.07}$$

The volume of the particle is a difficult quantity to measure, so a new term called *partial specific volume* $\bar{v}$, is used. $\bar{v}$ defined as the increase in volume when 1 g of dry solute is dissolved in large volume of solvent. The quantity $m\bar{v}$ is the incremental volume increase when one molecule of mass m is added to the solvent, that is, it is equal to v of the particle.

$$f\frac{dr}{dt} = \omega^2 rm - \omega^2 rm\bar{v}\rho$$

$$f\frac{dr}{dt} = \omega^2 rm(1-\bar{v}\rho) \quad \text{..... Eq. 5.08}$$

Rearrangement of Eq. 5.8 $f\frac{dr}{dt} = \omega^2 rm(1-\bar{v}\rho)$

$$s = \frac{dr/dt}{\omega^2 r} = \frac{m(1-\bar{v}\rho)}{f} \quad \text{..... Eq. 5.09}$$

$$s = \frac{M}{No}\left(\frac{1-\bar{v}\rho}{f}\right) \quad \text{..... Eq. 5.10}$$

Where M is the molar mass of the solute and No is Avagadro's number.

The frictional coefficient f is given by Stoke's law

$f = 6\,\Pi\,\eta\,r_s$ (r_s is the radius of the solute molecule)

$$M = \frac{sNo(6\Pi\eta r_s)}{(1-\bar{v}\rho)}$$

Diffusion coefficient $D = \frac{RT}{6 \Pi r \eta No}$

$f = \frac{KT}{D}$, $\boxed{M = \frac{sRT}{D(1-\bar{v}\rho)}}$ ..... Eq. 5.11

R = molar gas constant, T = absolute temp.

D = diffusion coefficient of the solute.

$\bar{v}$ = partial specific volume of the solute.

ρ = density of the liquid

s = svedberg constant / sedimentation coefficient.

5.7 Density - gradient ultracentrifugation : This method separates macromolecules and subcellular particles of a heterogeneous mixture, depending on the density gradient of the centrifuged solution.

(a) Preformed density - gradient ultracentrifugation : Successive volumes of progressively dilute sucrose solutions are poured gently into a plastic centrifuge tube. This establishes a sucrose density gradient rising progressively downwards in the tube ; but the sucrose density neither exceeds nor equals the density of the least dense macromolecules to be separated. The sample is then gently layered over the upper surface of the column of surcrose solution. On ultracentrifuging the tube, particles of each species of macro molecules descend according to their sedimentation coefficient and MW. Contents of the heterogeneous sample are thus separated into different layers or bands along the sucrose density gradient (Fig. 5.3).

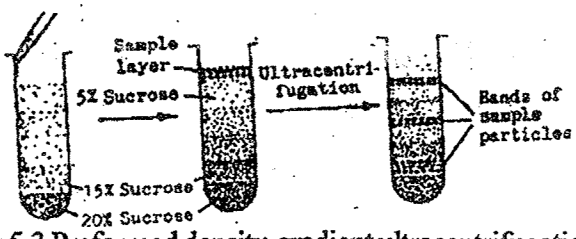


Fig 5.3 Preformed density-gradient ultracentrifugation

Particles of the highest and the lowest densities get concentrated in the lowest and the top most band respectively. Finally, the bottom of the tube is punctured to collect the contents of different bands successively in different tubes for further analysis.

(b) Equilibrium density gradient ultracentrifugation : This method is frequently used in speparating nucleic acids, viruses and subcellular particles like ribosomes, mitochondria. The sample is first mixed with a

concentrated solution of a fast - diffusing salt of a heavy metal such as the *Chloride of Cesium*. The mixture is then ultracentrifuged for several days until the *Sedimentation equilibrium* is attained and the bands of sample particles cease to migrate. The centrifugal force establishes a downward increasing concentration gradient of heavy metal ions. This results in a continuous liquid density gradient rising progressively in the direction of the centrifugal force (Fig. 5.4).

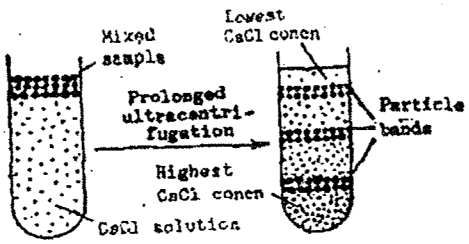


Fig 5.4 Equilibrium density gradient ultraccentrifugation

Each homogenous species of particles descends under the centrifugal force along this density gradient, but up to that layer of the liquid where the density of the latter coincides with the density of these particles. Thus, the particles of a heterogeneous sample are separated into different bands according to their bouyant density. For example, a heterogeneous mixture of DNA molecules may be separated into a lower band of GC - rich DNA and an upper band of AT - rich DNA by centrifugation at 5000 *rpm* for 3 days along a CsCl density gradient of 1.65 - 1.75 gm ml^{-1}. Finally, the bottom of the plastic centrifuge tube is punctured and the contents of separate bands are successively collected in separate tubes. But proteins cannot be separated by this method because of too close densities of different protein species and the salting out the denaturing actions of heavy metal ion (cesium).

Suggested Questions :

Q. 1. (a)What is Ultracentrifugation?
(b) Discuss briefly on the principle of an analytical ultracentrifuge.

Q. 2. (a) Discuss special features of moving boundary ultracentrifugation.
(b) What are sedimentation coefficents (s) and Svedberg unit(S)?

Q. 3. Give equation for determination of molecular weight from sedimentation coefficient and diffusion coeffient. Explain all terms.

Q. 4. (a) What are the *two* types of density gradient ultracentrifugation?
(b) Which method is used for separation of protein?

Keywords : Ultracentrifuge, density gradient, Sedimentation coefficient *rpm* , refractive index, Schlieran optical system, Centrifugal force, bouyant force, frictional force, frictional coefficient, Stoke's law, partial specific volume of solute, Diffusion coefficient, Svedberg constant, Sucrose density gradient.

Suggested Reading : At the end of module.

n **Topic : II. Properties of solution :**

6. OSMOSIS:

6.01 Semipermeable membrane: There are many membranes which preferentially allow the solvent molecules to pass freely through them but does not allow the solute molecules to pass through. Such a membrane is called a *Semipermeable membrane*. Examples of such semipermeable membranes are fish bladders, cellophane paper, parchment, collodion etc.

6.02 Osmosis : If a semipermeable membrane is placed such that it separates a solution from a solvent or a strong solution from a weak solution, it is found that water seeps through the membrane from the solvent side to the solution side (or, from the weak solution to the strong solution). *This spontaneous passage of solvent from a solution of lower concentration towards a solution of higher concentration when the two are separated by a semi permeable membrane is called OSMOSIS.*

6.03 Abbe' Nollet's Experiment : Osmosis i.e., passage of a solvent through membrane was first observed by Abbe' Nollet (1784). He used pig's bladder as the semipermeable membrane, which was stretched across the mouth of a thistle funnel (Fig 6.1) ρ. This funnel was filled with a strong sugar solution and inverted over a trough of water, as shown in the figure. The water flows across the membrane to the sugar solution, raising the fluid level in the stem of the invertled funnel and a position of equibrium is ultimately reached when the hydrostatic pressure equalises the pressure forcing the water in.This pressure developed as a result of osmosis is the *Osmotic pressure* of the solution and is measured by the difference in water levels inside and outside.

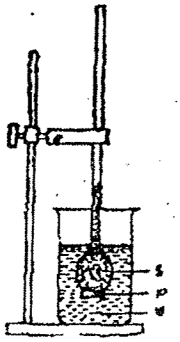


Fig. 6.1 : Abbe Nollet's Experiment.

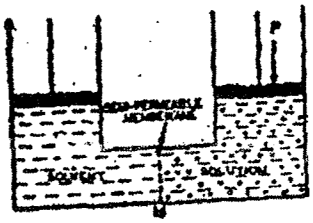


Fig. 6.2 : Definition of osmotic pressus

6.04 Definition of Osmotic pressure : Suppose we have an arrangement as shown in Fig. 6.2. It is a doubly bent tube fitted with a Semipermeable membrane, on one side of which there is the pure solvent while on the other is the solution, both arms being fitted with water tight pistons. This system cannot be in equilibrium, since the solvent will tend to pass to the solution unless we apply a pressure P on the piston. On the solution side to

counteract this tendency of solvent to flow in. This excess pressure on the solution's side is the Osmotic pressure of the solution at this concentration. So we may give the following definition of Osmotic pressure. *If a solution is separated from the solvent by means of a semi-permeable membrane, the excess pressure which has to be applied to the solution in order to check the inflow of the solvent and establish equilibrium in the systm, is called the Osmotic pressure of the solution* (Π).

The main point to realise about Osmosis and Osmotic pressure is that they are manifestations of the fact that a solution and a solvent (or two solutions of unequal concentrations) are not in *thermodynamic equilibrium.* Osmosis is merely a passage towards this eqilibrium.

6.05 Van't Hoff Laws of osmotic pressure : In dilute solution, solute molecules behave almost like gas molecules. So, van't Hoff laws of osmotic pressure resemble the gas laws.

Law - 1 : Temperature remaining constant, the Osmotic pressure of a solution is proportional to the molar concentration (c) of the solute .

$$\Pi \alpha c, \quad \Pi = k_1 c \quad \text{..... Eq.6.1}$$

If, V litres of the solution contain 1 mole of solute, then

$$c = \frac{1}{V} \qquad \therefore \Pi = k_1 c = k_1 / V \quad or \quad \Pi V = k_1 \quad \text{..... Eq.6.2}$$

This relation is comparable to Boyle's Law viz PV = constant, when the temperature is constant. If, V litres contain n moles of the solute, the expression turns to : $\Pi V = n\kappa_1$

Van't Hoff's First law implies that the more concentrated a solution, the higher is its osmotic pressure.

Law - 2 : The Osmotic pressure of a solution is directly proportional to the absolute temperature T as long as its concentration remains constant.

$$\Pi = \kappa_2 T, \text{ Or, } \quad \frac{\Pi}{T} = \kappa_2 \quad \text{..... Eq.6.3}$$

This relation is analogous to the expression for Gay -Lussac's law νi_3 P/T = constant, volume remaining constant.

Van't Hoff equation emerges from a combination of this two laws :

$$\Pi v = nRT \text{ Or, } \Pi = \frac{n}{V} RT \text{ . Or, } \boxed{\Pi = cRT} \quad \text{..... Eq.6.4}$$

Where R is the molar gas constant, T is the absolute temperature, n is the number of moles of solute in V litres of solution and c is the molar concentration of the solute.

Law - 3: *Equimolecular quantities of different solutes dissolved in the same volume of a solvent exert equal osmotic pressure under identical conditions of temperature.*

Since, equimolecular quantities of non ionized solutes contain an equal number of molecules, this law is equivalent to Avagadro's law for gases : Equal volume of all solution (nonelectrolytes) having same T and Π contain equal number of solute molecule.

Thus, the osmotic pressure of a solution containing 1 mole of solute in 22.4 litre is identical with the pressure of 1 mole of gas occupying 22.4 litres and both amounts to 1 atm at 0^0C.

s 6.06 The Van't Hoff equation for ionised solution : The osmotic pressure of a solution of an ionized solute is the sum of the partial osmotic pressure resulting from the molar concentration of all types of its ions, because each ion behaves as separate osmotically active partice. Thus -

$$\Pi = icRT \quad \text{..... Eq.6.5}$$

Where i is the isotonic coefficient of the ionizable electrolyte.

s 6.07 Units - Osmolarity : Osmotic pressure is expressed in mmHg, atm, or dynes cm^{-2}. Osmol unit give the number of osmotically active particles per mole of solute. Each mole of a nonionized solute is equivalent to 1 Osm or osmol.

Osmolarity of a solution is its solute concentration in osmols per litre of the solution.

Example - 1 : Calculate the osmotic pressure of a decinormal (N/10) solution of cane sugar at 0^0C.

using equation $\boxed{\Pi = cRT}$ where c = 0.1, $= 273^0$ Abs.

$$\Pi = 0.1 \times 0.0821 \times 273^0$$

$$\left[\frac{mole}{lit} \times lit - atm \deg^{-1} mole^{-1} \times \deg\right] \quad / \because R = 0.0821 litre - atm \text{ per degree per mole}$$

$$\Pi = 2.24 atm$$

6.08 Determination of Osmotic Pressure :

Barkeley and Hartley method : The apparatus consists of an inner, porous porcelain tube enclosed in an outer, wider and concentric gun-metal tube. The wall of the inner tube is made semipermeable by impregnation

with copper ferrocyanide $[Cu_2Fe(CN)_6]$. A Capillary tube (T) serving as a manometer and a stoppered funnel serving as a water reservoir are connected to the two ends of the inner tube . The outer tube is connected by the tube with a pump (P). The inner and outer tubes are filled respectively with water and the test solution (Fig. 6.3). The osmotic outflow of water from the inner tube through its sempiermeable wall to the outer tube cause, a fall in the fluid level in the manometer tube. Pressure is then applied by the pump on the solution in the outer tube until the water meniscus is maintained at the initial level in the manometer tube. This pressure read from the pressure gauge, gives the osmotic pressure of the test solution.

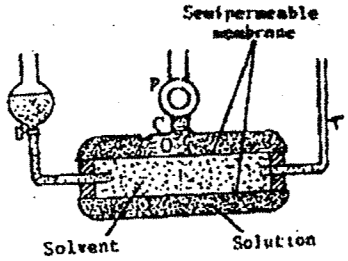


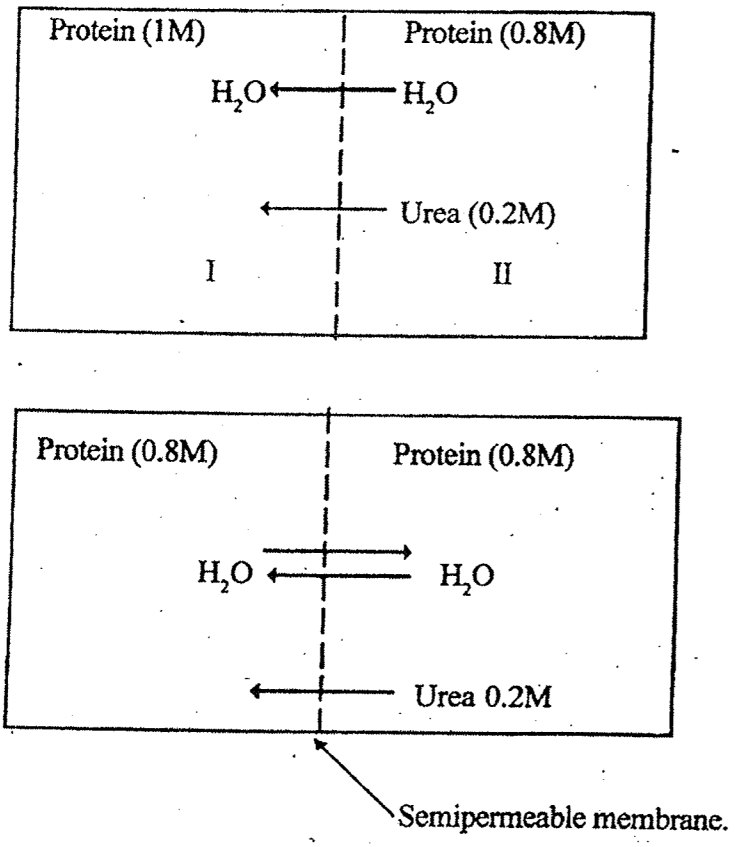
Fig. 6.3 Berkeley-Hartley apparatus.

6.09 (a) Tonicity and isotonic solutions : When a solution is separated from the pure solvent by a *perfectly semipermeable membrane* which allows the solvent particles but none of the solute particle to pass through, the osmotic pressure of the solution is proportional to the sum of the molar concentrations of all its solutes. Moreover, the osmotic pressure of this solution is identical with that of any other solution, having the same total molar concentration of solutes and remaining separated from the solvent by a perfectly semipermeable membrane. Solutions with such an identical osmotic pressure are called *Isosmotic solution.* A solution is called *hyperosmotic* or *hyposmotic* in comparison to another if the former has respectively a higher or lower osmotic pressure than the latter.

The plasma membrane, though semipermeable, is not perfectly semipermeable. It allows the diffusion of water and some solutes, but prevents the diffusion of some other solutes. This fraction of the total osmotic pressure is called the *tonicity* of the solution and is proportional to the sum of molar concentrations of only such solutes to which the membrane is impermeable. Two solutions of identical tonicity are called *isotonic solutions* though they are not necessarily *isosmotic.*

For example, if the plasma membrane, permeable to water and urea but not to proteins, separates a 1 M protein solution from another solution of 0.8M protein and 0.2 M area, the two solutions are isosmotic but not isotonic; the tonicity is higher in the first solution than in the second because tonicity depends on protein concentration only. (Fig 6.4). On the other hand, the first solution would have a lower osmotic pressure but an

identical tonicity with the second if the protein concentration is lowered to 0.8M in the former. The osmotic flow of water depends on the difference in tonicity across the membrane.



(A) I is isosmotic with but hyper-tonic to II

(B) I is hyposmotic to but isotonic with II

Fig. - 6.4 : Water movement across a membrane permeable to water and urea but not to proteins.

(b) **Hypertonic and hypotonic solutions :** In Fig. 6.4A. the solution in chamber - I is hypertonic to that in chamber II (higher tonicity), whereas the solution in chamber II is hypotonic to that in chamber I (lower tonicity).

6.10 Biological applications :

(a) **Hemolysis :** An animal cell placed in a hypotonic solution may undergo *Osmolysis* i.e. become leaky, due to osmotic inflow of water. So, if erythrocytes are placed in a 0.3% NaCl (normal saline cancentration in 0.9% NaCl) solutions, water passes into the cells from the hypotonic solution by osmosis (endosmosis) and makes the cells swell. This osmotic flow may ultimately so distend the erythrocyte that its membrane ruptures. Consquently, hemoglobin passes out to the surrounding medium (hemolysis).

(b) **Crenation :** It is the *shrunken* condition of an animal cell in a hypertonic solution owing to an osmotic outflow of water (exosmosis) from the cell.

(c) **Plasmolysis :** Plant cells are bounded by more or less firm cellulosic walls which are lined with a membrane permeable to water outward but impermeable to substances dissolved in the cell - sap, e.g. glucose, potassium salts etc. If such a cell is immersed in a solution of lower concentration and consequently of lower osmotic pressure than that of the solution inside the cells, no appreciable change in the size of the cell will be observed, since the rigidity of the wall will not allow the water from outside to come in. If, however, the cell is immersed in a solution whose osmotic pressure is greater than that of the cell -sap, there will be a diffusion of water from the interior of the cell to the surrounding solution. This will result in a partial collapse of the cell membrane and would appear under the microscope shrivelled or 'crenated'. This phenomenon is known as *Plasmolysis.*

Suggested Questions :

Q. 1. Define Osmosis and Osmotic pressure.

Q. 2. (a) State Van't Hoff's first and second laws of Osmotic pressure.

(b) What is Van't Hoff equation and how is it modified for ionised solution?

(c) What is the third law?

Q. 3. Example - 1 in the text.

Q. 4. Discuss how Osmotic pressure can be determined by Berkley - Hartley method.

Q. 5. For a biological membrane isosmotic solution may not be isotonic - explain with example.

Q. 6. What is plasmolysis?

Keywords : Semipermeable membrane, Osmosis, Osmotic pressure, gas law, Van't Hoff equation,Avagadro's law, Osmolarity, Isotonic, hypotonic, hypertonic,osmolysis, hemolysis, crenation, plasmolysis.

Suggested reading : At the end of the module.

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Topic : III. pH and BUFFERS :

7.1 The Ion product of water : The dissociation of water is an equilibrium process :

$$H_2O \rightleftharpoons H^+ + OH^-$$

for which we can write the equilibrium constant

$$K_{eq} = \frac{[H^+][OH^-]}{[H_2O]}$$

Where the concentrations in moles lit $^{-1}$. Since the concentration of pure water is very high (it is equal to the number of grams of water in a liter divided by gm molecular weight or $\frac{1000}{18} = 55.5M$) and the concentration of H^+ and OH^- ions are very low (1×10^{-7} M $at 25^0C$), the molar concentration of water is not signiciantly changed by its slight ionization. The equilibrium constant expression may thus be simplified to :

$$(55.5 \times K_{eq}) = [H^+][OH^-]$$

and the term $55.5 \times K_{eq}$ can be replaced by constant $K\omega$, called *ion product of water.*

$$K\omega = [H^+][OH^-]$$

The value of $K\omega$ at 25°C is 1.0×10^{-14}

$K\omega$ is basis for *pH scale.*

As each H_2O molecule dissociates into one H^+ and one OH^-, $[H^+][OH^-] = [H^+]^2 = K\omega$

$Or, [H^+] = \sqrt{K\omega} = \sqrt{10^{-14}} = 10^{-7}$ molo lit^{-1}

Thus, $[H^+] = 10^{-7}$ mole lit^{-1} in pure water.

7.2 pH : pH is the negative logarithm of the molar concentration of H^+ to the base 10.

$$pH = - \log [H^+]$$

For pure water at room temperature (25°C)

$$pH = - \log [H^+] = - \log [10^{-7}] = 7$$

Sorensen's pH scale is a logarithmic scale ranging fram O to 14 fordilute aqueous solutions. pH 7 is considered the neutral pH. If an acid is added to water, protons donated by the acid raise the H^+ of the solution above 10^{-7} mol lit^{-1}, if say $[H^+]$ now eguals to 10^{-5}, then mol. lit^{-1}

$$pH = - \log 10^{-5} = 5$$

Thus, pH lower than 7 indicates $[H^+]$ higher than 10^{-7} mol $lit.^{-1}$; the greater the acidity ; the lower the pH. If a base is added to water, some of the protons donated by water are accepted by base, lowering the $[H^+]$ below 10^{-7} mol $lit.^{-1}$; if say $[H^+]$ now amounts to 10^{-10} mol $lit.^{-1}$.

$$pH = - \log 10^{-10} = 10$$

Thus, a pH higher than 7 indicate $[H^+]$ lower than 10^{-7} mol $lit.^{-1}$; the greater the alkalinity, the higher is the pH.

7.3 Acids and Bases : According to the Bronsted - Lowry concepts, an acid is a proton donor and a bse is a proton acceptor.

Proton donor		**Proton acceptor**
CH_3COOH	$\rightleftharpoons$	$H^+ + CH_3COO^-$
Acid		**Base**

An acid-base reaction always involves a ***conjugate acid-base pair***, made up of a proton and the corresponding proton acceptor. For example, acetic acid (CH_3COOH) and the acetate ion (CH_3COO^-) form a conjugate acid-base pair. Each acid has a characteristic affinity for its proton. Those with high affinity for protons are weak acids and dissociates only slightly, those with low affinity are strong acids and lose H^+ ions readily. The tendency of any given acid to dissociate is given by its dissociation constant. For the acid HA, the dissociation constant at a given temperature is :

$$K' = \frac{[H^+][A^-]}{HA}, \qquad HA \rightleftharpoons H^+ + A^-$$

K' is called an apparent dissociation constant K' for CH_3COOH is 1.74×10^{-5}, while $\rho K = -\log_{10} K' = 4.76$. Strong acids have low $\rho K'$ value, and strong have high $\rho K'$ values.

7.4 Henderson - Hasselbach Equation : is a logarithmic transformation of the expression for dissociation constant K'.

$$K' = \frac{[H^+][A^-]}{[HA]}$$

$$[H^+] = K' \frac{[HA]}{[A^-]}$$

Take the negative logarithm of both sides :

$$-\log [H^+] = -\log K' - \log \frac{[HA]}{[A^-]}$$

Substituting pH = $pK' - \log \frac{[HA]}{[A^-]}$

If, we change signs, we obtain Henerson Hasselbalch equation :

$$pH = pK' + \log \frac{[A^-]}{[HA]}$$

Or, $$\boxed{pH = pK' + \log \frac{[\text{Proton acceptor}]}{[\text{Proton donar}]}}$$

This equation makes it possible to calculate the pK' of any acid from the molar ratio of proton -donor and proton -acceptor species at a given pH, to calculate the pH of a conjugate acid base pair of a given pK' and a given molar ratio and to calculate the molar ratio of proton donor and proton acceptor given the pH and pK'.

7.5 Acid Base Buffers : A buffer solution resists the change of pH when acids or alkaties are added to it. Ordinarily, an acid-base buffer consists of a mixture of either a weak acid (HA) and its conjugate (A^-), or a weak base (B) and its conjugate acid (BH^+). A mixture of two acid salts of weak polybasic acid also serves as a buffer. Examples of buffer solutions include acetate buffer (acetic acid : Na - acetate), bicarbonate buffer (H_2CO_3

: $NaHCO_3$) and phosphate buffer (KH_2PO_4 : Na_2HPO_4) .When a strong acid is added to a buffer solution, the H^+ ions donated by the acid are accepted by the base member (A^-) of the buffer to yield its acid member HA. On the other hand, a rise in OH^- ions in the solutions is neutralized by proton donated by the acid member (HA), with the simultaneous formation of its conjugate base (A^-).

For example, the acetate-acetic acid buffer neutralises acid (H^+) according to :

$$H^+ + CH_3COO \rightarrow CH_3COOH$$

and, counteracts alkalies (OH^-) as : $OH^- + CH_3COOH \qquad CH_3COO^- + H_2O$

and, tends to maintain a steady pH.

The pH of a buffer solution is determined by the ratio of molar concentrations of its acid and base members and dissociation constant (Ka) of the acid member : $pH = pKa + \log\frac{[A^-]}{[HA]}$

Evidently, addition of a strong acid or alkali changes the ration $[A^-]$ / [HA] of a buffer solution, and so, change the pH is far less that would have occurred in absence of any buffer.

The effective range of pH for a buffer extends roughly over a pH range of ± 1 around the pKa of its acid member.

7.6 Acid Base Indicator : Acid base indicators or pH indicators are weak organic acids or bases which change colour during the pH change of their solution over a specific pH range. Each indicator exists as a conjugate pair of either a weakacid and its conjugate base or a weak base and its conjugate acid. The two members of this conjugate pair bear sharply different colours. At acidic pH, an indicator accepts protons from the medium and exists in its protonated or acid form (Hln) ; at alkaline pH, Hln donates protons to the medium to change into the base form (In^-). Due to such structural interconversions the indicator solution changes colour with the pH change. The pH of an indicator solution is given by the Henderson - Hasselbalch equation :

$$pH = pK_{In} + \log\frac{[In^-]}{[HIn]}$$

7.7 Buffers in pH regulation in Biological System :

(a) Bicarbonate Buffer : It is the prinicplal buffer in extracellular fluids such as blood plasma. It comprises bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3) as the base and acid members respectively. The

bicarbonate buffer neutralizes stronger metabolic acid (HA), changing them to the corresponding weak conjugate bases (A^-); there is a simultaneous increase in H_2CO_3. Stronger base (B) are also changed to the corresponding weak conjugate acids (BH^+) with a concomitant rise in HCO_3^-.

$$HA + HCO_3^- \rightleftharpoons A^- + H_2CO_3$$

$$B + H_2CO_3 \rightleftharpoons BH^+ + HCO_3^-$$

and, Henderson - Harelbalch equation :

$$pH = pKa + \log \frac{[HCO_3^-]}{[H_2CO_3]}$$

The normal $[H'CO_3^-]$ / $[H_2CO_3]$ ratio amounts to 20 in the plasma, the buffer ratio of 20 gives a pH of 7.4 to the solutions of bicarbonate buffer.

pH = 6.1 + log 20 = 7.4

(b) **Phosphate Buffer :** The Phosphate buffer mainly comprises dibasic phosphate [HPO_4^-] and monobasic phosphate [$H_2PO_4^-$] as the base and acid members respectively. It is next to the bicarbonate buffer in importance in extracellular fluid other than blood. Plasma normally has a [HPO_4^-] [$H_2PO_4^-$] ratio of 4, therefore

$$pH = pKa + \log \frac{[HPO_4^-]}{[H_2PO_4^-]}$$

= 6.8 + log 4 = 7.4

7.8 pH determination by pH meter :

(I) **Two half-cells :** A pH meter consists of a concentration cell made of *two half cells* placed in a circuit with a potentiometer. The latter supplies an adjustable opposite EMF to balance and nullify the test cell EMF.

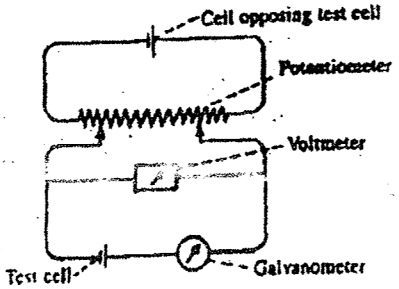

Fig. 7.1 : Potentiometric measurement of pH

The half cells consist respectively of a *reference electrode* (a silver - silver chloride or calomel electrode) and an *indicator electrode* (a glass or hydrogen electrode). The reference electrode is dipped in a standard solution of known H^+ concentration, while the indicator electrode is immersed in the unknown solution whose pH is to be determined. An EMF is generated in the test cell depending on the difference between the two solutions in H^+ concentration. The potentiometer is adjusted to supply an equal but opposite EMF to nullify the test cell EMF and to suspend all current flow in the circuit. The test cell EMF is obtained from the scale - reading for the nullifying EMF, and the pH of the unknown solution is computed therefrom **(Fig. 7.1).**

(II) Hydrogen electrode : This consists of a platinum black-coated platinum foil, dipping in a solution through which H_2 gas is bubbled to saturate the platinum black and *to maintain the constant H^+ activity* of the solution. The platinum black absorbs hydrogen and catalyses the attainment of equilibrium between hydrogen gas and hydrogen ions in the solution such that the electrode in effect functions as a hydrogen electrode (Fig. 7.2).

(III) Calomel electrode / Salt bridge : The other half cell is usually a normal calomel electrode which is usually the positive electrode of such set up. A calomel electrode consists of metallic mercury overlaid successively by a layer of Hg - $HgCl_2$ paste in KCl solution and then a layer of KCl solution saturated with calomel Hg_2Cl_2. A platinum wire inserted through the glass tube connects the mercury with potentiometer circuit ; the KCl solution of the calomel electrode is in turn connected to the unknown (test) solution by a *salt bridge* made of saturated KCl solution (Fig 7.2).

Thus, two half cells are joined through an intervening salt bridge. When equilibrium has been attained, the total EMF of the cell is measured by a potentiometer, which substituted to give pH value.

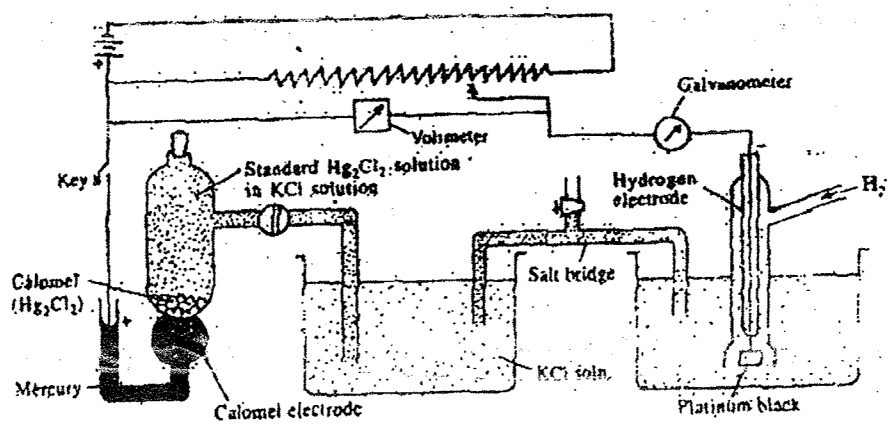

Fig. 7.2 Calomel and hydrogen electrodes.

(IV) Silver - Silver Chloride Electrode : This standard half cell is formed by immersing an AgCl coated silver wire into a saturated solution of KCl over some crystals of KCl in a glass tube (Fig 7.3).

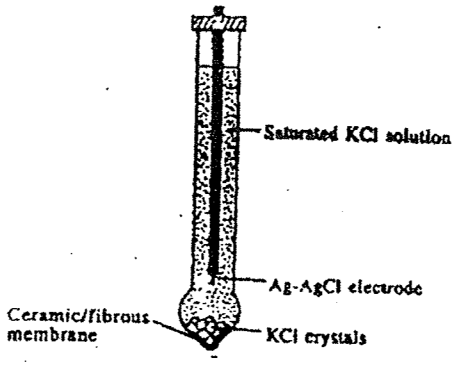

Fig. 7.3 Silver-silver chloride electrode

(V) Glass Electrode : A pH meter commonly carries glass electrode as the *indicator electrode* and a standard Ag - $AgCl_2$ electrode as *reference electrode*. A glass electrode consists of a high resistance glasstube which terminates at the bottom in a thin-walled low resistance pH sensitive glass tube (Fig. 7.4). The glass bulb is filled with an electrolytic solution of constant H^+ activity. An Ag - Agcl electrode possessing a definite electrode potential remain immersed in that solution. Electrical connection is made with this electrode by means of a wire passing through the glass tube.

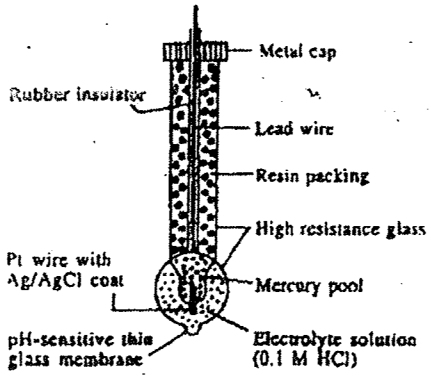

Fig 7.4 Glass electrode.

The glass electrode, joined to a reference electrode by a salt bridge, is immersed in the unknown solution. The membrane of the glass bulb allows only H^+ ions to cross that membrane. So, when the glass electrode is diped into a test solution differing in H^+ concentration from the standard electrolyte in side its bulb, H^+ crosses the membrane. This generates a potential difference between two sides of the glass membrane. This electrode potential is measured with reference to the adjoined reference electrode.

Suggested Questions :

Q. 1. (a) Give definition of pH.

(b) What is the range of pH?

Q. 2. (a) State Henderson -Hasselbach equation for acid and base.

(b) Define strong acid and strong base ; weak acid and weak base. Give examples.

Q. 3. What is a buffer solution? Cite examples of two buffer systems from physiological system.

Q. 4. Discuss measurement of pH by calomel and hydrogen electrode or by glass electrode and silver - silver chloride electrode.

Keywords : Euilibrium constant, ion product of water, pH, Sorensen's pH scale, acid - base pair, dissociation constant, strong and weak acid, proton donor, proton acceptor, buffer, acid base indicator, bicarbonate and phosphate buffer, pH meter, half cells, reference and indicator electrode, hydrogen electrode, calomel electrode, salt bridge, silver -silver chloride electrode, glass electrode.

Suggested Reading : At the end of the module.

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Topic : IV COLLOIDS :

8.01 Colloids and crystalloids : Thomas Graham (1861) observed that some substances would diffuse quite rapidly in solution and could easily pass through animal and vegetable membranes, while there were others which failed to do so. To the former class belonged almost all inorganic acid, bases and salts and many organic compounds, e.g. sugar, urea etc. Graham named them *Crystalloid* since most of the members of this class could be readily obtained in the crystalline state. The other class of compounds which diffuse rather slowly and could not pass through the membrane, was named *Colloid.* The colloid class comprised typical high molecular weight compounds, e.g. starch, gelatin, protein etc. (Kolla means glue in Greek).

8.02 Definition : Colloid is a particular state of matter. A colloidal system is a *two - phase heterogeneous* system consisting of two separate phases with distinct boundaries between them, viz. a *continuous phase or dispersion medium* made of one substance and a *disperse phase* of particles of another substance dispersed in the continuous phase. In contrast, a true solution is a single-phase homogeneous system without any physically demonstrable boundary between the solvent and the solute particles. In fact, dispersed particles of a colloidal system, though submicroscopic, are much larger than the solute particles of a true solution.

8.03 Classification : Colloids may be classified in several ways.

(a) By the state of the dispersion medium and the disperse phase :

(i) ***Sols or lyosols*** **:** Fluid colloidal systems – a liquid dispersion medium and solid disperse phase particles e.g., aqueous sols of agar, starch, gum acacia.

(ii) ***Emulsions*** **: A liquid disperse phase in a liquid dispersion medium :** e.g., emulsions of fats and lipids in aqueous media of milk or egg.

(iii) *Gels* **:** Semisolid, viscous colloidal systems formed from lyosols by gelation. Solid disperse phase particles of the sol aggregate into the solid dispersion medium of the gel; the liquid dispersion medium of the sol. gets scattered in the solid meshes as the liquid disperse phase of the gel ; e.g., gelatin gel, silica gel, fruit jellies

(b) By interfacial interaction :

(i) *Lyophilic sols* are characterized by a strong affinity between the disperse phase and the dispersion medium. So, each disperse phase particle is surrounded by an unmobilized layer (solvation layer) of the dispersion medium e.g., proteins in blood plasma, aqueous solution of starch. (solvent-loving colloids)

(ii) *Lyphobic sols.* show no affinity between the disperse phase and the dispersion medium (solvent-hating). So they possess no solvation layer around disperse phase particle : e.g., colloidal gold, colloidal sulphur.

8.04 Solvation : In lyophilic sols, each disperse phase particle holds around itself a relatively immobile shell-like layer of liquid dispersion medium. This phenomenon, solvation(hydration, if water is the dispersion medium), results from a strong affinity between the disperse phase and dispersion medium.

8.05 Tyndall effect : Ultramicroscope : If a streak of light is passed through a dark room, the dust particles in the path of the light become clearly visible, due to the scattering of light from the surface. The path of light through a *true* solution is invisible because both solute and solvent particles are too small to scatter light (Table : 8.1). But disperse phase particle of a liophobic sol. scatter a part of the incident light. If a colloid solution is placed under the instruments against a dark background, and a strong beam of light is passed at right angles to the line of view, the field becomes at once filled with coloured specks of light due to the scattered light from the colloidal particles. The individual particles could be thus observed and counted this phenomenon is known as *Tyndall effect* and the apparatus for viewing the colloidal particles is called *Ultramicroscope.*

Table : 8.1

Diameter of the disperse phase

True Solution	Colloids Solution	Suspension settles on standing turbid in appearance
1 – 10 A^0	10 A^0 – 1,000 A^0	> 1,000 A^0

8.06 Electrical double - layer : The surface of a disperse phase particles acquires charges due to (i) ionization of acidic and basic groups. (ii) adsorption of specific ions from the surrounding solution. (iii) dissolution of specific ions from the particle surface leaving behind the counterions.

The acquired surface charges on the disperse phase particle electrostatically hold and concentrate some counterions in the liquid medium surrounding the particle. *A fixed layer* of charges is thus constituted on the surface of each colloidal particle by the acquired surface charges on the solid phase of the particle and the opposite charges, concentrated and electrostatically held in the liquid dipersion medium immediately around. The potentials

of the solid and liquid phases of the fixed layer are counter balanced. In the liquid dispersion medium further away from the particle surface, *a diffuse* layer is formed by many layers of more mobile ions. The fixed and diffuse layers of charges together constitute the *Helmholtz - Guoy double -layer* around each disperse phase particle (Fig : 8.1).

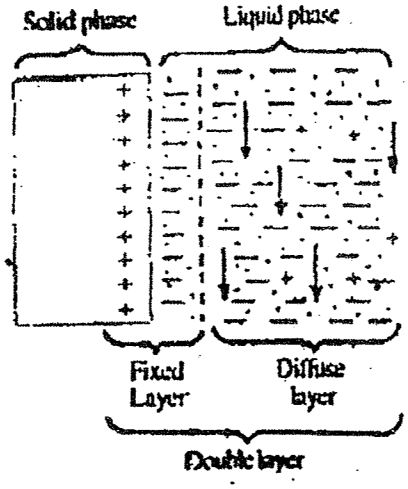


Fig 8.1 Electrical double-layer on disperse phase particle.

The electrokinetic or Zeta potential is the potential difference between the particle and the free-flowing **medium in which the diffuse part of the double layer over spreads itself. This potential is called the *Zeta Potential*, which is responsible for the stability of the colloid and its migration in an electric field.**

8.07 Salting in : The presence of small electrolyte ions, particularly di -and multi-valent ones, increases the stability of a sol, particularly a lyophobic sol. The disperse phase particles adsorb preferentially such ions. This enhances the like charges on the particles to keep them dispersed by increased electrostatic repulsion.

8.08 Salting out : Coagulation is the flocculation and separation of disperse phase particle from a sol. It may be caused by freezing, heating, mechanical agitation, electrolytes.

Sufficiently high concentration of small electrolyte ions may coagulate the disperse phase particles e.g. salting out of ovoglobulin by $(NH_4)_2SO_4$.

8.09 Dialysis : The process of separating a crystallloid from a colloid by means of diffusion through a membrane is called *dialysis*. Membranes made of cellophane, parchment, collodion are freely permeable to water and small solutes but are impermeable to colloid particles. A sac of such membrane is filled with the mixed solution and

suspended in a buffer solution of low ionic strength. Through the semipermeable membrane, water, minerals, glucose area, amino acids diffuse out freely from the sac to the surrounding fluid, but colloid particles are retained inside (Fig : 8.2).

The surrounding fluid is renewed periodically with fresh buffer solution to maintain steep outward concentration gradients of small solute. This method is extensively used in the purification of colloids.

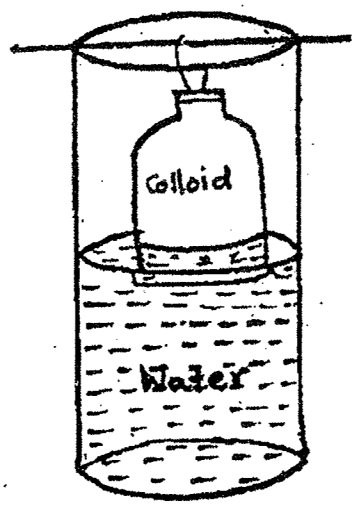


Fig 8.2 : Dialyser

8.10 Biological application :

(a) **Separation of proteins from small solute :** Dialysis is often used as the initial process for separating and concentrating macromolecules such as plasma proteins and cell homogenate proteins from small non-electrolytes and minerals.

(b) **Renal dialysis : In patients with acute renal failure and uremia, blood is dialyzed in *artificial kidneys*.** The artificial kidney has many minute channels bounded by thin cellophane, membranes, impermeable to blood cells and colloidal plasma proteins, but permeable to all small molecules and ions. Blood from the patient's artery is heparinized to prevent coagulation and then passed through the membrane -bound channels where it is separated by the cellophane membrane from a dialyzing fluid outside the membrane. The dialyzing fluid resemble normal plasma in glucose, lactate, Na^+, K^+, Mg^{++}, Ca^{++}, Cl^- and HCO_3^- concentration but no protein, urea, urate, $SO_4^=$, $HPO_4^=$ and creatinine. Due to dialysis through semipermeable cellophane membranes, large amount of urea, urate, creatinine, $SO_4^=$, $HPO_4^=$ diffuse from blood to the dialyzing fluid and are eliminated from plasma.

(c) **Electrodialysis :** Here the diffusion in dialysis is helped by establishment of an electric current through the colloid placed between two membranes. The small ions migrate out and colloid eventually becomes free of electrolytes. This is called electro dialysis. This principle is recently being utilized in producing pure water from sea water (desalination of sea water) on a large scale.

8.11 Adsorption : Adsorption is the concentration of one substance (adsorbate) on the surface of another substance (adsorbent). The absorbate does not penetrate uniformly into the interior of the absorbent; instead, its concentration on the surface of the adsorbent for surpasses that in the interior of the latter as also in the surrounding medium.

There are two principal types of adsorption.

(i) ***Physical adsorption*** or ***Vander Waals adsorption*** takes place at low temperature in particular and depends on Vander Waals forces (inter molecular attraction) for holding the adsorbate molecules on the surface of the adsorbent ; e.g. adsorptions of gases like O_2 and Cl_2 by charcoal. The adsorbed solute or gas is easily realeased from the adsorbent by a fall in the solute concentration or in the partial pressure of the gas in the surrounding medium.

(ii) ***Chemisorption*** or ***activated adsorption*** take place at high temperature and reqires specific adsorbents for a given adsorbate. The adsorbate binds with the surface molecule or atom of the adsorbent molecule by covalent bonds and exists probably in a form possessing enhanced chemical activity e.g. thin film of metal oxide on the surface of metals like zinc, manganese due to chemisorption of O_2 on metals.

8.12 Biological applications of adsorption :

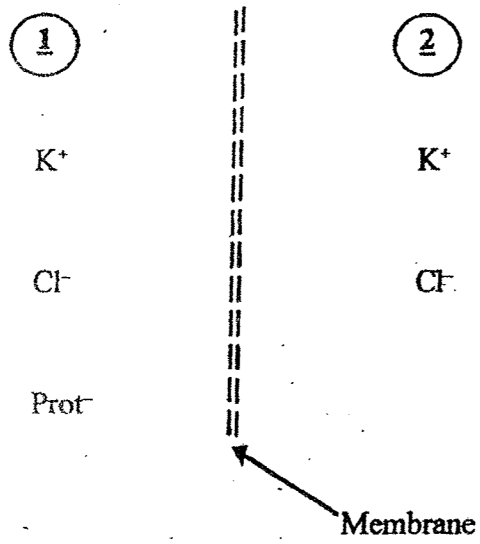
(a) Mineral ions, carbohydrates and lipids are often transported in the plasma, after being adsorbed on the colloidal plasma proteins.

(b) **Adsorption chromatography :** This is used in separating steroids, carotene, sugars, purine, pyrimidene, protein etc. from biological samples. It is based on the physical adsorption of the desired molecules on the surface of an inert and insoluble absorbent such as silica gel, alumina, activated charcoal, packed as a column or spread in a thin layer.

8.13 Donnan Membrane Equilibrium :

(1) **Donnan effect :** When there is an ion on one side of a membrane that cannot diffuse through the membrane, the distribution of other ions to whch the membrane is permeable is affected. For example, the negative

charge of a non-diffusible anion *hinders* diffusion of the diffusible cations and *favours* diffusion of diffusible anions. Consider the following situation :



in which the membrane between compartments (1) and (2) is impermeable to Prot $^-$ but freely permeable to K^+ and Cl.$^-$ Assume that the concentrations of the anions and of the cations on the 2 sides are initially equal. Cl^- diffuses down its concentration gradient from (2) to (1), and K^+ moves with the negatively charged Cl^-, maintaining electroneutrality on side (2). Therefore, at equilibrim, $[K^+]_1 > [K^+]_{(2)}$

Thus, on the side containing the non-diffusible ions, diffusible counterions are more concentrated while the like-charged diffusible ions concentrate more on the opposite side (Gibbs-Donnam affect).

Furthermore, $[K_1^+]+[cl_1^-]+[Pro^-]$ $[K_2^+]+[Cl_2^-]$

i.e. there are more osmotically active particles on side (1) than on side (2).

Donhan and Gibbs showed that in presence of a non-diffusible ion, the diffusible ions distribute themselves so that at equilibrium, there concentration ratios are equal :

$$\frac{\left[K^+_{(1)}\right]}{\left[K^+_{(2)}\right]} = \frac{\left[Cl^-_{(2)}\right]}{\left[Cl^-_{(1)}\right]}$$

Or, $$\left[K^+_{(1)}\right]\left[Cl^-_{(1)}\right] = \left[K^+_{(1)}\right]\left[Cl^-_{(2)}\right]$$

This is Giffs-Donnan Equation. It holds for any pair of cations and anions of the same valence.

(2) Factor affecting Donnan effect :

(a) Effect of salt concentration : Where the concentration of a diffusible electrolyte salt far exceeds the concentration of non-diffusible ion, each diffusible ion is then almost identically distributed on the two sides.

(b) Effect on pH : Donnan effect may lead to a difference in pH between two sides of a semipermeable membrane, because a non-diffusible cation or anion on one side of the membrane causes respectively a lower or a higher concentration of diffusible H^+ on the same side and reverse effects on the other side.

(c) Effect on Osmotic pressure : Osmotic-pressure (Π) is also affected by Giffs - Donnan effect. If, at equilibrium a difference (n) exists between the molar concentrations of solutes on the two sides of the semipermeable membrane, then

$\Pi = n R T$, more osmotically active particle on side (1). With a rise in the molar concentration on side (2) of a diffusible ion, the osmotic pressure declines and with a fall osmotic pressure declines.

(d) Effect on Transmembrane Potential : Non-diffusible anions or cations on one side ofthe membrane develop respectively a negative or a positive potential on that side relative to the other side due to the differential distributions of diffusible ions on both sides.

(3) Biological applications :

(a) Gibbs -Donnan effect of plasma protein anions enhances the outward diffusion or filtration of Cl^- and reduces that of Na^+ from the plasma. So, interstitial fluids, have lower Na^+ concentrations and higher Cl^- concentrations than the plasma.

(b) Because diffusible cations like Na^+ are held back in the plasma by the Gibbs-Donnan effect of protein anions, the contribution of the proteins to the plasma osmotic pressure is about 50% higher than predicted from their MWs and concentrations.

(c) Cells possess far higher K^+ concentrations (155 mM) and far lower Cl^- concentration (4mM) than their plasma concentration (5 and 120 mM respectively). That unequal distributions of these two ions between cells and plasma result almost solely from the Gibbs-Donnan effect of cellular protein anions, is indicated by the closeness of the resting membrane potential (— 90 mV) of mammalian muscle cells to the Nernst potential E for K^+, Cl^-

$$Ek = \frac{RT}{nF}\log_e \frac{[K^+]plasma}{[K^+]cell} = 61.5\log\frac{5}{155} = -91.7mV$$

$$E_{cl}^{\cdot} = \frac{RT}{nF}\log_e \frac{[Cl^-]plasma}{[Cl^-]cell} = -61.5\log\frac{120}{4} = -90.8mV$$

Where, n = +1 *for* K^+ *and* −1 *for* Cl^-

Suggested Questions :

Q. 1. Write on the differences between colloid and crystalloid.

Q. 2. Classify colloid on the basis of

(a) The dispersion medium and the disperese phase.

(b) By interfacial interaction.

Q. 3. Write on Tyndal effect and ultracentrifuge.

Q. 4. (a)What is Helmholty - Guoy electrical double layer?

(b)What is Zeta - potential?

Q. 5. (a) Write on the process of dialysis and describe a dialyser.

(b) Write on artificial kidney and electrodialysis.

Q. 6. (a) What is adsorption?

(b) Write briefly on the adsorption chromatography.

Q. 7. (a) What is Gibbs- Donnan effect?

(b) Cite two biological application of Donnan effect.

Keywords : Colloids, crystalloid, dispersion medium, disperse phase, sol, gel, emulsion, lyophilic and lyophobic sols, solvation, Tyndal effect, Ultramicroscope, electrical double layer, Zeta potential, salting in and selating out, dialysis, renal dialysis, artificial kidney, electrodialysis, adsorption, Donnan effect.

Suggested Readings for Module : 22.

1. Elementary Physical Chemistry by *S.R. Palit.*
2. Biochemistry by *D. Das.*
3. Principle of Biochemistry by *A.L. Lehninger* (3rd ed.)
4. Illustrated Bio-chemistry by *Harper* (26th ed.)
5. Circulation by *Folkow.*
6. Biophysics and Biophysical chemistry by D. Das (4th ed).
7. Biophysics, by G.R. Chatwal.
8. Biophysics, An Introduction by R.Cotterill.
9. Instant Notes : Biochemistry (2nd ed) by B.D. Hame & N. M. Hooper.

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M.Sc.
in
Zoology

Part - I
Paper - II, Unit - II, Group - B

Module No. 23

Topic – V : Radioactivity

1 **Introduction**
- 1.1 Atomic Structure
- 1.2 Isotope

2 **Types of radioactive emissions**
- 2.1 Beta rays
- 2.2 Alpha decay
- 2.3 Gamma rays

3 **Kunetis of radioactive decay**
- 3.1 Rate of decay
- 3.2 Activty 'A'
- 3.3 Half life of radio isotope
- 3.4 Unit of radioactivity
- 3.5 Some naturally occurring radioisotope

4. **Radiation detection and measurement**
- 4.1 Geiger-Muller Counter
- 4.2 Scintillation Counter

5. **Artificial radioisotope**
- 5.1 Production
- 5.2 Some artificial radio isotopes used is therapy and research

6. Biological application of radioactive tracers
- 6.1 Metabolic studies

6.2 Radio immuno assay
6.3 Autoradiography
6.4 Radio therapy
6.5 Carbon dating
7. **Biological effects of radioactivty**
7.1 Stochastic effects of radioactive
7.2 Non-stochastic effect of radiation
7.3 Effect on rare biomolecules
7.4 Radiosensitivity of tissue

1. INTRODUCTION

1.1 Atomic Structure

The nucleus is made up of neutron and proton. The proton provides the positive charge in an atom. The neutron has no charge and has slightly more mass than the proton. The number of protons in the nucleus is called the atomic number 'Z'. The number of neutrons in the nucleus is called the neuron number 'N'. The sum of proton and neutron in the nucleus is called the mass number 'A' (A=Z+N). Thus helium (He) is represented as number $_2H^4$, mass no =4 and atomic number = 2 or $_7N^{14}$, $_8O^{16}$, $_6C^{12}$

The atomic nucleus is made of gvien combination of proton and neutron. The nucleus is stable only if its neutron-proton ratio (n: p ratio) lies within a specific narrow range. Atoms with low mass number usually reguire equal number of proton and neutron for nuclear stability (see example given above), but atoms with high mass number require higher number of neutron than proton (n>p) for stability. In other words the stable n :p ratio rises slowly but progressively with the rise in the mas number. Examples are :

$_{30}Zn^{65}$, $_{47}Ag^{108}$, $_{79}Au^{197}$, $_{92}u^{238}$

Significant deviations from the stable n:p ratio makes the nucleus unstable and *radioactive.* Such a nucleus tends to change its n:p ratio to the nearest stable ratio through radioactive decay. If the n:p ratio exceeds that required for stability (n>>p), it is lowered by changing one or more neutrons into protons with emission of β^-

particle (n = p+ β^- + η when η = uncharged mass less nutrino). If the n : p ratio is lower than that for the stability (n<<p), it may be raised by ejecting α particle, or by capturing an orbital electron to convert a proton to neutron (proton + electron = neutron), or by ejecting a β^+ or positron from a proton to replace latter by a neutron.

1.2 Isotope

The atoms of an element having the same atomic number, same chemical properies but differ in the number of neutrons in the nucleus are called *isotopes* of that element. The *naturally occurring isotopes* (of C, O, H, N) have unstable combinations of proton and neutron in the nuclei and are radioactive. Since electrons determine the chemical properties, the isotopes of an elecment are identical in all chemical properties like density, rate of diffusion etc. Isotopes are represented by their mass numbers. For example, *Carbon* has three isotopes ${}_6C^{12}, {}_6C^{13}, {}_6C^{14}$ and *Oxygen* has three isotopes ${}_8O^{16}, {}_8O^{17}, {}_8O^{18}$ and Hydrogen has three isotopes ${}_1H^1, {}_1H^2, {}_1H^3$ [${}_1H^2$ is known as deuteriun or heavy water and ${}_1H^3$ is known as Tritium] Nitrogen has two isotopes ${}_7N^{14}$, ${}_7N^{15}$

2. TYPES OF RADIOACTIVE EMISSIONS

Radioactivity is the spontaneous emission of accelerated high energy particles and electro magnetic radiations from unstable atomic nuclei. The nucleus thereby loses energy and changes into the more stable nucleus of another element (deughter element). Radioactive emissions include mainly electromagnetic γ rays similar to X-rays, and α and β rays consisting of charged high velocity particles. They produce visible spots and tracks on photographic films (Fig 1)

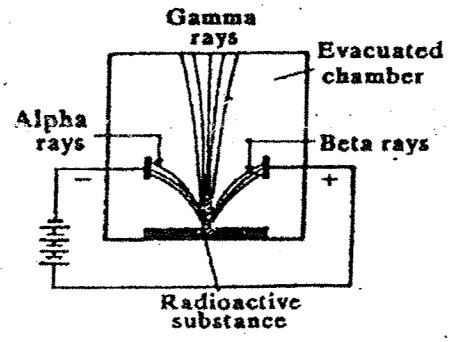

Fig - 1 Deflection of rays from radioactive substance in an electric field

2.1 Beta rays

Beta decays are nuclear transformation of one type of nucleon (mother) to another (daughter). They alter the atomic number and the n : p ratio of nucleus but do not change the mass number. They are the most frequent

radioactive decays in radioisotopes with low mass number. Beta decays include β^- emission, β^+or Positron emission and K capture

a) β^- emission or negative emission :

These are stream of very light, highly accelerated and negatively charged particles and are consequently deflected towards the anode in an electric field (Fig-1). Radioactive elements possessing surplus neutrons and thus too high n : p ratio (n>>p), emit β^-particle. A neutron is broken down into a proton, β^- particle and an uncharged massless nutrino ($n \rightarrow {}_1p + \beta^{-1} + n$). The proton retained in the nucleus, $\beta^-$and *n* emitted. Each β^- particle resembles an electron in possessing the same mass and charge but unlike orbital electrons, β^- comes from the nucleus, has initial velocities in the range of light rays. Emission of each β^- replaces a neutron by proton and lowers the n : p ratio. It raises the atomic number by 1 but leave the mass number unaltered. Example :

$$_{15}P^{32} \rightarrow {}_{16}S^{32} + \beta^- + \eta + 1.71\ \text{Mev}$$

b) β^+ decay or positron emission :

Neutron deficient radioisotope with too low n : p (n<p) ratios may raise the ratio through positron emission when α-decay is not possible energitically. During β^+ decay, a proton changes into a neutron by emitting β^+ which is a highly accelerated particle with a positive charge. ($p \rightarrow {}_1n + \beta^+ + \eta$) A nutrino is simultaneously emitted. β^+ emission replaces a proton by neutron and raises n : p ratio. The atomic number is lowered by 1 with no change in mass number. Example :

$$_{30}Zn^{65} \rightarrow {}_{29}Cu^{65} + \beta^+ + n$$

c) K- capture or electron capture :

This nuclear transformation occurs in neutron deficient radioisotope with low n : p ratio (n<P), provided neither Positron emission nor α-decay is possible. A proton changes into a neutron in the atomic nucleus by capturing an extra-nuclear orbital electron most frequently from the innermost K-shell of electron. This replaces a proton by neutron ($p + e^- \rightarrow n + \eta$) and raises the n: p ratio. The atomic number falls by 1 with no change in mass number. K - Capture is immediately followed by the fall of an electron from an outer shell to K-cell to occupy the position vacated by the captured electron. This is accompanied by the emission of a low energy X-ray photon (0.15 Mev). The net loss of a orbital electron counterbalance the fall in the atomic number. Every positron emitted, β^+ decay is annihilated on coming into contact with an electron, because positron is the antiparticle of electron. The amount of energy released in the annihilation of a positron is equal to the total energy of 1.022 Mev which is spent in its creation. This energy is generally released as two γ-rays each of 0.511 Mev energy. They are emitted in opposite directions.

2.2. Alpha decay

Alpha decay leads to the emission of alpha rays which are streams of high velocity alpha particles. It takes place in radio isotope with too low n : p ratio (n<<p) and consists of the ejection of α - particle from nucleus simultaneously with ejection of two electrons from extranuclear electron orbits. Each α - particle is a tight cluster of two neutrons and two protons and corresponds to the extremely stable Helium nucleus ($_2He^4 \rightarrow He^{2+}$). It bears two positive charges and is deflected towards cathode (Fig-1). An alpha particle decay lowers the mass number by 4 and thereby brings down the n : p ratio required for stability. α -decays are frequent in redioisotopes with high mass number. An α -decay also lowers the atomic number by 2.

$$_{92}U^{238} \xrightarrow{\alpha\text{-decay}} {}_{90}Th^{234},\ {}_{86}Rn^{222} \rightarrow {}_{84}P_o^{218} + {}_2He^4 + 5.58Mev$$

2.3 Gamma Ray

These are monochromatic electormagnetic radiations resembling *X*-rays. They are not deflected in an electric field. (Fig-1). In contrast to *X*-rays, γ - rays generally possess higher frequencies and shorter wavelengths and originate from nucleus instead of electronic parts. They originate in the following way. If an alpha, beta or positron particle is emitted with less than the maximum possible energy, the daughter nucleus is left with too large a share of the decay energy released. This 'excited' daughter nucleus immidiately drops down to its 'ground' state by emitting the excess energy as gamma ray.

3. KINETICS OF RADIOACTIVE DECAY

Each radioisotope is characterized by its own specific rate of decay which cannot be altered by any physico chemical process. The rate of radioactive decay of nuclei at any time is proportional to the total number of radioactive nuclei present at that time.

3.1 Rate of decay

Suppose that at a given time *t*, there are *N* atoms of a radioisotope present. Suppose further that is an extremely small time interval *dt*, the number of nuclei decaying is *dN*. The rate of decay is then represented by $\frac{dN}{dt}$, which is proportional to total number of atom *N*,

$-\frac{dn}{dt} \alpha N$ .... (1) minus sign is indication of decrease in number of nuclei with time.

or $-\frac{dn}{dt} = \lambda N$ ....(2) Where λ is the decay constant of a particular isotope.

or $-\frac{dn}{N}=\lambda\, dt$ .... (3)

By integrating between limits N_1 and N_2 and t_1 and t_2 we have $\int_{N_1}^{N_2}\frac{dN}{dt}=-\int_{t_1}^{t_2}\lambda dt$ ... (4)

$\ln\left(\frac{N_2}{N_1}\right)=-\lambda(t_2-t_1)$ ... (5) where N_1 is the number of radioactive nuclei at time t_1 and N_2 is the number of nuclei at time t_2

If we put $t_1 = 0$ and $N_1 = N_0$

and $t_2 = t$ and $N_2 = N$

then $\ln\frac{N}{N_0}=-\lambda t$ .... (6)

or $N = N_o e^{-\lambda t}$ .... (7)

or $\log\frac{N_0}{N}=\left(\frac{\lambda}{2.303}\right)t$ ..... (7[1])

3.2 Activity 'A'

Rate of disintegration $\left(\frac{dN}{dt}\right)$ is called activity A. The activity A at any time is given by

$A = -\frac{dN}{dt}=\lambda N$ Equation .... (2)

Substituting the value of N from Equation (7)

$A=\lambda\left(N_0 e^{-\lambda t}\right)$ .... (8)

The initial activity A_0 when t = o, $A_0 = \lambda N_0$

Thus substituting N_0. $A = A_0 e^{-\lambda t}$ .... (9)

or $\frac{A}{A_0}=e^{-\lambda t}$

Exponential relation beween relative activity (A/Ao) and time is given is Fig - 2.

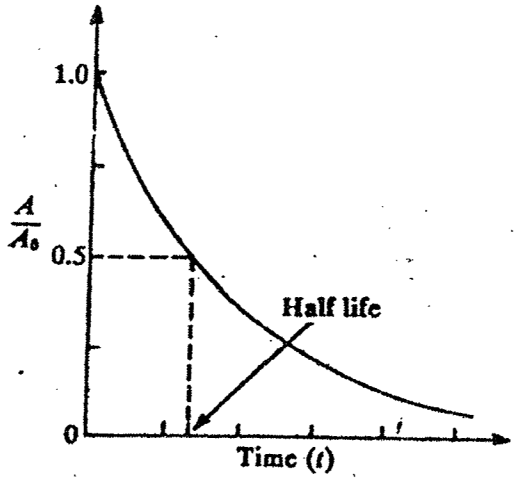

Fig-2 Exponestial relationship between relative activity (A/Ao) and t

3.3 Half - life of radio isotope

Half-life of a radioisotope is the time, at the end of which exactly half of its initial number of nuclei still remain unchanged. It indicates the speed of radioactive decay and is a measure of instability of a radioisotope. It is an unalterable characteristic of the specific radioisotope and is indepenent of the initial number of its nuclei, initial mass and intial radioactivity.It differs widely among different radioisotopes, ranging from a few μsec to several billion years.

When $N = N_0/2$ in equation (7^1)

$\log 2 = \frac{\lambda}{2.303} t_{½}$ Where $t_{½}$ = half time

$t_{½} = \frac{2.303 \log 2}{\lambda} = \frac{0.693}{\lambda}$ ($\because$ log2 = 0.3013)

3.4 Unit of radioactivity

Curie (Ci) was an international unit of radioactivity based on the radioactivity of 1g of radium. One curie equals 3.7×10^{10}Ci nuclear disintegration per second (1mCi = 10^{-3}Ci)

Becquercl (Bq) is now used as an international unit of radioactivity. It is the quantity of radioactive material in which one nuclear disintegration occurs in one sec 1Bq = 2.7×10^{-11} C_i.

3.5 Some naturally occurring radioistope

Isotope	emission	half life
Uranium $_{92}u^{238}$	α	4.5×10^9 years
Thorium $_{90}Th^{230}$	α,γ	8.0×10^4 years
Radium $_{88}Ra^{226}$	α,γ	1600 year
Radon $_{86}Rn^{222}$	α,γ	3.8 days

4. RADIATION DETECTION AND MEASUREMENT

4.1 Geiger Muller Counter (GM counter)

This is a gas ionizing counter operated at very high voltage drop.

Gas oxizing Counter detects and measures radiation on the basis of their effect on gas molecules in producing ion-pairs which lowers the potential difference between the anode and the cathode to generate a current pulse. Basically, a gas ionization counter consists of a cylindrical metal tube with one of its ends closed by a thin mica. The other end is closed by an insulated gas-proof plug through which a thin metal-wire runs inside the tube along its axis. A potential difference is maintained between the axial wire which serves as the anode (+) and the metal wall of the tube serving as *Cathode* (–), by connecting them to a source of voltage. The tube is filled with an inert, ionizable low presure gas mixture (eg. Argon-methane). A sensitive electronic indicator for pulse counting are also included in the circuit (Fig-3)

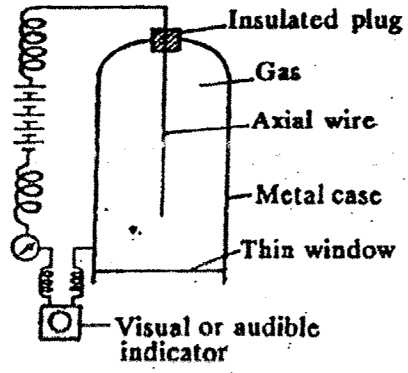

Fig. -3 Gas lonization counter for radiation measurement

Ionizing radiations enter throngh the mica membrane and ionize the gas molecules into ion pairs. The electrons and positive ions so generated pass respectively to the axial wire (anode) and the metal wall (cathode). As the

electrons get collected by the central anode, the wire potential suffers a drop. *The voltage drop is directly proportional to the number of electrons reaching it hence to the magnitude of the ionizing radiation.* This fluctuations of voltage constitute a voltage pulse. Pulses can be counted for ionizing actions or photon to serve as a measure of radioactivity.

Thus the GM counter may be placed with its mica window on the radioactive substance to detect or estimate radioactivity. This thin mica membrane prevents the entry of most low energy α and β particles (H^3, C^{14}, S^{35}). Most γ–photons with energy >100 kev pass through without ionizing their gases. So these gas ionizing counter possesses poor ability for detecting high energy γ–photon and low energy α and β partide. *They are therefor, applied for high* energy α, β particle, low energy γ–photon

4.2 Scintillation Counter

Scintllatior is the production of short duration visible light flashes when γ-photons, β and α particles strike crystals of scintillator or phosphors such as thallium - activated iodides of Sodium (NaI (Tl)).

Scintillation Counter is an energy transducer which converts the energy of an ionizing particle or of a γ-photon into scintillation flashes, each of which is then converted to an electric current pulse to be further amplified and counted.

If consists of a 'scintillation chamber' connected by a *perspex light tube* to a *photomultiplier tube*. The scintillatior *crystal* is housed in the scintillation chamber. (Fig-4)

Ionizing radiation enters into the scintillation chamber. The incident radiations 'excite' and ionize the atoms of the crystals which drops down to the ground state by emithing visible light photons. These light photons pass

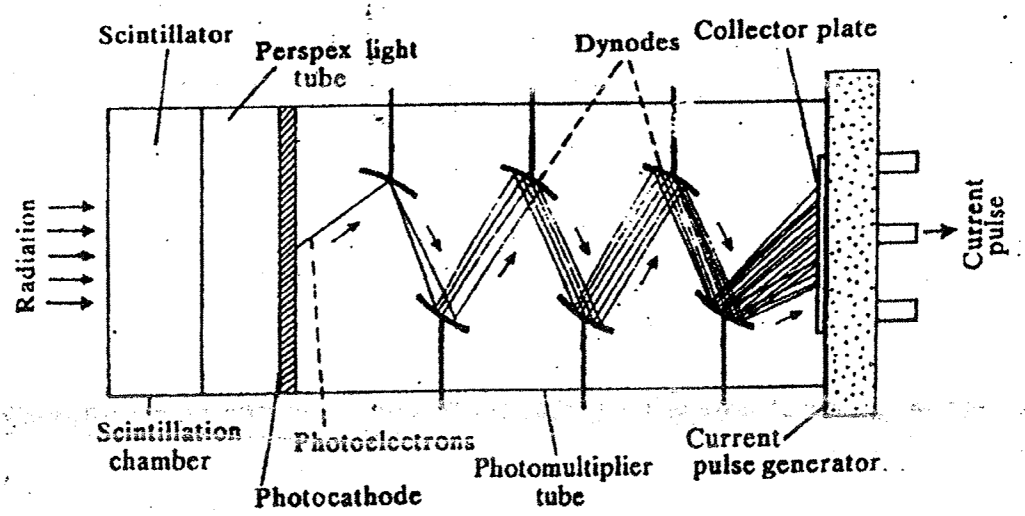

Fig. 4 Scintillation counter.

through the light tube to strike the surface of the *photocathode* in the photomultiplier. The electrons emitted from photocathode impinge successively on a series of about ten electrodes called *dynodes. Each* electron striking a dynode knocks off about 4 electrons and these go to strike the next. Repeatation of this process multiplies the number of ejected electrons. This showers of electrons finally impringes on a collector plate to generate a current pulse. After amplification the current pulse is recorded by an electric pulse counter.

Scintillation counters require far higher voltages than GM counter for operation. These are extensively used in counting γ–rays Unlike GM counter. Scintillation counter can distinguish between different ionizing radiations because the magnitnde of their output current pulses are proportional to the energies of the primary lonizing particles and photons.

5. ARTIFICIAL RADIOISOTOPES

Many man made radioisotopes are used extensively in research, industry and therapy. They also possess specific half lives and obey the law of radioactive decay.

5.1 Production of artificial radioisotope.

(a) In cyclotron

High voltage ion accelerator such as cyclotrons accelerates charge particles like proton, α -particle and deuterons to very high energies. These highly accelerated charged particles bombard natural non radioactive to produce radioisotope. For example, cyclotron bombardment with a deuteron changes Mg^{24} to $_{11}Na^{22}$ and $_{10}Ne^{20}$ to F^{18} with the emission of an α-particle in each case

$$_{12}Mg^{24} \xrightarrow{{}_1H^2} {}_{11}Na^{22}$$

$$_{10}Ne^{20} \xrightarrow{{}_1H^2} {}_{9}F^{18}$$

Bombardment with proton changes N^{14} and Ni^{58} to C^{11} and Co^{57} respectively with the emission of α particle in the 1st case and two protens in the second

$$_{7}N^{14} \longrightarrow {}_{6}C^{11}$$

$$_{28}Ni^{58} \longrightarrow {}_{27}Co^{57}$$

(b) By nuclear fission in nuclear reactor

In nuclear reactor the atomic nucleus of a nuclear fuel such as U^{235} and Rn^{239} is made to capture a high energy neutron. The nucleus thereby undergoes fission with two radioactive daughter nuclei called *fission fragments,* and several fast neutrons and γ–photons. The fission fragments decay through chains

of several radioactive products called fission products, which include some medically and biologically important β emitters such as Sr^{90}, I^{131} and Xe^{133} or γ–emitter. For example

$_{16}S^{32} \rightarrow {}_{15}P^{32}$ with emission of proton

or $_{3}Li^{6} \rightarrow {}_{1}H^{3}$ with emission of α -particle

$_{27}Co^{59} \rightarrow {}_{27}Co^{60}$ with γ - emission

(c) Nuclear fusion in neutron generation

Nuclear fusion e.g. fusion of accelerated deuteron with H^3 to produce H^4 generate very high energy neutron in neutron generator

s 5.2. Some artificial radioisotopes used in therapy and research. They are as follows :

Isotope	emission	half life
H^3	β^-	12.26 year
C^{14}	β^-	5730 year
Na^{22}	β^-, γ	2.58 hour
Na^{24}	β^-, γ	15.1 hour
P^{32}	β^-	14.3 days
S^{35}	β^-	87.1 days
Cl^{36}	β^-	3×10^5years
K^{42}	β^-, γ	12.4 hour
Ca^{45}	β^-	165 days
Co^{57}	β^+, γ	270 days
Fe^{59}	β^-, γ	45 days
Sr^{90}	β^-	28 days
I^{125}	γ	57 days
I^{131}	β^-, γ	8.07 days

6. BIOLOGICAL APPLICATIONS OF RADIOACTIVE TRACERS.

Radioisotope have been extensively used in the medical and biological research, in the dignostic and treatment of disease.

6.1. Tracer in metabolic studies

Radioisotopes commonly used in biological research are $\beta\square^-$ emitters because the emitted $\beta\square^-$ particle can be easily detected, located or estimated using GM counter or Scintllation Counter. Radioisotopes should also possess fairly long half lives to retain detectable measurable amount of radioactivity during the study time [C^{14} with half life 5730 year, H^3 with half life 12.26 year are widely used. For example :

(a) **In pentose phosphate shunt :** when glucose labelled with ^{14}C at C_1 position, $^{14}CO_2$ were liberated. However if C-C_6 position of glucose are labelled with ^{14}C very little $C^{14}O_2$ were liberated

(b) In the conversion of C^{14}-Oxaloacetate plus acetate to citrate in TCA Cycle, $C^{14}O_2$ liberated comes from oxaloacetate.

(c) In the biosynthesis of cholesterol from acetate CH_3COOH, carbon labelled at carboxyl and methyl group it was obserred that C-12 of cholesteral comes from carboxyl group of acetate while C-15 comes from methyl group.

(d) P^{32} labelled (DFP) di-isopropyl fluorophosphate binds co-valently with the active site of trypsin or acetyl choline esterase enzymes to inhibit it irreversibly. After fragmenting the di-isopropyl bound amino acid can be detected and subsequently identified as serine residue.

(e) Thyroid function and the metabolic pathways of its hormone. Thyroxine have been investingated after adminis tering I^{131} which is concenterated by the gland used in thyroid hormone. synthesis & secretion in the blood.

6.2. Radioimmuno assay (RIA)

The principle of RIA involves competitive binding of radiolabelled antigen and un-labelled antingen to a high affinity antibody. The antigen is labelled with γ - emitting isotope such as I^{125} (half life = 57 days). The labelled antigen is mixed with antibody at a concentration that just saturate the antigen binding sites of the antibody molecule. Then increasing amounts of unlabelled antingen are added. The antibody does not distinguish labelled from unlabelled antigen and so the two types of antigen compete for available binding sites on the antibody. With increasing concentration of unlabelled antigen, more labelled antigen will be displaceed from the binding sites. By measuring the amount of labelled antigen free in solution, it is possible to determine the concentration of unlabelled antigen

6.3 Autoradiography

Autoradiography is a technique for locating radioactive compounds within cells. It can be observed with light or electron microscopy. Living cells are first exposed to the *radio active precursor* of some intracellular component. The labelled precursor is a compound with one ar more hydrogen atom (H^1) replaced by the radioisotope tritiun (H^3) (example H^3 - thymidine which is a precursor of DNA and H^3 uridine which is a precursor of RNA). The labelled precursors enter the cells and are incorporated into the appropriate macromolecules. The cells are then fixed and the samples are embedded in wax/resin and are sectioned into thin slices.

The sections containing the specimen are placed on microscope slide or electron microscopic grid and are coated with a thin film of photographic silver halide emulsion. After the preparation dries, the preparations are stored in the dark box to permit the radioactive decay to expose the overlying emulsion. The length of exposure depends on the amount of radioactivity in the sample but is typically several days or few weeks for light microseopy and upto several months for electron microscopy. During this period, β^- particles from the specimen strike the silver halide crystals of the overlying photographic emulsion to change them to silver. When the photographic emulsion is subsequently developed, all exposed silver halide crystals are dissolved out, leaving dark silver grains overlie regions of the cell that contain radioactive molecules.

6.4. Radiotherapy

6.4.1 Therapeutic

a) Destruction of malignant cell

A radioisotope may be implanted in a malignant tissue. Controlling the dose of the radioactivity and period of implantation, ionizing radiations can destroy the malignant cells with mimmum permanent damage to healthy cells (healthy cells are generalhy less radio sensitive than malignant cells). A radioisotope with a short half life (example Rn^{222} with half life 3.8 days) is often used; alternatively, a long living radio isotope like Ra^{226} (radium) with a half life of 1620 years is implanted for a specified short period.

b) RBC destruction in polycythemia

Frequently the patient is injected with a radioisotope which is selectively concentrated by the tissue to be irradiated. For example when P^{32} (half life = 143 days) is administered to polycythemia patient (an increase in red cell mass), it gets rapidly concentrated by bore marrow cells leading to their selective destruction by the β^- emitted rays from ^{32}P.

(c) Destruction of thyroid tumour

I^{131} (ualf life = 8 days) is selectively concentrated by thyroid cells and helps to destroy thyroid tumour by its β^- and γ– rays.

6.4.2. Diagnostic

a) I^{123} was used to monitor the *Performance of the thyroid gland.* The patient would simply drink a glass of water containing I^{123} and with an appropriate detector placed on the skin near the thyrord gland, the γ- rays given off by this radioisotope can be deteeted. I^{123} is useful due to its short half life. However, it has been superseded by the use of Technetium-99 ($_{43}Tc^{99}$) produced in the following way.

$$_{42}Mo^{99} \longrightarrow {}_{43}Tc^{99} + {}_{-1}e^{o}$$

b) **Positron emission tomographic scan (PET Scan)**

This diagnostic method uses biomolecules labelled with positron (β^+) emitter radioisotopes such as C^{11}, N^{13}, O^{15} to produce images of tissue, revealing the 3D distribution, concentration, circulation and metabolism of the administered biomolecules. The desired biomolecules such as CO_2, ammonia, sugars and aminoacids are labelled with positron emitters. The labelled substance is administered to the patient. As it circulates is the body, its radioisotope emits positron which on coming is contact with an electron gets annihilated, because positron is the antiparticle of electron. This energy is generally released as two γ-rays (each of 0.511 mev energy) emitted in opposite directions. These γ–rays are recorded at specife angles throughout the subject's body and the information is computer processed for reconstructing the tomographic distributon of the isotope throughout the body.

PET scanning is very much helpful for detection of epileptic foci because the areas show less metaboliom and the visualized areas get activated during scizures. Also PET is useful for delection of ischemic areas of myocardium.

[Tomography - an apparatus for moving the X-ray tube through an arc during exposure thus showing in detail a predetermined plane of tissue while blurring details of other planes]

6.5 Carbon dating

Carbon occurs in nature mainly as non radioactive C^{12} and in small amount as C^{14} (half life = 5730 years), the latter produced from N^{14} due to the bombardment with fast neutrons from cosmic rays. Living organisms continuosly take both the carbon isotopes in the same proportion in which they occur in nature. After death, fresh C^{14} no longer enters the body while preexisting C^{14} decays exponentially with time in the dead tissue.

Hence, if $\varnothing^o$ and $\varnothing$ are the amount of radioactivity of a given amount of C^{14} in the freshly died organism

and in the fossil of a dead organism respectively, the age of the fossil can be determined by the time t required for the observed decline of the radioactivity of the fossil relative to that of the freshly dead organism

$$t = 3.3t_{1/2} \log \frac{\varnothing^{\circ}}{\varnothing}$$

7. BIOLOGICAL EFFECTS OF RADIOACTIVITY

7.1. Stochastic or zero threahold effects of radiation : Include mainly the genetic effect and malignancies. They have no obvious relationship with radiation exposure, although the probability of their incidence rises in direct proportion to the magnitude of the radiation dose. No minimum dose can be identified. Stochastic effects seem to result from changes in some essential biomolecules like nucleic acids and enzymes due to direct ioniging actions of radiation.

7.2 Non-stochastic or threshold effects of radiation : include most of its other biological effects. They have cause and effect relationsships with the exposure to radiation and rise in intensity with increasing doses of radiation. Non-stochastic effects seem to result from the 'indirect' action of radiation on water (H_2O), the most abundant biomolecule in the organism that produce free hydrogen (H^+, OH^-, $^*HO_2$) and H_2O_2 which is turn effect other biomolecule like nuclieic acid or enzyme.

7.3 Effect on rare biomolecules

(a) Chromosomal DNA may suffer radiational damage in three ways.

(i) Charged particle or photons of radioactive emission may *directly* eject electrons from DNA molecule to ionize them.

(ii) DNA molecules may be *indirectly* ionized by fast moving electrons ejected from the molecule. Ionization of a base in one of the DNA strands frequently leads to mutation.

(iii) A DNA molecule may suffer a single stranded or double stranded break resulting fron binding with free radicals. Chromosomal injury may stop or delay DNA replication and consequently lower the mitotic rates of cells. This results in structural and functional abnormalitis of cells having high mitotic rates, example - hemopoietic cells and granulopoietic cell of bone marrow, lymphopoietic cell of lymph nodes, intestinal mulosal cell, cutaneous germinating cell.

(b) Erzyme may lose their active site due to changes in the 3-D structure. Chronosomal damage and mutation may affect the synthesis of new enzyme molecules. Both adversley affects vital metabolic pathways

such as replication and repair of DNA; transcription of RNA, carrier mediated transport and mitochondrial respiration.

(c) Specific tissne damage : (i) *Bone marrow*, lymph and blood. These tissues show acute symptoms from early stages after exposure. Edema, hemorrhage, ulcer and loss of germinal centre occur in lymph glands. Hemopoietic syndrome (Characterised by an ablation of bone marrow) appears on exposure to γ–rays. Blood changes include thrombocytopenia - prolonged clotting time and hemorrhage, fall in granulocyte counts and erythrocyte counts, hematocrit values and hemoglobin concentration; lymphopenia and low serum immunoglobulin which become evident within withis few hours. Fall in thrombocyte and granulocytes appear within a day or two.

(ii) Gastrointestinal tract : moderately high doses of radiation may cause esophagal damage, edema and painful deglutation, cessation of gastric secretion. Intestinal crypt cell suffer progressively. The denudation of intestinal mucosal cells is extensive after high dose exposure of γ–rays. This produces 'gastrointestinal syndrome' - anorexia, vomiting and diarrhea, edema, hemorrhage and ulcer in the GI. tract.

(iii) Skin : Low energy β, γ and Xrays affect the skin more severely than most of the deeper tissne (Hair loss, vasodilatation). With higher dose pigmentation, dermatitis and blisters appear.

(iv) Gonads : They are very susceptable to radiatior damage. Temporary sterility results. Hypospermia, anovulation, amenorrhoea occur - can produce permanent sterility.

(v) Eye : cataract, conjunctivitis, corneal keratinization

7.4. Radiosensitivity of tissues

1) The greater the ionizing action the higher is the damage. The radiation damage depends on the dose and the period over which the radiation spread. The radioisotope with greater half life produce more harm.

2) Exposnre of a part of the body is less damaging than whole body - healthy cells can repair damaged cells.

3) Radioisotope produce selective damage to certain tissues of selective organs eg. I^{131} damages thyroid gland, Ra^{226} and Sr^{90} are concentrated in bone, cause bone cancer

4) The absorbed dose falls uniformly with the depth of the uniformly dense tissue.

5) Fetus, infants and children are far more sensitive to radiosiotope than adults.

6) Malignant cells possess higher mitotic rates and radiosensitivity than normal cells

Suggested Questions

Short Questions

1. How β^- ray emission differes from α -ray radition?
2. What is γ-ray ?
3. What is the main unit of radioactivity ?
4. Name some naturally occurring radioisotope.
5. What is meant by half-life of radioisotope ?

Long Question

1. Describe how Geiger Muller Counter measures or detects radioactivity.
2. Discuss the principle of Scintillation counter.
3. What is artificial radioisotope? give one method of preparations of artificial radioisotope
4. Give two biological applications of radioactive tracer technuque.
5. Effect of radioactive emission on chromosomal DNA.

Keywords

atomic strncture, neutron-proton ratio (n : p ratio), radioactive decay, isotope, β rays, α–rays, γ–rays, rate of decay, half-life, radioactivity, unit of radioactivity, Geiger Muller Counter, Scintillation counter, artifcial radioisotop, cyclotron, nuclear reactor, neutron generater, biological application of radioisotope, metabolic studies, radioimmuno assay, autoradiography, radiotherapy, carbon dating, biological effect

Select Readings

1. Biophysics and biophysical chemistry (4th ed) by Debojyoti Das
2. Fundamental of nuclear science by P. N. Tiwari.

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M.Sc. in Zoology

Part – I

Paper – II : Group – B : Unit – 02

Module No. 23/A

(Biophysics)

6. Photometry and polarimetry

Principle of colorimetry and photometry, application of photometry in biological experiment, optical activity of organic substance, principle of spectrophotometry, dextrorotatory and levorotatory isomers, polarometer, polarization microscopy – Principle.

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1. Principal of Colorimetry

1.1 Absorption Spectra / Electronic Spectra

Electrons are distributed about the atom in certain fixed positions. But they are not stationary particles. The electron energy level of a molecule under normal conditions is known as the ground state (E_0) and the electronic levels with the higher energies are called the first, second etc. excited states (E_1, E_2....) (Fig. 1). When an atom absorbs (or emits) some energy ΔE (E_0–E_1) it jumps from one stable state to another stable one. The energy of the absorbed (or emitted) quantum is equal of the systems energy.

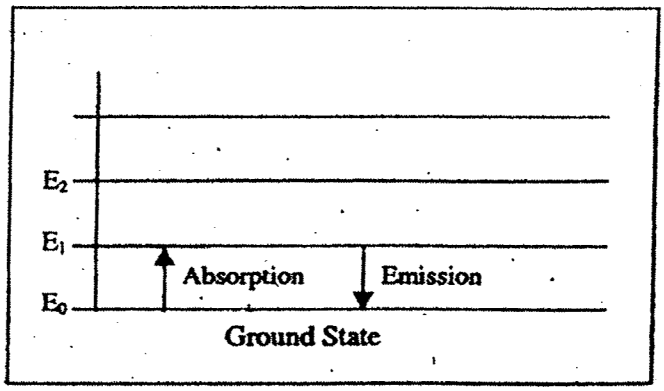


Fig. 1

Plank Equation : $E = h\upsilon = h\frac{c}{\lambda}$ where h = Plank's constant = 6.67×10^{-27} *erg. sec.*

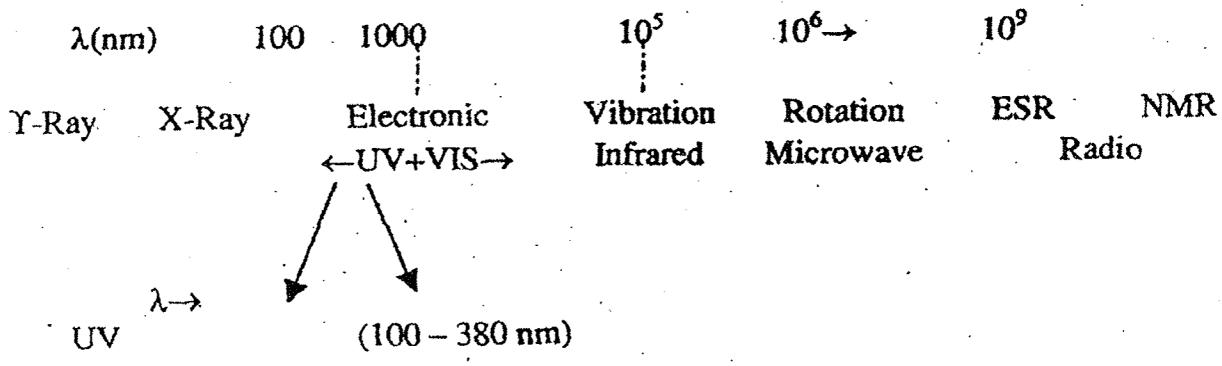
Absorption (or emission) of radiation can occur only if the energy of the absorbed only if the energy of the absorbed (or emitted) quantum or photon is equal to the difference between two energy level

= 6.6×10^{-34} joule.sec.
ν = Frequency of light
c = Velocity of light
3×10^8 m/Sec.
λ = wave length of light

$$\Delta E = E_1 - E_0 = h\upsilon = h\frac{c}{\lambda}$$

or $\Delta E \alpha \frac{1}{\lambda}$ i.e., Energy is inversely proportional to wave length.

Table-1 :



Visible	(380 – 760 nm)	N.B. : it is to be noted that the lower
Violet	380 – 450 nm	the wave length (λ) the higher the
Indigo	450 – 480 nm	energy (E) uv>violet>Red>IR
Blue	480 – 510 nm	
Green	510 – 550 nm	
Yellow	550 – 575 nm	
Orange yellow	575 – 585 nm	
Orange	585 – 620 nm	
Red	620 – 760 nm	

1.2 Absorption of Light

Visible light (λ= 380-760 nm) (Table –1) is also form of energy and it interacts with matter. The amount of energy is sufficient to affect the electrons of an atom in many cases. The energy is sufficient to push electron to next orbit (i.e., next high energy level). The energy absorption takes place in small discrete packet called quanta. Molecules which have the capacity to absorb 'visible' light are called chromophore.

1.3 Colorimetry

Colorimetry is the estimation of concentration of a coloured solution. When white light passes through a coloured solution, some wave lengths are absorbed more than others (Fig. 2). Many compounds are not themselves coloured, but can be made to absorb light in the visible range by reaction with suitable reagents.

The widely used method for determining the concentration of biochemical compound is colorimetry which make use of this property.

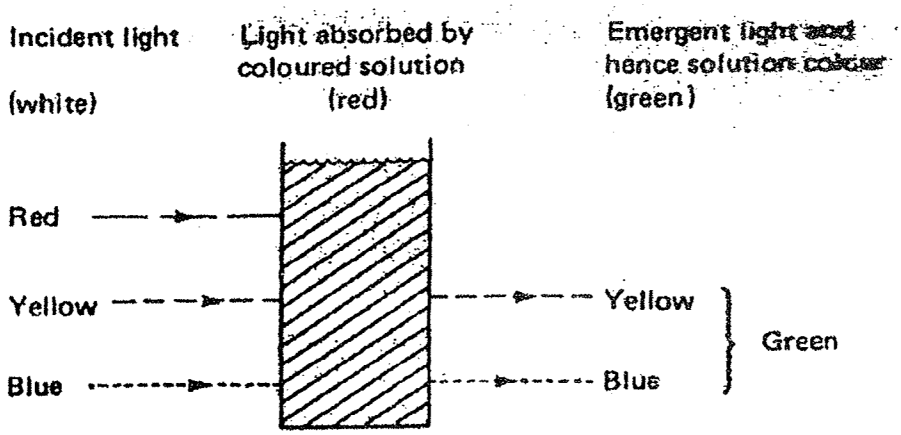


Fig. 2 Why solution appeared coloured.

Monochromatic Light

If a beam of light is passed through a *red filter*, the light coming out will comprise waves with wave length in the range of 600 nm) (Red) – a mono-chromatic light.

A monochromatic light of precise wavelength, can be generated by means of grating or prisms. Consider a solution containing a substance (chromophore) which absorbs in the range of 600nm. If now a monochromatic light beam of 600 nm λ is passed through it, *the energy will be absorbed.* And the amount of absorption depends on the number of absorbing molecules present in the solution (i.e., concentration of chromophore).

Thus,

(a) A substance absorbs light energy, if its chemical structure is designed for it (chromophore).

(b) The maximum absorption is at specific wave length (λ max).

(c) The extent of absorption at the specific wave length is dependent on the concentration (Absorption ∝ concentraton).

1.4 The Beer-Lambert Law :

When a ray of monochromatic light of intensity I_0 passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light I is less than I_0 ($I<I_0$) (Fig.-3). The relationship between I and I_0 depends on path length 1 of the absorbing medium and concentration c of the absorbing solution. These factors are related in the laws of Lambert and Beer.

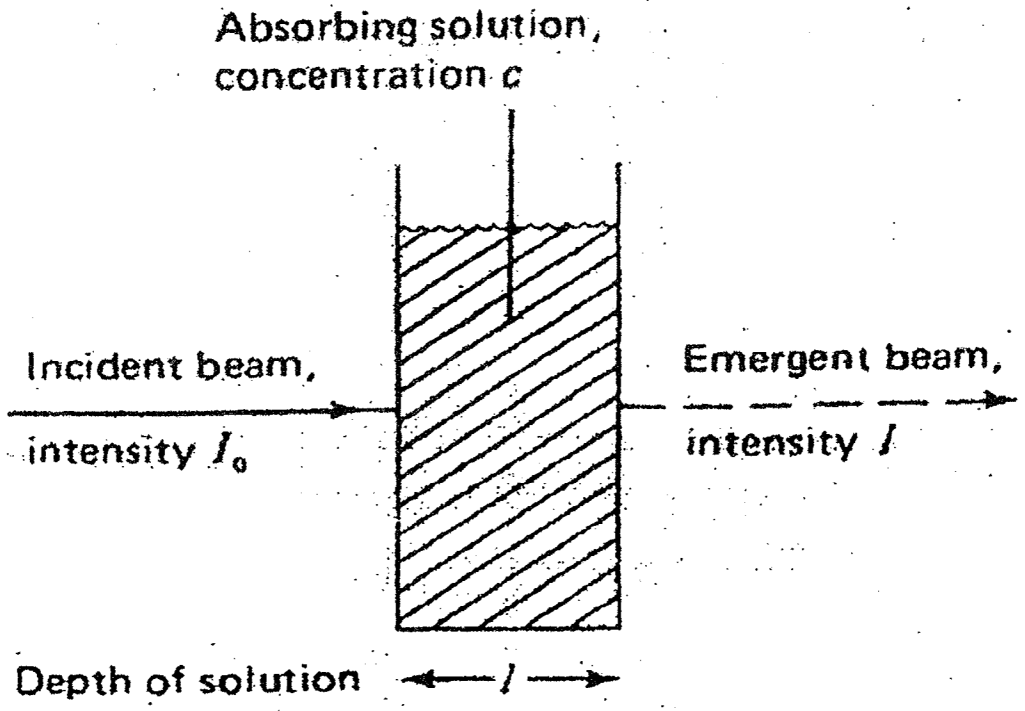


Fig. 3 : The absorption of light by a solution.

Lambert's Law

When a ray of monochromatic light passes through an absorbing its intensity decreases *exponentially* as the length of the absorbing medium (l)

Increases $I = I_0 e^{-kl}$

Beer's Law

When a ray of monochromatic light passes through an absorbing medium its intensity decreases exponentially as the concentration (c) of the absorbing medium increases $I = I_0 e^{-K_2 C}$

[N.B. The Law only holds upto a threshold maximum concentration for a given substance].

These two laws are combined together in *Beer-Lambert Law*

Beer-Lambert Law

$$I = I_0 e^{-k_3 lc}$$

The ratio of intensities is known as transmittance (T)

$$T = \frac{I}{I_0} = e^{-K_3 Cl} \text{ and } T = \frac{I}{I_0} \times 100 = e^{-K_3 Cl}$$

This is not very convenient since a plot of percent T against C gives a negative exponential curve (Fig. 4(a)).

Extinction (ϵ) : If logarithms are taken of the equation of a ration then,

$$\log_e \frac{I}{I_0} = -k_3 Cl \text{ or } \log_e \frac{I}{I_0} = k_3 Cl$$

$$\text{or } \log_{10} \frac{I_0}{I} = \left(\frac{k_3}{2.3}\right) Cl \text{ or } \log_{10} \frac{I_0}{I} = \epsilon Cl$$

$$\log_{10} \frac{I_0}{I} = A = \epsilon Cl$$

Optical density O.D. = A = ϵCl

Where ϵ = Extinction coefficient and A = absorbance.

If Beer-Lambert Law is obeyed then a plot of Absorbance against Concentration gives a straight line passing through the origin (Fig. 4(b)) which is more convenient than the Trnsmittance curve (Fig. 4a).

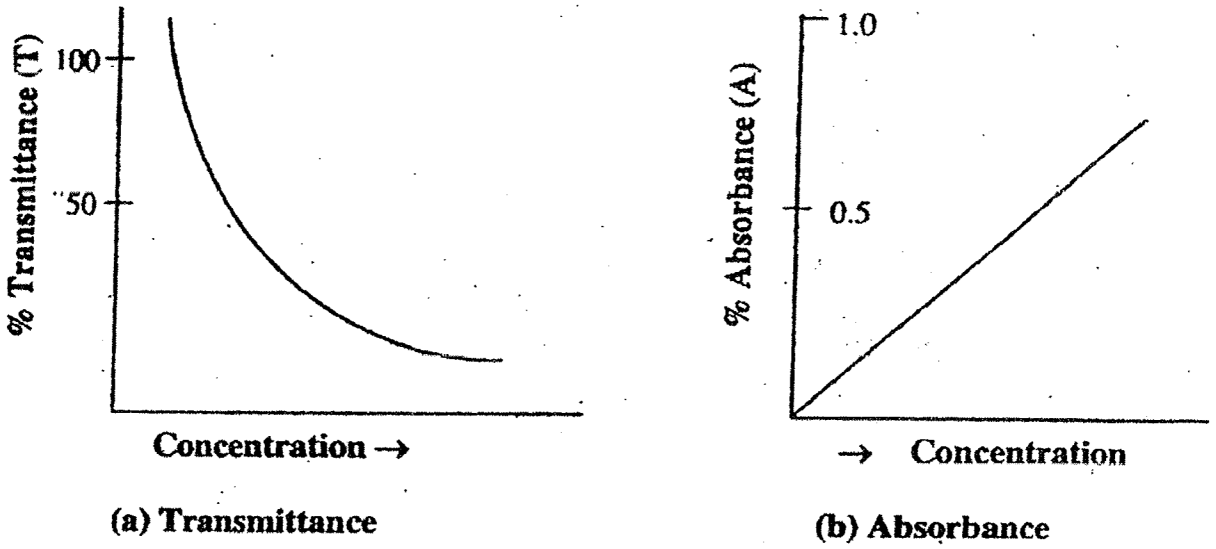


Fig. 4 : The relationship between the absorption of light and he concentration of absorbing solution

1.5 Molar Extinction coefficient :

If length is 1 cm and c is 1 mole lit^{-1} then $\in$ is molar extinction coefficient which is the characteristics of a compound and it has the dimension of litre mol^{-1} cm^{-1}

Specific Extinction Coefficient :

The molecular weights of some compounds such as proteins or nucleic acids in a mixture are not readily available and in this case, the specific Extniction Ciefficients used. This is the extinction of 10 gm/litre of compound in the light path of 1 cm.

1.6 Limitation of Beer-Lambert Law :

1. Light must be a narrow wave length range and preferably monochromatic.
2. The wave length of light used should be at absorption maxime (λ_{max}) of the solution.
3. The law only holds upon a *threshold maximum concentration* for a given substance. The solution should not be too concentrated, giving an intense colour.
4. There must be no ionization, association, dissociation of the solute with concentration or time.

1.7 Measurement of Absorption :

I. The Photoelectric Colorimeter : A diagram of the basic arrangement of a typical colorimeter is given in Fig.-5, white light from a *tungsten lamp* passes through a slit, then a *condenser lens,* to give a parallel beam which falls on the *solution* under investigation contained in an *absorption cell or cuvette*. The cell is made of glass with the sides facing the beam cut parallel to each other. In most cases, the cells are 1 cm square and hold 3 ml of liquid comfortably. Before the absorption cell is the *filter* which is selected to allow maximum transmission of the colour to be absorbed. The filter gives narrow transmission bands. If a blue solution is under examination, the red is absorbed and a red filter is selected (complementary colour (see table-2). The light then falls on to a photocell which generates an electrical current in direct proportion to the intensity of light falling on it. The small electrical signal is increased in strength by the amplifier, and the amplified signal passes to a galvanometer, or digital readout, which is calibrated with a logarithmic scale so as to give absorbance reading directly. The blank solution is first put in the colorimeter and the reading adjusted to zero.

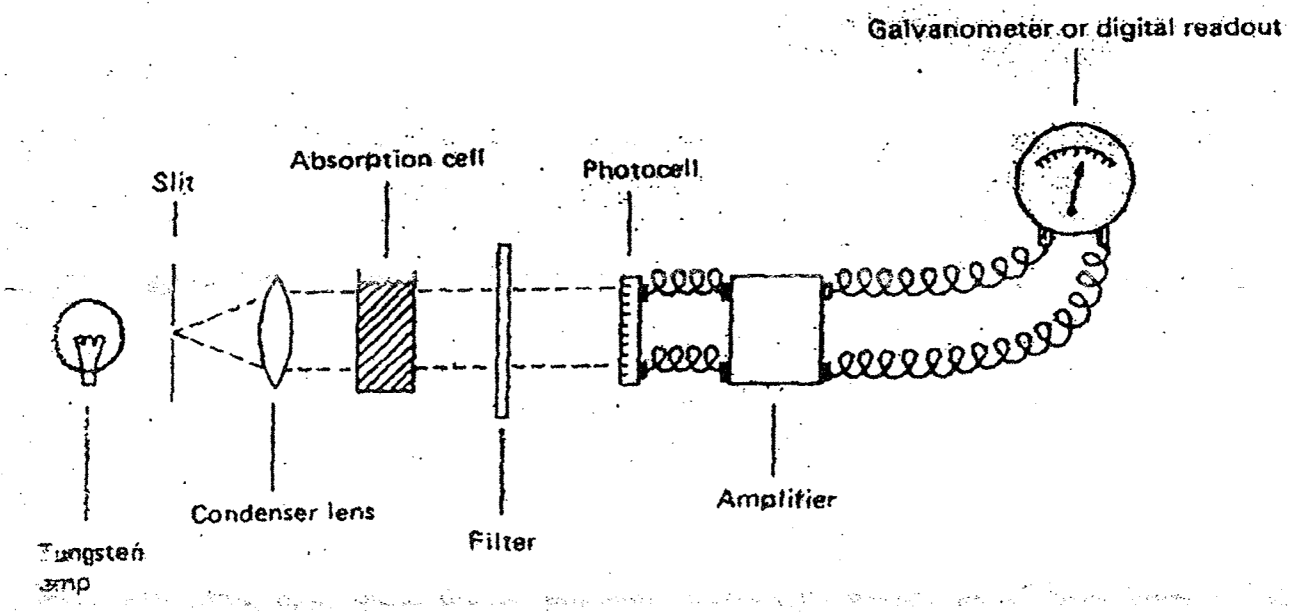


Fig. 5 : A Diagram of Photoelectric Colorimeter

Table-2 : The relationship between the colour of the solution examined and the filter chosen (complentary colour)

Colour of Solution	Filter
Red-Orange	Blue-blue green
Blue	Red
Green	Red
Purple	Green
Yellow	Violet

II. UV and Visible spectrophotometry

The principal components of spectrophotometry : A *light source* emits light along a broad spectrum, then monochromator selects and transmits light of a particular wavelength (λ_{max}). The monocluomatic light passes through the sample in a cavette of path length 1 cm and is absorbed by the sample in proportion to the concentration of the absorbing species. The transmitted light is measured by a detector. A spectrophotometer is a sophisticated type of colorimeter where monochromatic light is provided by a grating or prism.

Some *colorimeters* and spectrophotometer have two scales, a linear one of percent transmittance and a logarithm one of absorbance. It is this latter scale that is related linearly to concentration and is the one used in the construction of a standard curve (Fig.-6).

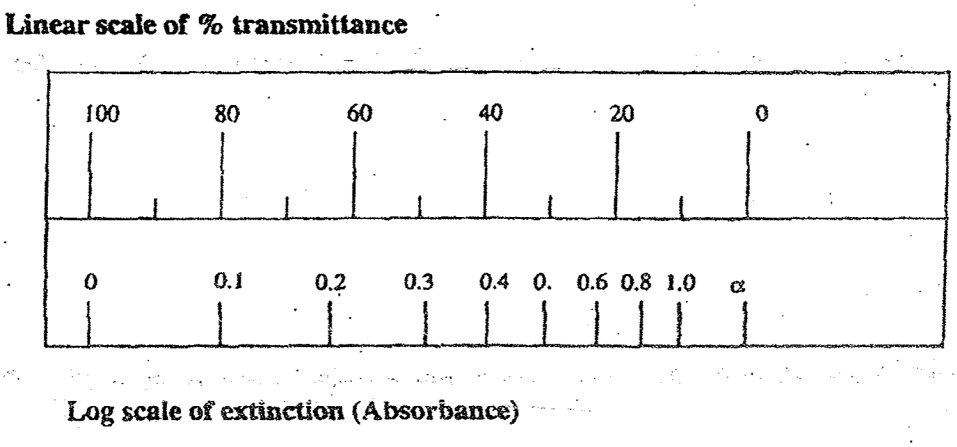
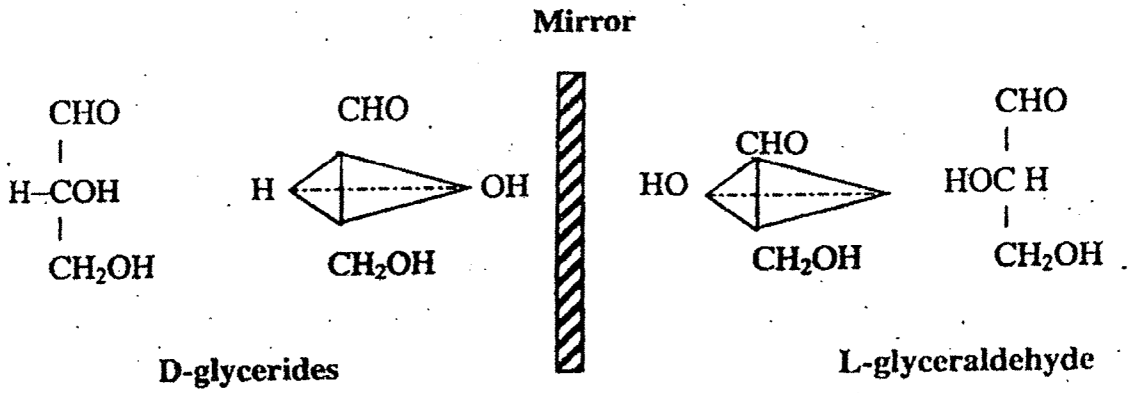


Fig 6 : The relationship between percent transmittance and absorbance (extinction)

2. Optical Activity of Organic Substance

2.1 Optical Activity :

Many biological molecules, including sugars, contains one or more asymmetric carbon atoms or chiral centres and because of this, a number of *stereoisomers* are possible. Compounds composed of some elements, in the same numbers, but organized differently by three dimensional arrangements are known as *siomers*. For example, glyceraldehydes, one of the simplest sugars, has one asymmetric carbon atom and there are two possible arrangements of the four groups around this carbon. The formulae of these two isomers are given below where the asymmetric carbon is placed at the centre of the tetrahedron, the four groups being situated at each corner and the dotted line shown as lying below the plane of the paper. These structures are mirror images of each other and are referred to as *enantiomers*. They have identical physical and chemical properties.



These two configurations are optical isomer or enantiomers and *rotate the plane of polarized light* to the same extent but in the opposite direction. When the plane of polarized light is rotated to the right, the compound is dextrorotatory and is labelled as (d) or (+). Likewise when the plane of polarized light is rotated to the left, the compound is levorotatory (1) or (–).

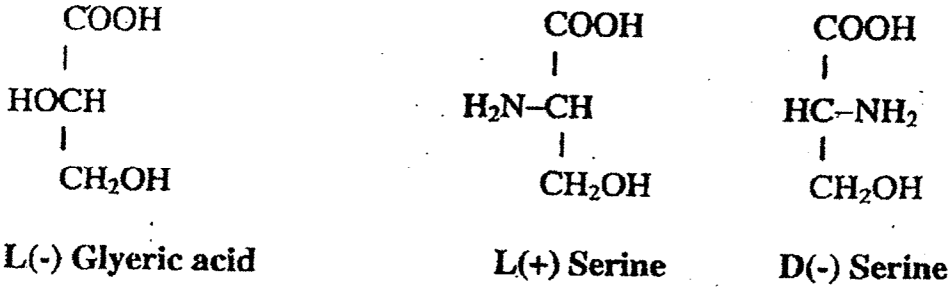
2.2 The D and L forms :

Aldoses are derived from the parent compounds, glyceraldehydes, by the addition of successive secondary alcohol group (–CHOH). Since glyceroldehyde can exist in two forms, two distinct families

of aldolase emerge : those derived from D-glyceride known as D-sugars and those from L-glycerides called L-sugars. The letter D and L do not give an indication of the optical activity, but refer to the configuration of the carbon atom next but one furthest removed from the end of the chain containing the aldehyde or ketone group, when the –OH group of this carbon atom is on the right, the sugar is a member of the D-series, when it is on the left, it is a member of L-series. Thus D-sugar could be dextrorotatory D(+) or Levorotatory D(–) depending on the configuration of the other carbon atoms present.

2.3 Stereochemistry of amino acids :

The α-carbon atom is asymmetric and is a chiral centre for all amino acids except glycine, so that, apart from glycine, all aminoacids show optical activity. If Serine is taken as the parent compound, then it can be compared with L(–) glyceric acid, the parent compound for the L series of sugar.



L(-) Glyeric acid **L(+) Serine** **D(-) Serine**

All the aminoacids present in proteins are of L-configuration, although the D form is found in antibiotics and bacterial cell walls. The L and D forms refer to the absolute configuration about the chiral centre and not to be optical activity.

2.4 The Polarimeter :

The rotation of the plane of polarized light can be demonstrated and measured with a polarimeter (Fig. 7). It consists of a light (monochrometic light) source in which light is oscillating in all possible directions. This is passed through a *polarizer* (a Nicol prism) and the light merging travels in one plane. This polarized beam then passes the sugar sample placed in the *polarimeter tube*, which rotates the plane of light. At the end of the tube is an *amalyzer* which detects the extent and direction to which the light has been rotated (optical rotation).

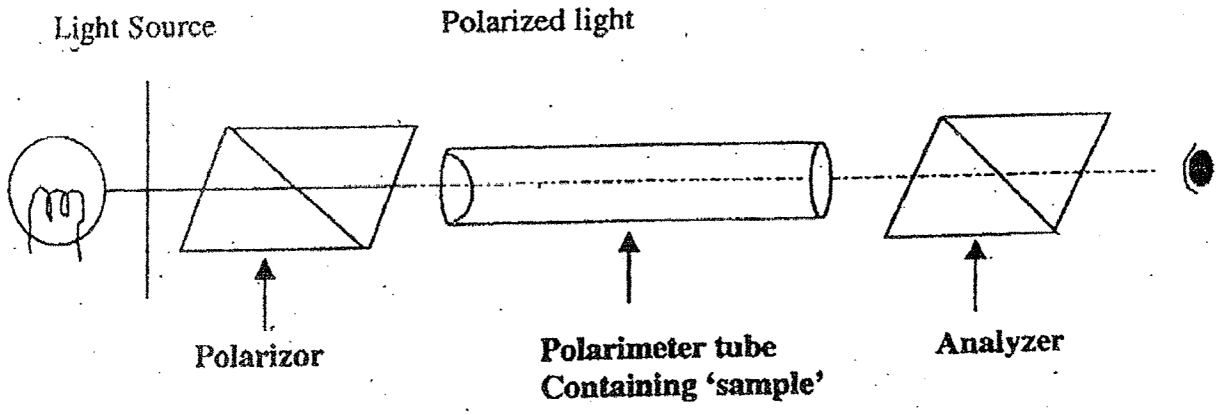


Fig. 7 : Essential Features of Polarimeter.

If the angle of rotation is clockwise then the compound is dcxtrorotatory (+) and if *anticlockwise* then levorotatory (–).

Specific Rotation :

The degree to which plane polarized light is rotated by an optically active compound is referred to as the *optical rotation* and is given the symbol α. However, in addition to the actual nature of enantiomeric compound there are several factors which can influence α. These are (1) the wave length of the original light source; (2) the temperature, (3) the *sample concentration* (4) ty;e of solvent used and (5) the length of the polarimeter tube. By definition, the *specific rotation* is the rotation detected using a 10 cm tube, the sample concentration as 1 gm ml^{-1}, at a fixed temperature 25°C, the light source being sodium D-line (λ- 589 nm).

Then specific Rotation $[\alpha]_D^{25^0} = \frac{a}{c \times l}$ where is the concentration is gm ml^{-1} and 1 is the path length in 1 dm (10 cm). Whent he specific rotation of an optically active solute is known, its concentration (gm ml^{-1}) in a solution can be estimated from the degree of rotation of the plane polarizede light passing through that solution.

$$c = \frac{a}{l \times [\alpha]_D^{25^0}}$$

2.6 Optical Purity :

A sample of a single enantiomer is said to be optically pure. Nature for most part, uses and generates optically pure compounds. If the mixture is 1:1 enantiomers, a racemic mixture is said to be the result and *no net optical rotation will be detected.*

'Specific rotation' of some bio-molecules

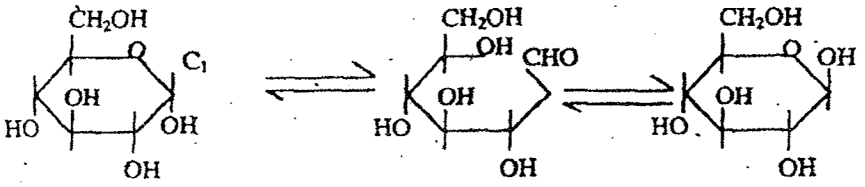
Substance	$[\alpha]_D^{25^0}$
Amino acid :	
L-Arginine	+ 12.5^0
L-Leucine	– 10.4^0
L-Proline	– 86.2^0
Carbohydrate	
D-Ribose	–23.7^0
D-Frucose	– 92.3^0
D-Glucose	+ 52.5^0
Glycogen	+ 197.0^0
Lactose	+ 52.5^0
Sucrose	+ 66.5^0

2.7 Inversion of Sucrose :

On hydrolysis dextrorotatory sucrose {(α)=+66.5^0] yields an equimolecular mixture of glucose and fructose. This mixture, called the invert sugar, is levorotatory ([α]=–21.2^0) because the levorotation due to fructose (α= –92.3^0 exceeds in magnitude of the dextrorotation of glucose (α=+52.5^0). Because of this changé in the direction of optical activity, the hydrolysis of sucrose is called the 'Inversion'.

2.8 Mutarotation of glucose :

When D(+) glucose is dissolved in water, a specific rotation of +1113 degree is obtained, but this slowly changes, so that at 24 hr. the value has become +52.5^0. This phenomenon is known as *mutarotation* and is shown by a number of pentoses, hexoses and reducing disaccharides. The reason for this change in rotation is that glucose exists in solution mainly in the *ring form.*



αD-glucose specific rotation +113° (36%) | Open-form (0.02%) | β-D-gliucose +19.7° (6%)

The ring form creates another asymmetric carbon atom (C_1) so that two ring forms (α and β) are now possible and these are known as *anomers*. The open chair form (and this) is responsible for the reducing properties of the sugar.

2.9 Biological application :

Optical activity of biomolecules : Most carbohydrates amino acids, proteins and nucleic acids and some vitamins are optically active. Usually *only one of the optical isomers of each of these substances* is *biologically active.* In animals, for example only the dextrorotatory isomers of glucose galactose, lactose, glycogen, alamine, arginine and the levorotatory isomers of fructose, ribose, proline, leucine are biologically active.

3. Polarization Microscopy – Principle :

Isotropic structures have their molecules randomly oriented in different directions. Because of the lack of well-oriented structure, they possess almost the same refractive index in different directions and do not rotate the plane of vibration of polarized rays.

Ansotropic structures, however, are made of well-oriented distributions of molecules. Because of this, such structures are doubly refractive or brefringent – they possess different refractive indices for light rays traversing them lengthwise and for the rays crossing them perpendicular to their long axis. Thus *anisotropic structurs rotate the plane of vibration of polarized light* traversing them. Polarizing microscope bring the birefringent anisotropic cellular and tissue structures into view using plane polarized light,

Such microscope are particularly useful in studying microtubules, mitotic spindles, striated muscle fibres, collagen fibres and bone matrix in unstained tissue preparations against a dark back ground.

Because the use of polarized light is associated with considerable decrease in the intensity of illumination, a high intensity of illumination, a high-intensity light source is used for polarization microscopy. Either a Nicol prism or a polaroid film is placed below the substage condenser. This serves as a *polarizer*. As the ordinary light beam passes through the polarizer, only plane polarized rays emerge from it and reach the condenser which converges the rays to the specimen held on the stage. Another Nicol prism or a second Polaroid film is placed between the objective and the eye piece that serves as an *analyzer*. It can be rotated so that it may be placed crosswise to the polarizer. Plane polarized light traversing anisotropic structurs in the specimen, suffer from a rotation of their plane of vibration and consequently pass through the corssed analyzee to reach the observer through the eye piece. This produces bright images of the anisotropic structures against the dark background.

Suggested Questions on Photometry :

Q1. A solution of bilirubin in chloroform at 25°C measured at a wave length of 453 nm in a 1 cm light path curve gave an absorbance (A) of 0.528. The concentration of bilirubin solution was 5 mg/lit. Calculate the Molar Extinction Coefficient. (Given MW of bilirubin = 582).

Q2. Molar extinction coefficient of tyrosine in Water is 1420 lit $mole^{-1}$ cm^{-1} at 275 nm wave length, waht is the concentration of tyrosine in a solution of path length 1 cm for which A=0.71.

Q3. What is the Beer – Lambert Law and its limitation? (Give equation for Beer-Lambert's law stating all units)

Q4. What is molar extinction coefficient? What is its significance? What is specific extinction coefficient?

Q5. Write on principle components of spectrophotometry.

Suggested Questions on Optical Activity :

Q1. Write notes on

1) Stereosomerism and optical isomerism.

2) Specific rotation.

3) Mutarotation of glucose.

4) Inversion of sucrose.

Q2. Write on the main features of Polarimeter.

Q3. Write on the principle of polarization microscopy.

Key Words :

Absorption / Amission radiation, visible spectra, chromophore, monochromatic light, The Beer Lambert Law, Absorbance, Transmittance, Molar extinction coefficient, Photoelectric colorimeter, spectrophotometry, Optical activity, asymmetric carbon atom, isomer, enantiomer, stereoisomer, optical isomer, dextrorotatory levorotatory, optical rotation, specific rotation, Inversion of sucrose, mutarotation, anomers, anisotropic birefringent, polarization microscopy.

Select Readings :

1. Biophysics and biophysical chemistry by Debojyoti Das (4th Edn.).

2. An introduction to practical biochemistry by D.T. Plummer (3rd Edn.).

3. Harper's illustrated Biochemistry (26th Edn.).

Paper II : Group B : Module No. –23 : Unit II

Biophysics

7. Thermodynamics

Thermodynamics systems – isolated, closed and open. First and second laws of thermodynamics, Enthalpy and Entropy. Biological Steady State and its Maintenance, Gibbs Free Energy

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2.6 Phosphate pool and energy cycle

Paper-II : Group B : Module No. – 23 : Unit II

THERMODYNAMICS (Energy Flow)

1. INTRODUCTION

1.1 Thermodynamic Analysis of energy exchange begins by *specifying* a SYSTEM, the collection of matter under study. All other matter in the UNIVERSE apart from the specific system is called the SURROUNDINGS (Fig. 1).

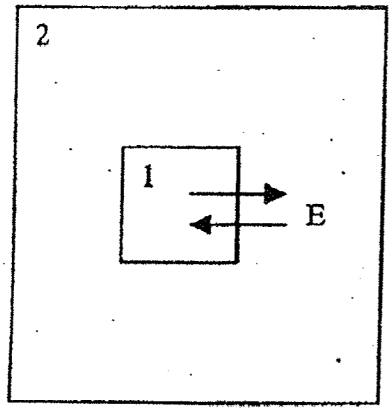


Fig. 1 :

1 = SYSTEM

2 = SURROUNDINGS

1+2 = UNIVERSE

$\rightleftarrows$ **E = ENERGY FLOW**

During the process under study energy may flow the system to surroundings or from surrounding to the system. Processes of energy flow are analyzed in terms of the energy content of the *initial state* of the system and the surroudning and the *final state* of the system and the surrounding after equilibrium has been reached.

[**Example** :Cooling of water to room temperature from boiling temperature]

The energy content of each state (initial or final) is a function of various measurable attributes – *temperature, pressure, volume, mass*. From the measurements of the changes in the energy content of the system and the surroudning as the system proceeds from its *initial* to its final equilibrium state, an energy balance sheet can be prepared. This formulates an *equation of state*. Since the analysis of energy exchange by thermodynamics is made by considering properties of matter in the bulk such as temperature, pressure, etc., it requires no knowledge of molecular composition of the system or its surroundings or of the molecular mechanism by which a process takes place. Furthermore, thermodynamic analysis of energy exchange is independent of the rates of processes, it depends only on the *initial* and *final* state of the system.

Three types of system are defined :

Open system : a system in which *both matter and heat* can be exchanged with the surrounding.

Closed system : a system in which only *heat* can be exchanged with the surroundings.

Isolated system (adiabatic) : a system in which neither matter nor het can be exchanged with the surroundings.

1.2 The First Law of Thermodynamics

This is the principle of the conservation of energy. In any process *the total energy of the system plus the surrounding remains constant.* Although the energy is neither created nor destroyed during a chemical or physcial process it may undergo transformation from one form to another. The forms of energy are *heat, light, electrical, mechanical* and *chemical.*

Formulation of First Law :

Suppose we put some amount of heat in a system. The amount of heat energy remains either wholly or partly as internal energy in the system or can be wholly or partly used up by the system in doing mechanical work. In general case, when the heat absorbed goes *both* to increase the internal energy and to produce some mechanical work, we have,

Heat absorbed = Incrase in internal energy + Work done by the system

[q] [ΔE] [w]

or, $\Delta E = q - w$ (1)

If the change is very small we can write

$$dE = dq - dw \quad (2)$$

If the system is under a constant pressure P and increases by a small volume dV, the work done is PdV, the equation becomes

$$dE = dq - PdV \quad (3)$$

at Constant P, i.e., change in internal energy is equal to heat absorbed by he system minus the PdV work at constant pressure.

Example : One gram of water at 100°C requires 436 caloreis of heat for conversion to steam. Calculate the increase in internal energy per mole of water (assume water as ideal gas)

q = heat absorbed = 536 cal x 18 mole gm = 9648 cal.mole^{-1}

w = work done > $PV = RT$ (ideal gas for 1 mole)

$= 1.98 \times 373$ cal mol^{-1} $\qquad R = 1.98$ cal mole^{-1} deg^{-1}

$= 746$ cal mole^{-1} $\qquad T = (100 + 273) = 373$

$\therefore$ Internal energy change = $\Delta E = q - w$ (9648 – 746) cal. mole^{-1}

= 8902 cal mole^{-1} Ans.

1.3 The heat content or 'Enthalpy' of a system

It is often convenient in dealing with system at constant pressure to use a function H, called the *heat content* or *enthalpy* in place of internal energy, the two being related by the equation,

Definition of Enthalpy : $H = E + PV$

and ΔE would depend on the initial and final state of the system. If the final heat content is H_2 and the initial value in H_1, then

$$\Delta H = H_2 - H_1 = (E_2 - P_2V_2) - (E_1 + P_1V_1)$$

$$= (E_2 - E_1) + (P_2V_2 - P_1V_1)$$

$$\Delta H = \Delta E + \Delta (PV) \qquad (4)$$

If pressure is constant this reduce to

$$\Delta H = \Delta E + P(V_2 - V_1) = \Delta E + w \text{ (work done)}$$

$$\Delta H = \Delta E + P\Delta V \qquad (4)"$$

From equation (1) $\Delta E = q - w$ or $(\Delta E + w) = \Delta H = q$ (het absorbed)

$\therefore \Delta H = q_p$ (het absorbed at constant pressure) (5)

Therefore, ΔH, i.e., increase in H is equal to the heat absorbed at constant pressure 'q_p' and this explains the other name, *heat content*, for Enthalpy function. Positive and negative values of ΔH indicate that heat must be respectively supplied (+) to or liberated by (–) the system for conducting the reaction.

Exothermic and Endothermic Reactions : Reaction which take place with liberation of heat are called exothermic reactions, whereas reactions which take place with absorption of heat are endothermic;

e.g., Exothermic : $H_2 + Cl_2 = 2HCl + 43,600$ Cal

Endothermic : $H_2 + I_2 = 2HI - 12,680$ Cal

2. THERMODYNAMICS IN CHEMCIAL REACTION

2.1 The Second Law of Thermodynamics

The first law of thermodynamics cannot beused to predict whether a reaction can occur spontaneously. Therefore a function *entropy* (S) is developed which is a *measure of the degree of randomness or disorder of the system.* The entropy of a system increases when the system becomes more disordered.

The second law states that a process can occur spontaneously only if the sum of the entropies of the system plus its surroundings increases (or that the universe tends towards maximum disorder), that is :

$\Delta S_{system} + \Delta S_{surrounding} > 0$ for a spontaneous process

Thus entropy provides the driving force and gives direction to all processes, i.e., in which direction a given process is likely to occur. All processes tend to proceed in a direction until *an equilibrium* is reached at which the entropy is maximum. An *equilibrium* is a state in which *no further net chemical or physical change is taken place and in which temperature, pressure and concentration are uniform throughout the system.* A system at equilibrium has exhausted its capacity to do work on its surroundings. It cannot reverse to return to the initital stae spontaneously – known as *Irreversible process.*

2.2 Gibb's free energy

However, using entropy is a criterion of whether a biochemical process can occur spontaneously is difficult, as entropy changes of chemical reactions are not readily measured. This difficulty is overcome by using a different thermodynamic function, *free energy (G)* proposed by Gibb's which combines the first and second laws of thermodynamics

$$\Delta G = \Delta H - T\Delta S \qquad (6)$$

in which ΔG is the *free energy* of a system undergoing a transformation at *constant pressure (P) and temperature (T),* ΔH is the change in enthalpy (heat content) of this sysem, and ΔS is the change in the entropy of this system. The enthalpy change (equation 4)

$$\Delta H=\Delta E+P\Delta V$$

the volume change (ΔV) is small *for chemical reactions,* so ΔH nearly equal to ΔE. Therefore

$$\Delta G=\Delta E-T\Delta S \qquad (7)$$

Thus, *the ΔE of a reaction depends both on the change in internal energy and on the chane in entropy of the system.* The change in free energy ΔG of a reaction is a valuable criterion of whether that reaction can occur spontaneously. Total energy ΔE of the system is, thus, a sum of the terms – the change in free energy (ΔG) and the entropy change TΔS (at constant P, T).

$$\Delta E=\Delta G\downarrow+T\Delta S\uparrow$$

As the system approaches equilibrium the free energy decreases to a minimum and the entropy of the universe increases to a maximum.

Please note,

* a reaction can occur spontaneously if ΔG is negative.
* a system is at equilibrium if ΔG is zero.
* a reaction cannot occur spontaneously if ΔG is positive. An input of energy is required to drive such a reaction.

* The ΔG of a reaction is independent of the path of the transformation.
* ΔG provides not information about the rate of the reaction.

2.3 The free energy change occurring during chemical reaction is calculated using an equation derived from the law of chemical equilibrium. For general equation of reaction.

$$A + B \rightleftharpoons C + D$$

(Reactants) (Products)

The free energy change (ΔG) is given by the equation

$$\Delta G = \Delta G^0 + RT \ln \{[C][D]/[A][B]\}$$

where ΔG^0 is the standard free energy change.

The standard free energy change (ΔG°) *is that for converting 1 mole.lit^{-1} of reactants into 1 mole.lit^{-1} of products at 1 am pressure, 25°C and pH 7.0.*

When the reaction is at equilibrium regardless of the starting concentration of A, B, C, D, the condition of minimum free energy exists and no further change is possible. ΔG is then equal to zero therefore at equilibrium,

$$O = \Delta G^\circ + RT \ln \{[C][D]/[A][B]\}$$

and since $K_{eq} = \{[C][D]/[A][B]\}$

$$\boxed{\Delta G^\circ = -RT \ln K_{eq}}$$ at equilibrium

2.4 Thus the standard free energy change (ΔG°) *of a chemical reaction can be calculated from its equilibrium constant K_{eq} at any given temperature*

$$\Delta G^\circ = -RT \ln K_{eq}$$

$$\Delta G^\circ = -2.3\, RT \log_{10} K_{eq}$$

when R = 1.98 calmole^{-1} deg^{-1}

$T = 25°C = (25 + 273) = 298°K$

Then $\Delta G^\circ = (-2.3 \times 1.98 \text{ calmole}^{-1} \text{ deg}^{-1} \times 298 \text{ deg}) \log_{10} K_{eq}$

$\Delta G^\circ = -1363 \log_{10} K_{eq} \text{ calmole}^{-1}$

Example 1 : Consider the following interconversion in glycolysis

$\text{Fructose-6-PO}_4 \rightleftharpoons \text{Glucose-6-PO}_4$

If $K_{eq} = 1.97$ what is ΔG° at 25°C

Ans. $\Delta G^\circ = -1363 \times \log 1.97$ $\log 1.97 = 0.297$

$\Delta G^\circ = -1363 \times 0.297 = -400 \text{ cal.mole}^{-1}$

It is a spontaneous reaction,

If $K_{eq} = 1$, $\Delta G^\circ = 0$, that is no change in free energy

$K_{eq} <$ i.e., concentration of reactants is more than the concentration of products, ΔG° is positive

$K_{eq} > 1$ i.e., concentration of reactants is less than concentration of products, ΔG° is negative

Chemical reaction with a negative standard free energy chane are termed *exergonic* (release of energy) and are spontaneous reaction, whereas chemical reaction with positive standard free energy chane are called *endergonic* (absorption of energy) and are not spontaneous.

Example 2. From equilibrium data on the enzyme phosphoglucomutase in the following reaction at 25°C

$\text{Glucose-1-PO}_4 \rightleftharpoons \text{Glucose-6-PO}_4$

Start	0.02 M	
at equilibrium	0.001M	0.019 M

$$K_{eq} = \frac{0.019}{0.001} = 19; \quad \Delta G^\circ = -1363 \log 19 = -1363(1.28)(\log 19 - 1.28)$$

$\Delta G^\circ = -1745$ cal. mole^{-1}, spontaneous reaction.

The standard free energy of chemical reactions have another valuable property, they are additive for sequential reactions.

If 1) $A \rightarrow B$ and ΔG_1°

2) $B \rightarrow C$ and ΔG_2°

3) $C \rightarrow D$ and ΔG_3°

Sum Σ $A \rightarrow D$ and ΔG_S°

For the sum of these reactions $A \rightarrow D$, whose fre energy change ΔG_S° is algebraic sum of all ΔG°'s

$$\Delta G_S^\circ = \Delta G_1^\circ + \Delta G_2^\circ + \Delta G_3^\circ$$

Example 3:

(1) Glucose-1-$PO_4 \rightarrow$ Glucose-6-PO_4 $\Delta G_1^\circ = -1.74$ Kcal.mole^{-1}

(2) Glucose-6-$PO_4 \rightarrow$ Fructose-6-PO_4 $\Delta G_2^\circ = +0.40$ Kcal.mole^{-1}

Σ Glucose-1-PO4 $\rightarrow$ Fructose-6-PO_4

$\Delta G_S^\circ = -1.74 + (+0.40) = -1.34$ Kcal.mole^{-1}

Example 4 :

Hydrolysis of ATP : $ATP + H_2O \rightleftharpoons ADP + P_i$

Direct measurement of K_{eq} of this reaction is not practical since the reaction yields large amount of energy (–7300 Cal.mole$^-$). It can be measured in steps :

(1) $ATP + Glucose \xrightarrow{\text{Hexokinase}} ADP + Glucose\text{-}6\text{-}PO_4$

$K_{eq} + 661$, $\Delta G_1^\circ = -4.00$ Kcal.mole^{-1}

(2) Glucose-6-PO_4 $\xrightarrow{\text{Phosphatase}}$ Glucose + P_i

+ HOH K_{eq} =171, ΔG_2° = –3.30 Kcal.mole^{-1}

Overall ATP + HOH $\longrightarrow$ ADP + P_i

$\Delta G_S^\circ = \Delta G_1^\circ + \Delta G_2^\circ = -4.00 + (-3.3) = -7.3$ Kcal.mole^{-1}

ATP allows the coupling of thermodynamically unfavourable reaction to favourable ones:

(1) Glucose + $P_i \rightarrow$ G1-6-PO_4 + H_2O ΔG° = +3.3 Kcal.mole^{-1}

cannot proceed under physiological condition. The reaction must be coupled with another high exergonic reaction in order to take place such as hydrolysis of ATP

(2) ATP + HOH $\rightarrow$ ADP + P_i ΔG_2° = –7.3 Kcal.mole^{-1}

the reaction occurs sum $\Delta G_1^\circ + \Delta G_2^\circ$ = +3.3 + (–7.3) = –4 Kcal.mole^{-1}

(overall Glucose + ATP $\rightarrow$ G1-6-PO_4 + ADP)

From the standard free energy of formation of reactants and products ΔG° for a given chemical reaction can be calculated.

The results of many thermodynamic measurements have shown that each type of organic functional group has a characteristic free energy of formation from its elements and that such values are additive.

The standard free energy of formation (ΔG_f°) for many biologically occurring compounds are given in the following table :

Substance	ΔG°_f (Kcal.mole^{-1})
H^+ (reference)	0
Fumerate	– 144.40
α-D-Glucose	– 219.20
α-Keto glutarate	– 190.60
Lactate	– 123.80
L-malate	– 201.98
Oxaloacetate	– 190.50
Pyruvate	– 113.40
Succinate	– 164.90
Water	– 56.69

and, $\Delta G^\circ = \Sigma \Delta G^\circ_{f\,product} = - \Sigma \Delta G^\circ_{f\,reactants}$

Example 5 :

Calculate ΔG° of the following reaction from their ΔG_f°

Fumerate + $H_2O \rightleftharpoons$ malate

ΔG° = –201.98 – {–144.4 – 56.7}

= –0.88 Kcal.mole^{-1}

Example 6 :

Calculate ΔG° for the alcohol fermentation of glucose

D-glucose → 2-ethanol + $2CO_2$

Given ΔG_f° of D-glucose = –219.22 Calmole^{-1}

ΔG_f° of CO_2 = –94.45 Calmole^{-1}

ΔG_f° of Ethanol = – 43.39 Calmole^{-1}

ΔG° = –2 x 43.39 + (2 x 94.45) – (–219.22)

= –86.78 – 188.9 + 219.22

= –275.68 + 219.22

= –156.46 KCalmole^{-1}

2.5 Biological Systems – Equilibrium vs Steady State

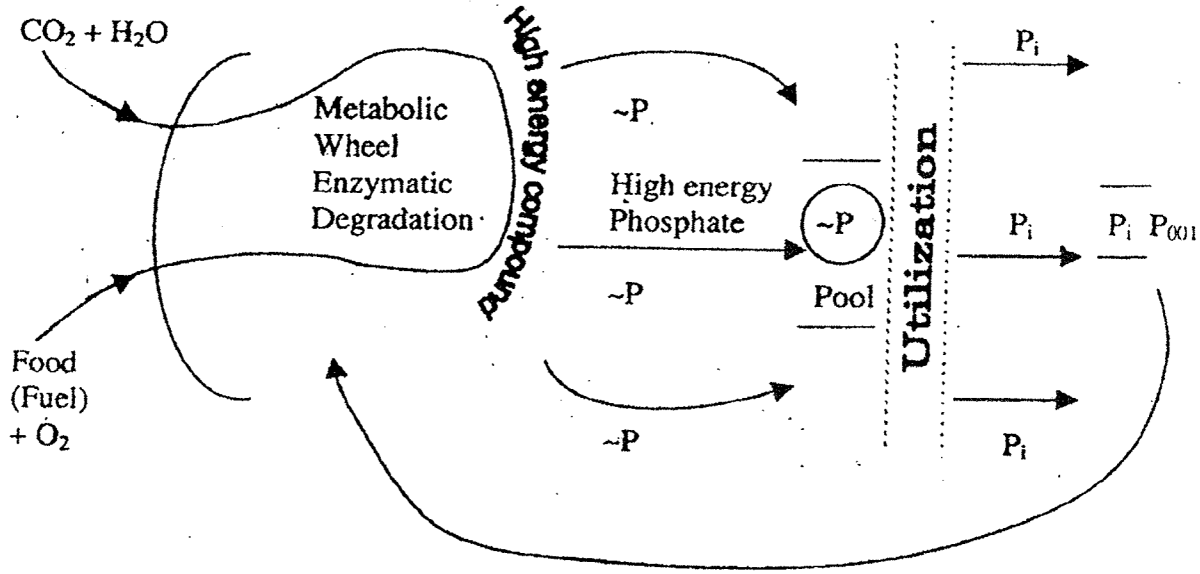
All living organisms constitute open, non-equilibrium system that derive energy and matter from their surroundings, using them for the life processes and eliminate both energy and matter to the surroundings and maintain a *steady state.* Living organisms constantly produce increase in entropy in their surroundings as a necessary cost of maintaining their own internal order.

Photosynthetic plants trap the photons of sunlight with the help of chloroplasts, gather CO_2 and H_2O from the surroundings utilize them to synthesize biomolecules and simultaneously eliminate O_2 to the surroundings.

Other organisms including animals and man take nutrients in the form of complex biomolecules from plants or other animals, catabolize them using O_2 from the surroudnings and release CO_2, H_2O and other metabolites produced, to the surroundings along with some heat while the rest of the energy is utilized in the life processes to delay the decay towards the *thermodynamic equilibrium state of death.* Thus life is maintained as a steady state of non-equilibrium by a specific and irreversible energy flow through the living organisms that work at constant temperature.

2.6 Phosphate Pool and Energy Cycle

How do the living organisms utilize the chemical energy from their environment to carry out chemical and physical activities? In the cell, both anabolic (synthetic) and catabolic (degradative) processes to on simultaneously. Energy released from the degradation of some compounds may be utilized in the synthesis of other compounds. Thus the concept of energy cycle has developed.



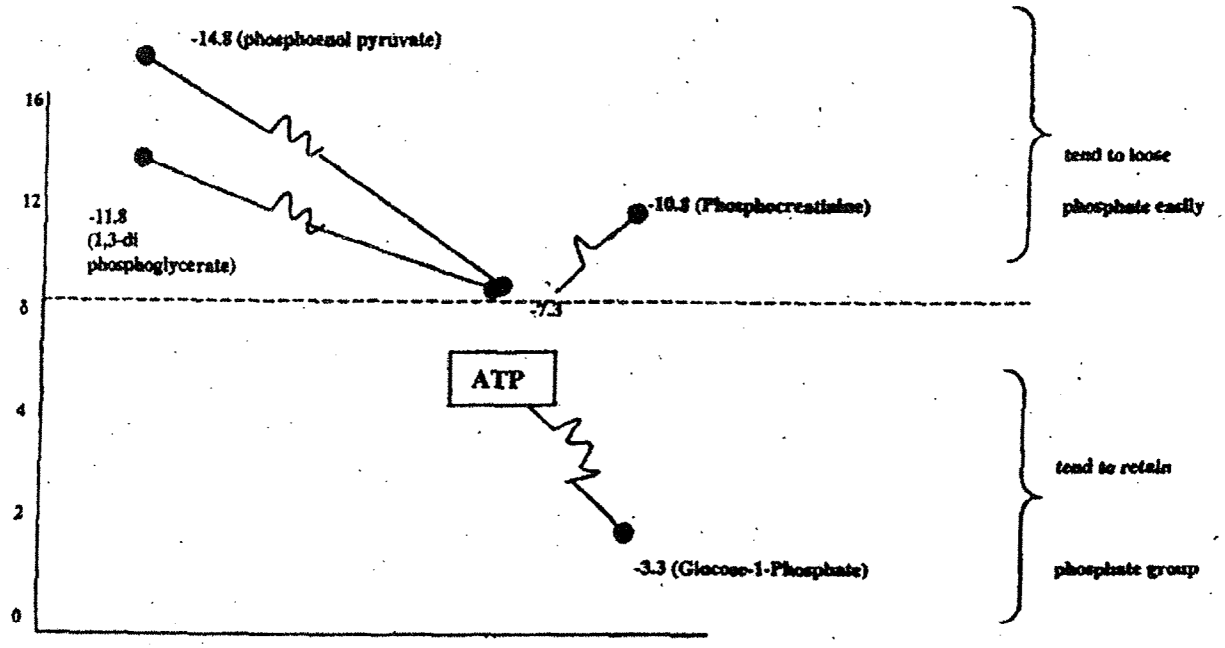
Fuel molecule representing a source of potential chemical energy is degraded through enzymatic reaction to *produce a few energy-rich compounds* playing a key role in the energy cycle. The ATP-ADP system occupies an intermediate value in the energy system. Some high energy phosphates are:

Phosphoenol pyruvate	14.80 Kcal	High energy phosphate
3-diphosphoglycerate	11.80 Kcal	compound
Phosphocretinine	10.30 Kcal	
ATP	7.30 Kcal	
G1-1-PO_4	5.00 Kcal	Low energy phosphate
Fr-6-PO_4	3.80 Kcal	compound
G1-6-PO_4	3.30 Kcal	

Compounds with the more negative value undergo *more complete hydrolysis at equilibrium*, i.e., have a high equilibrium constant than those lower in the scale, in other words those high in the scale tend to lose phosphate groups readily and those low in the scale tend to hold on to them. ATP has an intermediate value in this thermodynamic scale. It is the whole function of the ATP-ADP system to serve as an obligatory intermediate carrier of phosphate group originating from high energy phosphate

compounds above ATP on the themodynamic scale to acceptor molecule that form low-energy compounds below ATP on the scale.

Following figure is a flow sheet of enezymatic phosphate transfer reaction in the cell.



The ATP-ADP system is the obligatory connecting link between high and low energy phosphate compounds. Phosphate groups are first transferred by action of specific phosphotransferase from high energy compounds to ADP as in the following example :

$$\text{Phosphoenol pyruvate} + \text{ADP} \xrightarrow{\text{Pyruvate Kinase}} \text{Pyruvate} + \text{ATP}$$

(1)

The ATP thus formed then becomes the specific phosphate donor in a second enzymatic reaction to form a low-energy phosphate compound.

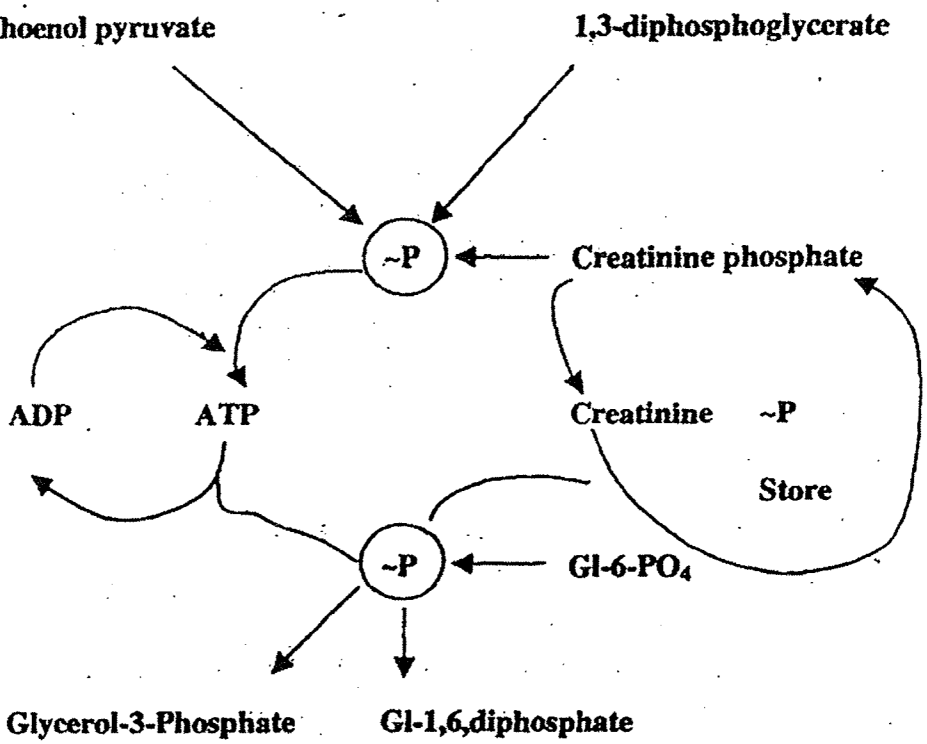
(2) $\text{ATP} + \text{D-glucose} \rightarrow \text{ADP} + \text{G1-6-PO}_4$

The Overall Reaction :

$$\text{Phosphoenolpyruvate} + \text{D-glucose} \rightarrow \text{Pyruvate} + \text{G1-6-PO}_4$$

The net result being the transfer of a phosphate group from a high energy donor to a low energy acceptor through ATP-ADP system.

Role of ATP/ADP cycle in transfer of high-energy phosphate :



SUGGESTED QUESTIONS :

Thermodynamics :

Q1. (a) In a chemical reaction how does the free energy change (ΔG) expressed?

(b) What is standard free energy change (ΔG°)? How does it expressed?

(c) (i) Enzyme phosphoglucomutase catalyses interconversion of 2-phosphoglycerate to 3-phosphoglycerate in glycolytic metabolism at 25°C. The K_{eq} is found to be 6.0. Calculate the standard free energy change [Given R = 1.98 Cal.mole^{-1}deg^{-1}, $\log_{10}6=0.778$]

(ii) Now if at any instant (before equilibrium is reached) the concentration of 2-phosphoglycerate = 1×10^{-4} M and the concentration of 2-phosphoglycerate = 5×10^{-4}M, what is the fee energy change? at 25°C [Give $\log_{10}5=0.70$]

Ans. (i) $\Delta G^\circ = -RT \ln K_{eq} = -1363 \log 6.0 = -1363 \times 0.778 = -1060$ Cal.mole^{-1}

(ii) $\Delta G = (-1060) + RT \ln \frac{5\times10^{-4}}{1\times10^{-4}}$

$= (-1060) + 1363 \times \log_{10} 5 = -1060 + 954.1 = -105.9$ Cal.mole^{-1}

Q2. Find ΔG° at 25°C of the following reaction :

$$\text{Glutamate} + \text{Oxaloacetate} \xrightarrow{\text{Transminase}} \text{asparatate} + \alpha\text{-ketolgutarate}$$

If $K_{eq} = 6.8$ (log 6.8 = 0.835)

Ans. $\Delta G^\circ = -1363 \times \log 6.8 = -1363 \times 0.835 = -1138.1$ Cal.mole^{-1}

Q3. (a) 'All living matter of universe constitute an open, non-equilibrium system and maintain a steady state'– explain.

(b) Staet 2nd Law of Thermodynamics. What is entropy?

Q4. ΔG° For a given chemical reaction can be calculated from the standard free energy of formation (ΔG°_f) of the reactants and products. Calculate the ΔG° value for the alcoholic fermentation of glucose (D-glucose $\rightarrow$ 2-ethanol + $2CO_2$) from standard table on the standard free energy of formation (given below)

	ΔG°_f
D-glucose ⟶	–219.22
Carbondioxide	– 94.45
Ethanol	– 43.39

Ans. $\Delta G^\circ = \Sigma \Delta G^\circ_{f\,products} - \Sigma \Delta G^\circ_{f\,reactants}$

$\Delta G^\circ = \{-(43.39 \times 2) + (-94.45 \times 2)\} - (-219.22)\}$

$= \{(-86.78) + (-188.9)\} - (-219.22)$

$= -275.68 - (-21922) = -56.46$ Kcal.mole^{-1}

Q5. If $1x10^{-1}$M solution of glucose-1-phosphate is incubated with catalytic amount of phosphoglucomutase enzyme, Glucose-1-Phosphate is converted to Glucose-6-Phosphate. At equilibrium concentrations of Glucose-1-Phosphate and Glucose-6-Phosphate are $4.5x10^{-3}$ M and 9.6×10^{-2} M respectively. Calculate K_{eq} and ΔG° at 25°C. Is it a spontaneous reaction? (log 21.3 = 1.328)

Ans. $K_{eq} = \frac{9.6\times10^{-2}}{4.5\times10^{-3}} = 21.3$

$\Delta G^{\circ} = -1363 \times \log 21.3 = -1363 \times 1.328 = -1800$ Cal.mole

Key words :

System, surrounding, universe, energy flow; open, closed as isolated systems, initial and final state, equilibrium, enthalpy, entropy, irreversible process, Gibbs free energy, Equilibrium constant (K_{eq}), Biological system – steady state, phosphate pool and energy cycle.

Select Readings :

(1) Lehninger's Principle of Biochemistry (3rd ed).

(2) Harper's Illustrated Biochemistry (26th ed).

(3) Biophysics and Biophysical Chemistry of Debajyoti Das (4th ed).

M.Sc. in Zoology Part–I

Paper III : Group–B :Module-24A : Unit –I

Biochemistry

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1. ENZYME

1.1 Enzyme Kinetics is the field of biochemistry concerned with the quantitative measurement of the rates of enzyme-catalyzed reactions and systemic study of factors that affect these raes. A key factor affecting the rate of a reaction catalyzed by a purified enzyme in vitro is the concentration of substrate, [S]. However, studying the effects of substrate concentration is complicated by the fact that [S] changes during the course of a reaction as substrate is converted to product. One simplifying approach in kinetics experiments is to measure the initial rate (or initial velocity), designated v_0, when [S] is generally much greater than the concentration of enzyme, [E]. Then, if the time is sufficiently short following the start of a reaction, changs in [S] are negligible and [S] can be regarded as a constant.

The effect on v_0 of varying [S] when the enzyme concentration is held constant is shown in Fig. 1. At relatively low concentrations of substrate, v_0 inceases almost linearly with an increase in [S]. At higher substrate concentrations, v_0 increases by smaller and smaller amount in response to increase in [S]. Finally a point is reached beyond which increases in v_0 are vanishingly small as [S] increases. The plateau-like v_0 region is close to the *maximum velocity*, V_{max}. The shape of the resulting graph when v_0 is plotted against [S] is called a hyperbolic curve (Fig.1).

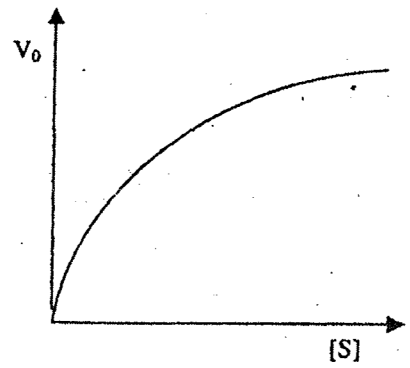


Fig. 1 : The relationship between [S] and initial reaction velocity v_0

The ES complex is the key to understanding this kinetic behaviour. Combination of an enzyme with its substrate molecule to form an ES complex is a necessary step in enzyme catalysis. This idea was expanded into a theory of enzyme action by *Michaelis and Menten.*

1.2 Michaelis and Menten Equation :

They postulated that the enzyme first combines reversibly with its substrate to form an enzyme–substrate complex, and then breaks down in a slower second step to yield the free enzyme and the reaction product P.

$$E + S \underset{k-1}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

the rate constants k_1, k_2 and k_{-1} describe the rates associated with each step of the catalytic process. Because the slower second reaction must limit the rate of the overall reaction, the overall rate must be proportional to the concentration of the species that reacts in the second step, that is, E.S. (ES) remains approximately constant until nearly all the substrate is used, that is, [ES] maintains a steady state. From the observation of the properties of many enzymes it was known that the initial velocity v_0 at low substrate concentrations is directly proportional to [S], while at high substrate concentrations the velocity tends towards a maximum value, that is the rate becomes dependent of S (V_{max}) Michaelis and Menten derived an equation to describe these observations, the Michaelis–Menten equation :

$$v_0 = \frac{V_{max}[S]}{Km + [S]}$$

The equation describes a *hyperbolic curve* of the type shown for the experimental data (Fig. 1). In Deriving the equation, Michaelis and Menten defined a new constant, K_m, the Michaelis constant:

$$km = \frac{k_2 + k_{-1}}{k_1}$$

K_m is a measure of the stability of the ES complex, being equal to the sum of the raes of breakdown of ES over its rae of formation. K_m becomes a measure of the affinity of an enzyme for its substrate since its value depends on the relative values of k_1 and k_2+k_{-1} for ES formation and dissociation respectively. A high K_m indicates weak substrate binding (k_2+k_{-1}) predominant over (k_1) and a low k_m indicates strong substrate binding (k_1 predominants over k_3+k_{-1}). Note that K_m has a unit of concentration.

Because V_m(V_{max}) is achieved at infinite substrate concentration, it is possible to estimate V_m from a hyperbolic plot (Fig. 2).

1.3 Michaelio Menton Plot, a hyperbola

An important numerical relationship emerges from the Michaclis – Menten equation in the special case when v_0 is exactly half of V_m, then

$$v_0 = \frac{V_m}{2} - \frac{V_m[S]}{K_m[S]}$$

On dividing by V_m, we get

$$\frac{1}{2} = \frac{[S]}{K_m + [S]}$$

Solving for k_m, K_{m+1}+[S]=2 [S] or

$$K_m = [S] \; at \; v_0 = \frac{V_m}{2}.$$

K_m is the substrate concentration at half maximum velocity.

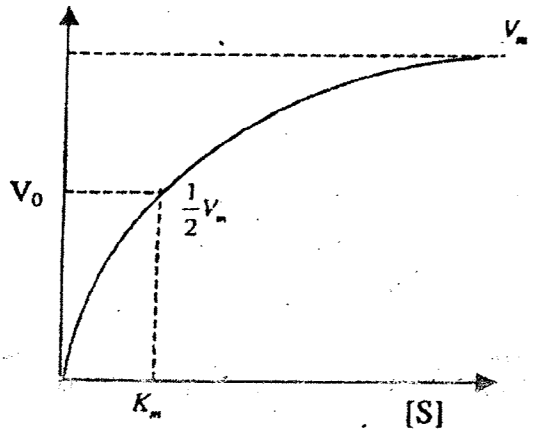


Fig. 2 : Dependence of initial velocity (v_0) on substrate concentration

1.4 Transformation of the Michaelis-Menlen Equation : The double–reciprocal plot

The Michaclis–Menten equation $v_0 = \frac{V_m[S]}{K_m + [S]}$ can be algebraically transformed into equation that is more useful in plotting experimental data. One such transformation is derived simply by taking the receiprocal of both sides of Michaclis-Menten equation:

$$\frac{1}{v_0} = \frac{K_m + [S]}{V_m + [S]}$$

Separating the components of the numerator on the right side of the quation.

$$\frac{1}{v_0} = \frac{K_m}{V_m[S]} + \frac{1[S]}{V_m[S]} = \frac{K_m}{V_m}\frac{1}{[S]} + \frac{1}{V_m}$$

This form of the Michaclis–Menten equation is called the Lineweaver-Burk equation. A plot of $\frac{1}{v_0}$ versus $\frac{1}{[S]}$ (the double reciprocal plot) yields a straight line (Fig. 3). This line has a slope of K_m/V_m, an intercept of $-1/K_m$ on the 1/[S] axis. This double-reciprocal presentation, also called a Lineweaver-Burk Plot, has a great advantage of allowing a more accurate determination of V_m which can only be approximated from a simple plot of v_0 versus [S] (Fig. 2).

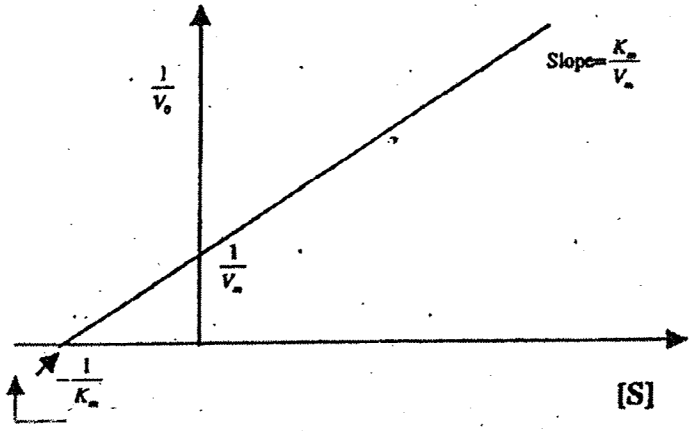


Fig.-3 : A double receiprocal or Lineweaver – Burk Plot

3.1 Effect of Temperature on Enzyme Activity

Temperature affects the rate of enzyme-catalyzed reactions in two ways. First, a rise in temperature increases the thermal energy of the substrate molecules. This increases the rate of the reaction. Howver, a second affect comes into play a higher temperatures. Increasing the thermal energy of the molecule which make up the protein structure of the enzyme itself will increase the chances of breaking the multiple weak, noncovalent interactions which hold the 3-dimensional structure

of the enzyme together ultimately this will lead to the *denaturation* of the enzyme. A graph of temperature plotted against v_0 will show a curve with a well defined temperature optima (Fig.4a). For many mammalian enzymes this is around 37°C.

2.1 Effect of pH on Enzyme Activity

Each enzyme has an optimum pH at which the rate of the reaction that it catalyzes is maximum small deviations in pH from the optimum value lead to decreased activity due to changes in the ionization groups at the active site of the enzyme larger deviations in pH lead to the *denaturation* of enzyme protein itself. A graph of v_0 plotted against pH will usually give a bell-shaped curve (Fig. 4b). Many enzymes have a pH optima of around 6.8, but ther is great diversity in the pH optima of the enzymes, due to the different environments in which they are adapted to work. For example digestive enzyme *pepsin* is adapted to work at the acidic pH of the stomach (around pH 2.0)

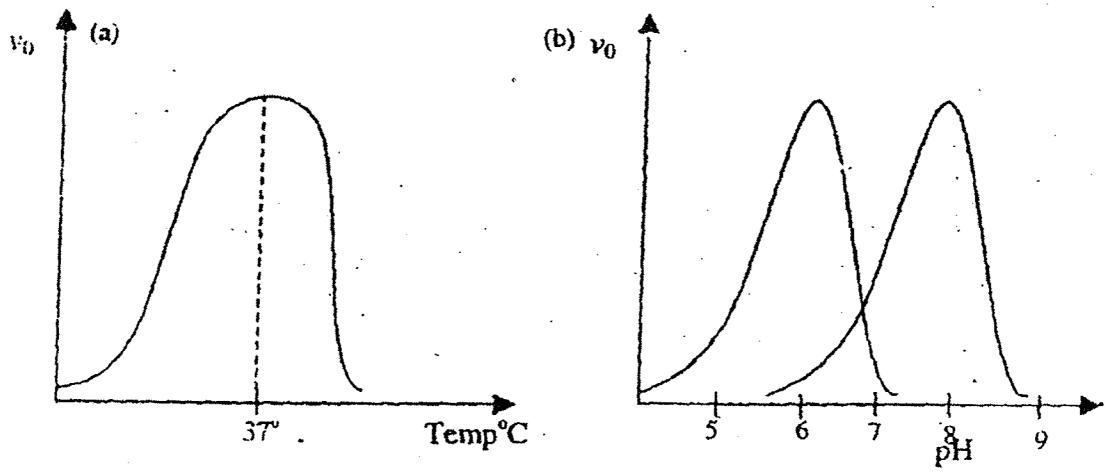


Fig. 4 : The effect of (a) temperature and (b) pH on enzyme activity.

2.1 Enzyme inhibition

Many types of molecule exist which are capable of interfering with the activity of an individual enzyme. Any molecules which acts directly on an enzyme to lower its catalytic activity is called an ***inhibitor***. Some enzyme inhibitors are normal body metabolites that inhibit a particualar enzyme as part of the normal metabolic control of a pathway. Other inhibitors may be foreign substance such as drugs or toxins, where the effect of enzyme inhibition could be either therapeutic or, at the other

extreme, lethal. Enzyme inhibition may be of two main types : *irreversible or reversible,* with reversible inhibition itself being subdivided into *competitive* and *noncompetitive* inhibition.

2.2 Irreversible inhibition :

Inhibitors which bind irreversibly to an enzyme often form a covalnt bond to an amino acid residue at or near the active site, and permanently inactivate the enzyme. Susceptible amino acid residue include Ser and Cys residue which have reactive –OH or –SH groups respectively. The compound *diisopropylphosphofluoride* (DIPF), a component of nerve gases reacts with Ser residue in the active site of the eznyme *acetylcholinesterase,* irreversibly inhibiting the enzyme. *Iodoacctamide* modifies Cys residues which may react with essential–SH groups of the enzyme forming a co-valent bond and destroys its activity. The antibiotic *penicillin* irreversibly inhibits the glycopeptide-transpeptidase enzyme by covalently attaching to a Ser residue in the active site of the enzyme.

3.3 Reversible Competitive inhibition :

A competitive inhibitor typically has close structural similarities to the normal substrate for the enzyme. Thus it compete with substrate molecules to bind to the active site (Fig. 5a). The enzyme may bind either a substrate molecule or an inhibiotor molecule, but not both at the same time. The competitive inhibition binds reversibly to the active site. At high subsrate concentrations the action of a competitive inhibitor is overcome because a sufficiently high substrate concentration will successfully compete out the inhibitor molecule in biding to the active site. Thus there is no change in the V_m of the enzyme but the apparent affinity of the enzyme for its substrate decreases in the presence of the competitive inhibitor, and hence k_m increases. A Lineweaver–Burk plot shows the effect of a competitive inhibitor on V_m and K_m (Fig. 5c).

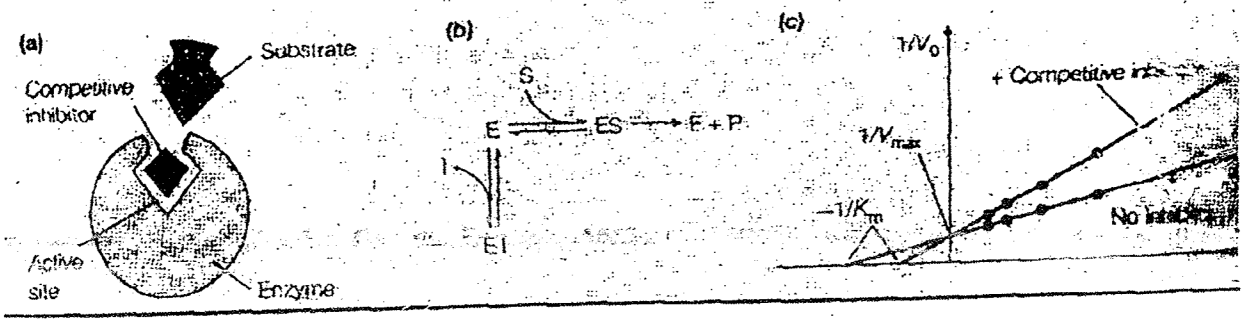


Fig. 5 : The characteristics of competitive inhibition

A good example of competitive inhibition is provided by *succinate ehydrogenase*. This enzyme uses succinate as its substrate and is competitively inhibited by *malonate* which differs from succinate in having one rather then two methylene groups (Fig. 6).

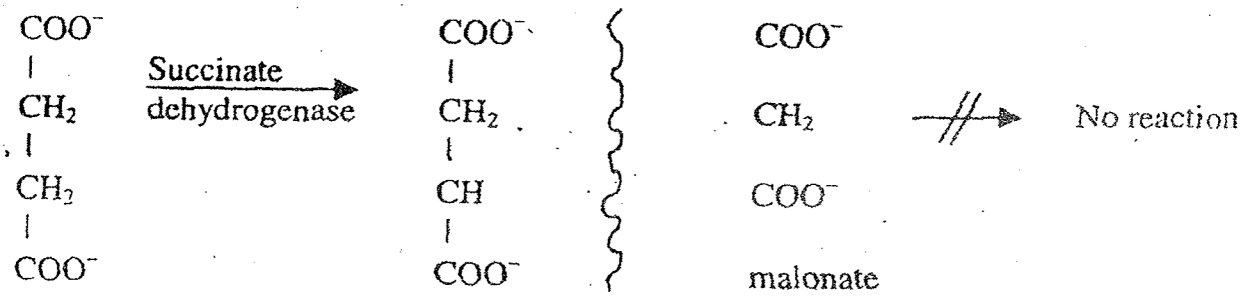


Fig.6: Inhibition of succinate dehdrogenase by malonate.

A competitive inhibition can be recognized by using Lineweaver – Burk Plot, v_0 is measured at different substrate concentrations in the presence of a fixed concentration of inhibitor. A competitive inhibitor increases the slope of the line and alters the intercepts on the x-axis (Since K_m is increased) but leaves the intercept on the y-axis unchanged (since v_m remains constant).

3.4 Reversible noncompetitive inhibition :

A noncompetitive inhibition binds reversibly at a site other than the active site (Fig. 7a) and causes a change in the overall three-dimensional shape of the enzyme that leads to a decrease in catalytic activity. Since the inhibitor binds at a different site to the substrate, the enzyme may bind the inhibitor, the substrate or both together. The effects of a non-competitive inhibitor cannot be overcome by increasing the substrate concentration, so there is a decrease in V_m. In noncompetitive inhibition the affinity of the enzyme for the substrate is unchanged and so K_m remains the same. Noncompetitive inhibition can be recognized on a Lineweaver – Burk pot since it increases the slope of the line and alters the intercept on the y-axis (since V_m is decreased), but leaves the intercept on the x-axis changed (since K_m remains constan) (Fig. 7c).

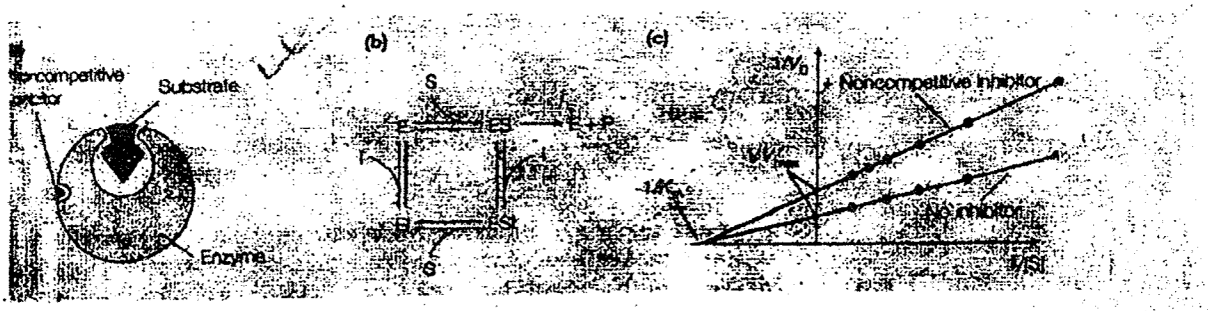


Fig. 7 : Characteristics of non-competitive inhibition.

An example of non-competitive inhibition is the action of *Pepstatin* on the enzyme *rennin.*

4.1 Active Site of an Enzyme :

The distinguishing feature of an enzyme catalyzed reaction is that it occurs within the confines of a pocket on the enzyme called the *active site.* The substrate molecule binds at the active site and acted upon by the enzyme. The active site has one or more amino acid residues whose substituent groups bind the substrate and catalyze its chemical transformation.

Koshland postulated that a major function of the active site of an enzyme is to produce orbital steering i.e., precise orientation of he substrate and the catalytic group of the enzyme with respect to each other. This simultaneously aligns portions of the substrate that will undergo change with the chemcial functional groups of peptidyl aminoacyl residues. Many amino acyl residues drawn from diverse portions of the polypeeptide chain contribute to the extensive size and three-dimensional character of the active site.

4.2 Enzymes employ multiple mechanisms to facilitate cataysis :

Four general mechanisms account for the ability of enzymes to achieve dramatic catalytic enhancement of the rates of chemical reactions.

Catalysis of Proximity :

For molecules to react, they must come within bond – forming distance of one another. When an enzyme binds substrate molecules in its active site, it creates a region of high local substrate concentration. This environment also orients the substrate molecular spatially in a position ideal for them to interact, resulting in rate enhancements of at least a thousand fold.

Acid-Base Catalysis

The ionizable functional groups of aminoacyl side chains and (where present) of prosthetic groups contribute to catalysis by acting as acids or bases.

Catalysis by Strain

Enzymes that catalyze lytic reactions which involve breaking a covalent bond typically bind their substrates in a conformation slightly unfavourable for the bond that will undergo cleavage. The resulting straing stretches or distorts the targeted bond, weakening it and making it more vulnerable to cleavage.

Covalent Catalysis

The process of covalent catalysis involves the formation of a covalent bond between the enzyme and the substrate. The modified enzyme then becomes a reactant. Covalent catalysis introduces a new reaction pathway that is energetically more favourable–and therefore faster–than the reaction pathway in homogenous solution. The chemical modification of the enzyme is, however, transient. On completion of the reaction, the enzyme returns to its original unmodified state. Its role thus remains catalytic.

4.3 Illustration of reaction mechanism for one enzyme, Chymotrypsin :

Chymotrypsin (MW 25,000) is a protease, an enzyme that catalyzes the hydrolytic cleavage of peptide bonds. These enzymes cannot be synthesized at the ribosome as such, as they are self destructive. They are synthesized as inactive precursor – Chymotrypsinogen which are converted to active enzyme Chymotrypsin just beofre use (Fig. 8).

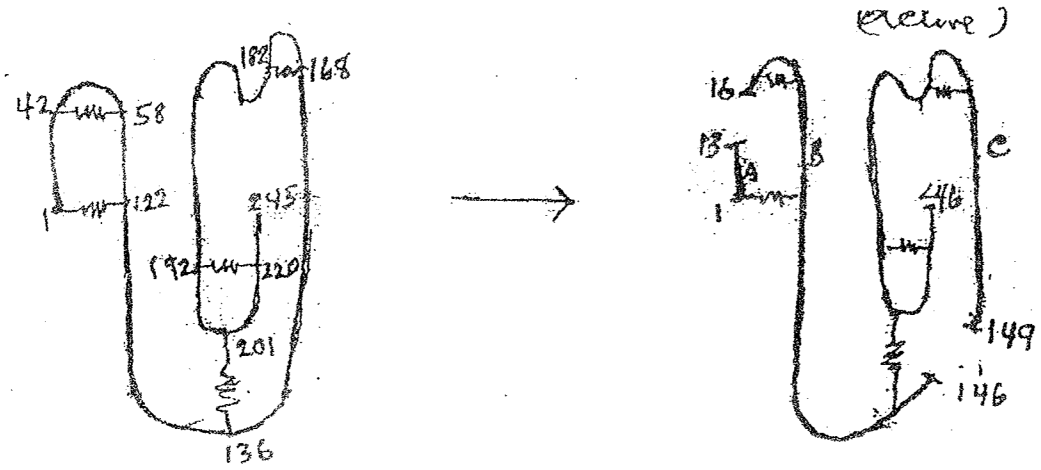
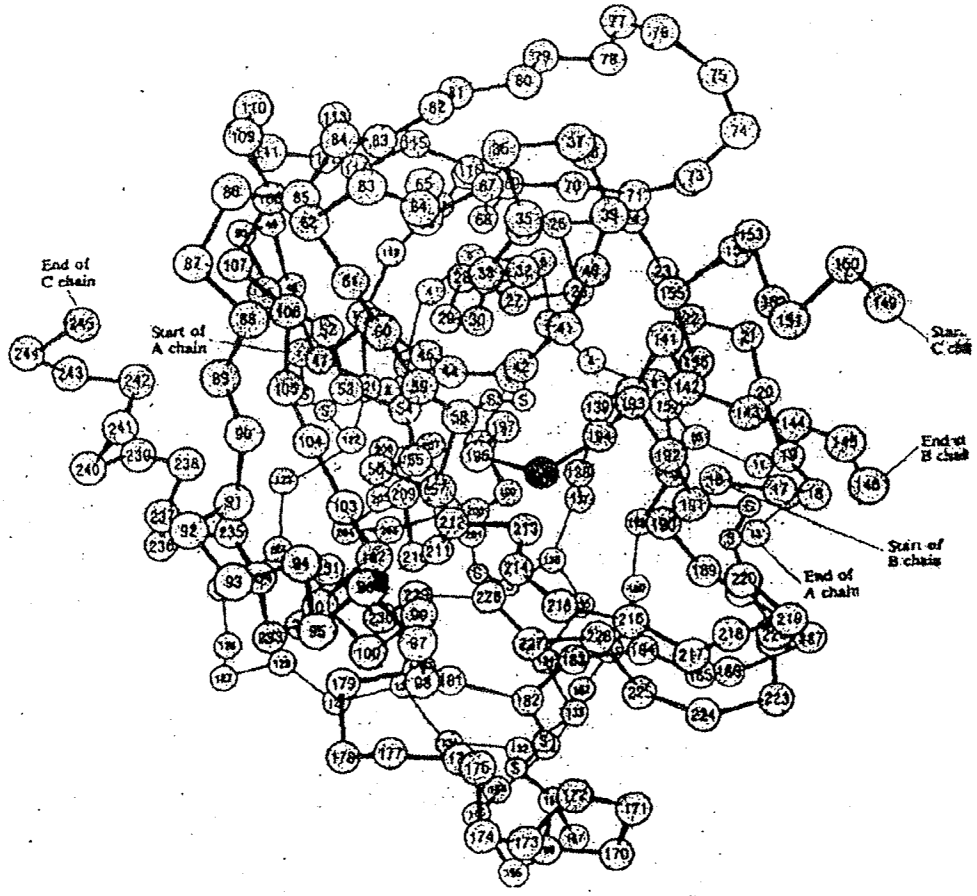


Fig. 8 : Chymotrypsinogen is a single polypeptide chain of 245 amino acid residues held by 5 S-S bonds. It is converted to active chymotrypsin by trypsin with a release of 3-polypeptide chains (A,B,C) held by 2 S-S- bonds.

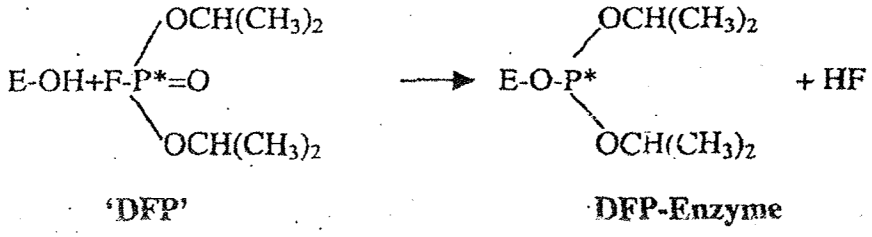
4.4 3-dimensional structure of Cymotrypsin (Fig. 9)

X-Ray crystallography method is used to prepare a space-filling model of chymorypsin. The three polypeptide chains A, B, C folds back into a spherical shape. Also three key amino acids of the enzyme, serine–195, Asp-102 and His-57, come together at the active-site of the enzyme these three amino acids were not in close contact in the primary structure. Only after 3-dimensional structure is formed they come in close vicinity to form the active site, known as *Catalytic triad*. These three amino acids take part in breaking the peptide bond of protein (Substrate) by acid-base catalysis.



4.5 Chemical modification at the active site :

Diisopropyl flurophosphate (DFP*) binds with the –OH group of Ser-195 residue of chymotrypsin enzyme. The reaction is as follows:



The Ser-195 residue forms a complex with DFP and is no longer active.

5.1 Regulatory Enzyme

In biological system the rates of many enzymes are altered by the presence of other molecules such as activators and inhibitors, collectively known as *effectors.* Such specialized forms as caleld regulatory enzyme. Regulatory enzymes are generally oligomers (more than one subunits) and have high molecular weight. In some cases the regulatory site (s) and the active site are on separate subunits.

There are *two* major classes of regulatory enzymes in metabolic pathways.

Allosteric enzyme function through reversible, noncovalent binding of regulatory compounds called *allosteric modulators.*

Other enzymes are regulated by *reversible co-valent* modification.

5.2 Allosteric enzyme :

Allosteric enzyme's catalytic activity is modulated through the non-covalent binding of a specific metabolite at a *site other than* the catalytic site. Allospteric enzyme nay be 'stimulated' or 'inhibited' by their modulator. An activator is often the substrate itself, regulatory enzyme. For which substrate and modulator are identical are called *homotropic.* Binding of the sustrate causes conformational changes that affect the subsequent activity of other site, on the protein. When the modulator is a molecule other than the substrate, the enzyme is said to be *heterotropic.*

5.3 Feedback inhibition (Heterotropic allosteric inibition) :

In some multienzyme sysems, the regulatory enzyme is specifically inhibited by the *end product* of he pathway whenever the concentration of the end product exceeds the cell's requirement. This type of regulation is called *feedback inibition.* Buildup of the pathway' end product ultimately slows the entire pathway. One of the example of allosteric feedback inhibition is the enzyme system that catalyzes the conversion of L-threonine to L-isoleucine in Five steps (Fig. 10). In this system, the first enzyme, *threonine, dehydratase,* is inhibited by isoleucine, the product of the last reaction

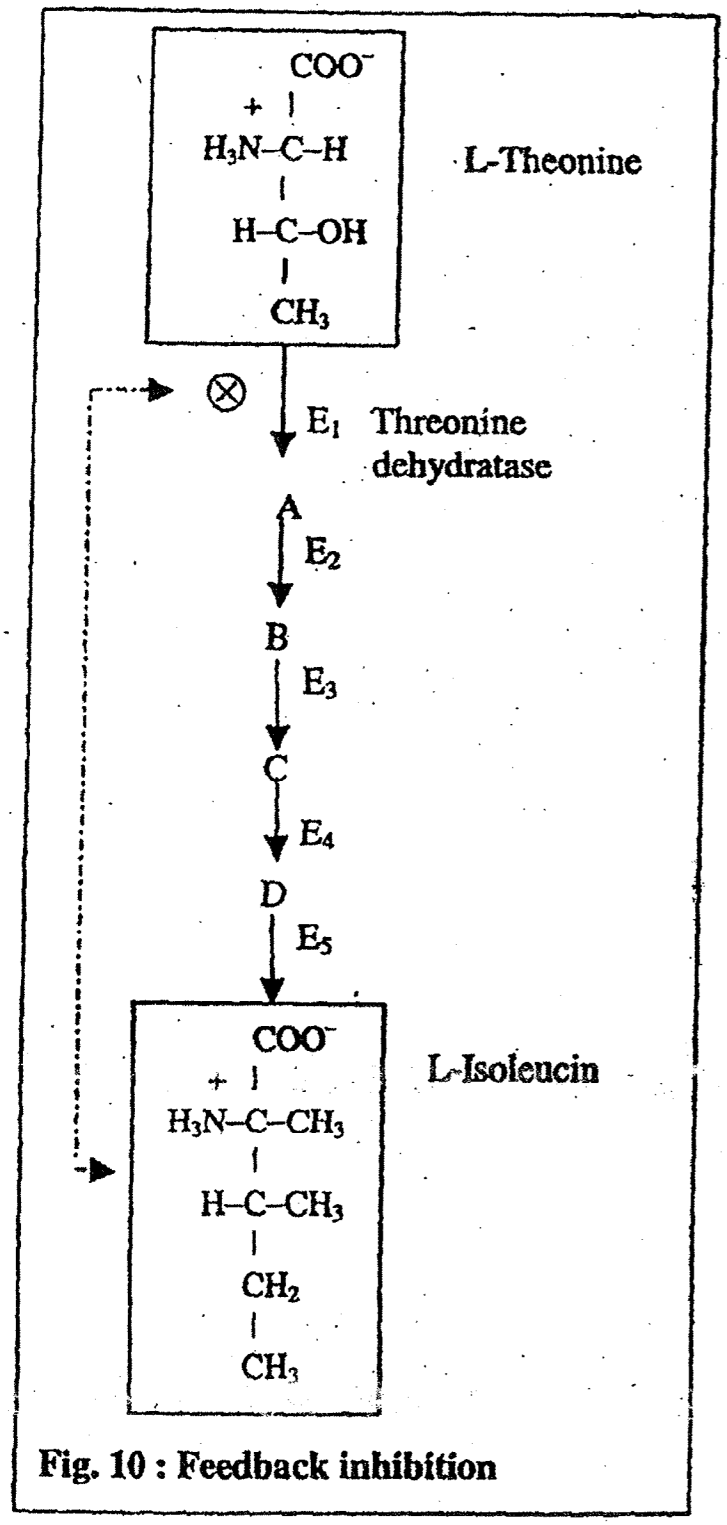


Fig. 10 : Feedback inhibition

of the series. This is an example of heterotropic allosteric inhibitor. Isoleucine is quite specific as an inhibitor. No other intermediate in this sequence inibits threonine dehydratase, nor is any other enzyme in the sequence inhibited by isoleucine. Isoleucine binds not to the active site but to another site, the regulatory site.

5.4 The Kinetic Properties of Alosteric Enzyme

Allosteric enzymes show relationships between v_0 and [S] that differ from Michaelis – Menten Kinetics. They do exhibit saturation with the substitute when [S] is insufficiently high, but for some allosteric enzymes, when v_0 is plotted against [S] a sigmoid saturation curve results rather than the hyperbolic curve (Fig. 11). Although we can find a value of [S] on the sigmoid saturation curve at which v_0 is half maximal, we cannoo refer to it with the designation K_m because the enzyme does not follow the hyperbolic Michaelis-Menten relationship. Instead the symbol $K_{0.5}$ is often used to represent half maximal velocity of the reaction catalyzed by an allosteric enzyme.

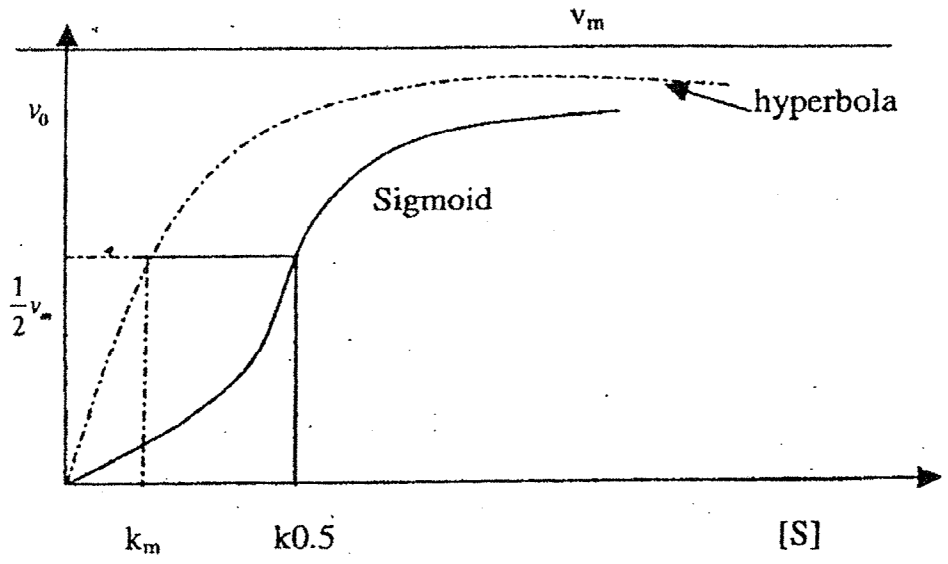


Fig. 11 : Kinetics for allosteric enzyme

Sigmoid kinetic behaviour generally reflects co-operative interactions between multiple protein subunits. In otherwords, changes in the structure of one subunit are translated into structural changes in other. For homotropic allosteric enzyme's positive modulator the binding of one substrate molecule to binding site alerts the enzyme's conformation and ehance the binding of subsequent substrate molecules. This accounts for the sigmoid rather than hyperbolic increase in v_0 with increasing [S]. One characteristic of sigmoid kinetics is that small changes in the concentration of a modulator can be associated with large change in activity.

5.5 An allosteric enzyme may have both positive and negative modulator

The best understood allosteric enzyme with both positive and negative modulator is the *Aspartate Transcarbomoylase (ATCase) enzyme,* the catalyst for the first reaction unique to pyrimidine biosynthesis.

ATCase catalyses the formation of N-Carbamyl-aspartate from aspartate and carbamyl phosphate.

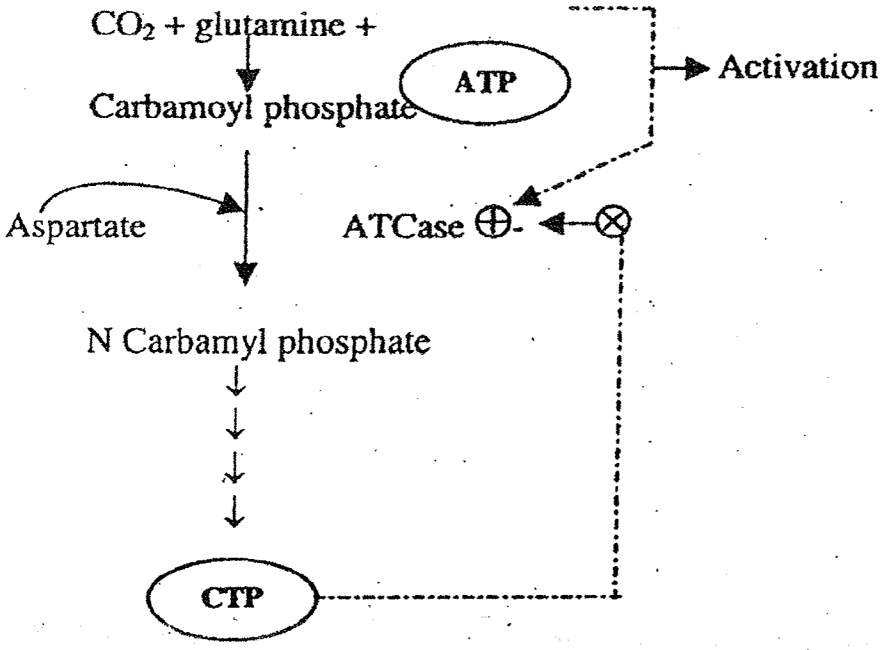


Fig. 12 : Steps in pyrimidine biosynthesis and the control points

CTP, the end product of this biosynthesis sequence is the specific negative modulator of ATCase also has a positive modulator, ATP, which reverse the effect of CTP, acts as an allosteric activator (Fig. 12).

Fig. 13 shows the effect of aspartate concentration on the activity of ATCase alone and in the presence of its modulations ATP and CTP. CTP increases the apparent k_m whereas ATP decrease it.

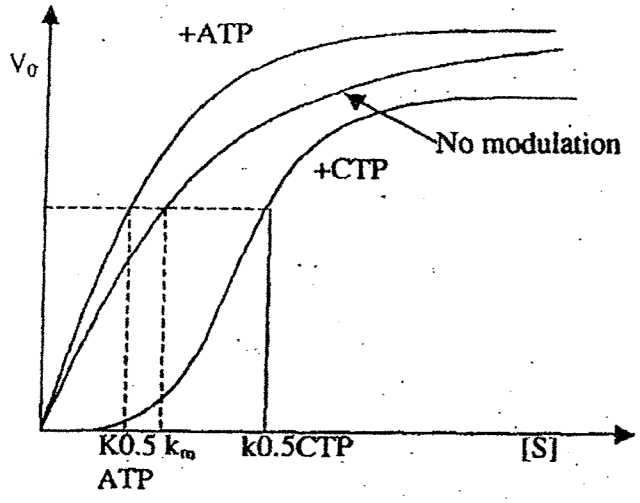


Fig. 13: Plot of v_0 against [S] for allosteric enzyme ATCase with positive and negative modulators.

5.6 Reversible covalent modification of regulatory enzyme:

In the second class of regulatory enzyme the active and inactive forms are interconverted by covalent modification of their structure that are catalyzed by other enzymes.

The classical example of this type of regulatory enzyme is *Glycogen Phosphorylase* which catalyzes the breakdown of glycogen to yield glucose-I-phosphate. This enzyme occurs in two forms, phosphorylase a-the more active form and phosphorylase b-the less active form.

Phosphorylase a is an oligomeric protein with four major subunit. Each subunit contains a serine residue that is phosphorylated at the hydroxyl group; these phosphate groups are required for catalytic activity the phosphate groups in phosphorylase a can be hydrolytically removed by he enzyme *phosphorylase phosphatase* enzyme to form phosphorylase b, the inactive form. Phosphorylase b, the inactive form. Phosphorylase thus is modulated reversibly by trnasfer of phosphate group from ATP to the enzyme by the enzyme *phosphorylase Kinase* to yield active phosphorylase-a (Fig. 14).

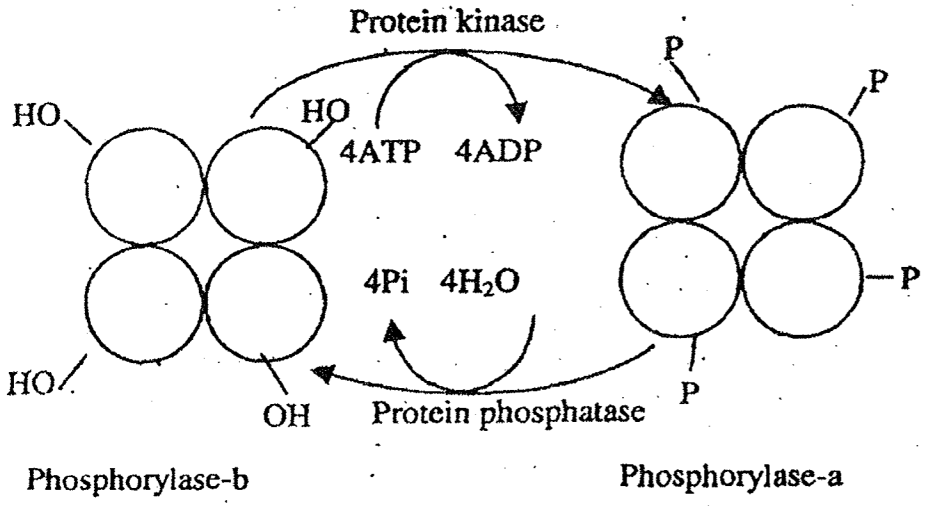


Fig.14: The reversible phosphorylation and dephosphorylation of phosphory

Suggested Questions

Enzyme

Q1. (a) Write down the Michaelis – Menton Equation for enzyme kinetics. Define all terms.

(b) Discuss the following conditions when

(i) $[S] = K_m$

(ii) $[S] >> K_m$

(iii) $[S] << K_m$

(c) Write on the significance of K_m.

Q2. (a) Write on the importance of doubel reciprocal plot (Lineweaver–Burke Plot) over Michales Menten Plot.

(b) What are the two main types of enzyme inhibition? How can you differentiate the two types with the help of Kinetic analysis?

Q3. (a) Discuss non-covalently regulatory enzyme with suitable example from

(i) Positive homotropic co-operative binding.

(ii) End product inhibition.

(b) Glycogen phosphorylase enzyme of animal tissue is co-valently modulated regulatory enzyme,-discuss.

Q.4. Discuss 'active site' of an enzyme with the help of an example (use following points)

(i) 3D structure of the eznyme.

(ii) Amino acid residues at the active site.

(iii) Reaction at the active site (ES complex).

Key words :

Enzyme kinetics, initial velocity, maximum velocity, hyperbolic plot, Michaelis and Menten equation and plot, K_m, Double reciprocal plot, effect of temperature and pH, enzyme inhibition, irreversible and reversible, competitive and non-competitive inhibition, Active site, Acid base catalysis, Covalent catalysis, Chymotrypsin, chemical modification at the active site, Regulatory enzyme, Allosteric enzyme, feedback inhibition, ACTase enzyme, reversible covalent modification.

Select Readings :

(1) Lehninger's Principle of Biochemistry (3rd edn.).

(2) Instant Notes, Biochemistry 92nd end.) by B.D. Hames and N.M. Hooper.

(3) Discovering Enzymes by D. Dressler and H. Potter.

10. Biological Oxidation

Redox potential, mitochondrial electron shuttle, mitochondrial respiratory chain, redox loop model and mechanistic model, oxidative phosphorylation.

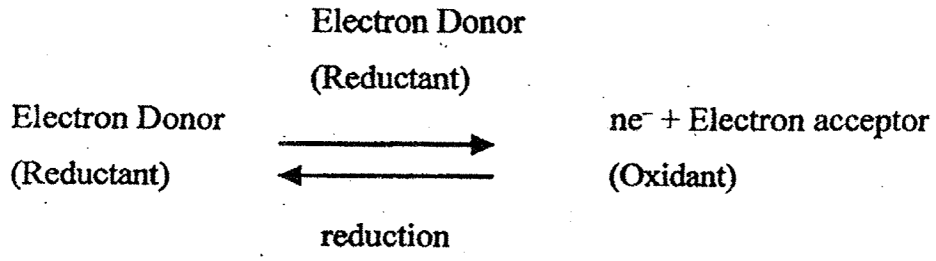
1. **Introduction** **pp-55**
   - 1.1 Oxiation – Reduction reaction
   - 1.2 Redox Pair
   - 1.3 Redox Potential
   - 1.4 Standard Redox Potential
   - 1.5 Electrical Work
   - 1.6 Gibb's free energy
2. **Electron Transport and Oxidative Phosphorylation** **pp-57**
   - 2.1 **Electron transport (ET)**
   - 2.2 Components of electron transport
   - 2.3 The pathways of ET
   - 2.4 Four ET Complexes
   - 2.5 Evidence
   - 2.6 Approximate sites for ATP synthesis
   - 2.7 P/O Ratio
   - 2.8 **Oxidative Phosphorylation**
   - 2.9 **F_0F_1 ATP Synthase**
3. **Summary**

10. BIOLOGICAL OXIDATION

1. INTRODUCTION

1.1 Oxidation-Reduction Reaction

Oxidation-Reduction reactions are those in which there are transfer of electron from an electron donor to an electron acceptor. *Oxidation* is loss of electron and *reduction* is gain of electron. Electron donor is a reducing agent or reductant and electron acceptor is an oxidizing agent or oxidant.



In some oxidation-reduction reaction the transfer of electron is made via the transfer the hydrogen atom, thus dehydrogenation is equivalent to oxidation.

1.2 Redox Pair

Oxidising agent and reducing agnets function as conjugate redox pair.

Example : In a Daniell Cell, two electrodes, one Zn and other Cu dipped into zinc sulphate and copper sulphate respectively, are connected by a voltmeter. The zinc sulphate contain Zn^{++} and in equilibrium : $Zn^{++} + 2e \rightleftharpoons Zn$, where e is the electron. A definite electron pressure is reached.

Similarly a definite electron pressure is also reached at the Cu-end $Cu^{++} + 2e \rightleftharpoons Cu$ (Fig. 1). Since the electron pressure on the Zn-electrode is higher than that of the Cu-electron, a steady stream of electrons flow from Zn to Cu. Here Zn is reductant and Cu is oxidant.

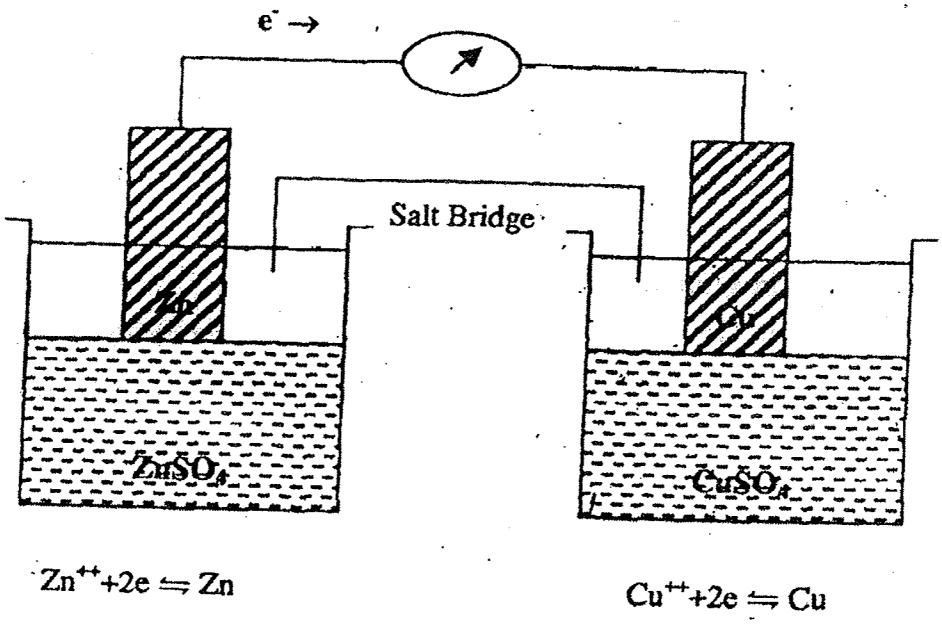
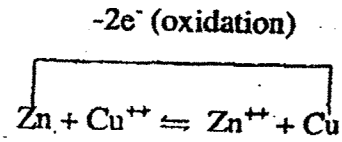


Fig. 1 : Daniell Cell.



1.3 Redox Potential

Redox potential gives a measure of the readiness of the substrance to give off electron. If a substance has high negative electrode potential it will be very reactive i.e., ready to give off electron. The intensity of the oxidizing or ruducing action of a redox pair is determined by its *Redox Potential* (E_0') in volt. A reductant can react with any oxidant that is below it in Redox potential table (a table of standard Redox potential is prepared where H^+, H_2 is kept at 0.0 volt at 25°C, pH = 0.0).

In the example of E_0' of Zn = –0.76 volt and E_0' of Cu = +0.35. Thus Zn has higher negative Redox potential than Cu, and electron flows from Zn to Cu. (Zn is electron donor (reductant) and Cu is electron accept or (oxidant)). For a system the tendency of a reducing agent to lose electron is defined as electromotive force (emf) in volt. In case of Zn-Cu cell, the emf can be calculated as

$$\Delta E_0' = E'_{0\,[oxidant]} - E'_{0\,[reductant]}$$

E_0' = +0.35 – (–0.76) volt = +1.11 volt

1.4 Standard Redox potential in biochemical system

When the previous redox potential is corrected to pH 7.0 [H^+=1 x10^{-7} M], the standard reduction potential of H^+, H_2 system becomes –0.42 volt.

1.5 Electrical work in a chemical reaction

Whenever a quantity of electricity is transported across a potential difference, electrical work done equals to the product of the quanity of electricity (Q) and potential difference ($\Delta E_0'$) (W=Q.$\Delta E_0'$). Since 1 Fraraday of electricity F is associated with one chemical equivalent, nF quantity of electricity needs to pass if the cell reaction concerns n chemical equivalent. If emf of the cell is $\Delta E_0'$, the electrical work is nf$\Delta E_0'$.

1.6 Gibbs free energy change of chemical reaction

Thus the electrical work in a chemical reaction that is expressed as Gibbs free energy change can be measured as follows

$$\Delta G^\circ = -nF\Delta E_0'$$

where, n = number of electron tansfer

F = Faraday's constant = 23063 calmole^{-1}volt^{-2}= 96500 Joules mole^{-1} volt^{-1}

$\Delta E_0'$ = Standard difference of emf

$$= E'_{0\,[oxidant]} - E'_{0\,[reductant]}$$

For Zn-Cu reaction $\Delta E_0'$ = +1.11 volt

ΔG° =–2 x 23063 calmole^{-1} x 1.1 volt = –50738.7 calmole^{-1}.

2. ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATION

In eukaryotes, electron transport and oxidative phosphorylation occur in the inner membrane of mitochondria. These processes reoxidize the NADH and $FADH_2$ that arise from the citric acid cycle (located in the mitochondrial matrix), glycolysis (located in the cytoplasm) and fatty acid oxidation (located in the mitochondrial matrix) and trap the energy released as ATP.

2.1 Electron Transport

Pair of electron derived from intermediary metabolism flow down the respiratory chain to molecular oxygen which is the ultimate electron acceptor in respiration. This process is known as electron transfport (ET).

The inner membrane of mitochondria and its involutions, the crystal have shown to be involved in the series of reactions involved in the transfer of electron between NADH and oxygen and coupling of this transfer to ATP formation (Fig. 2).

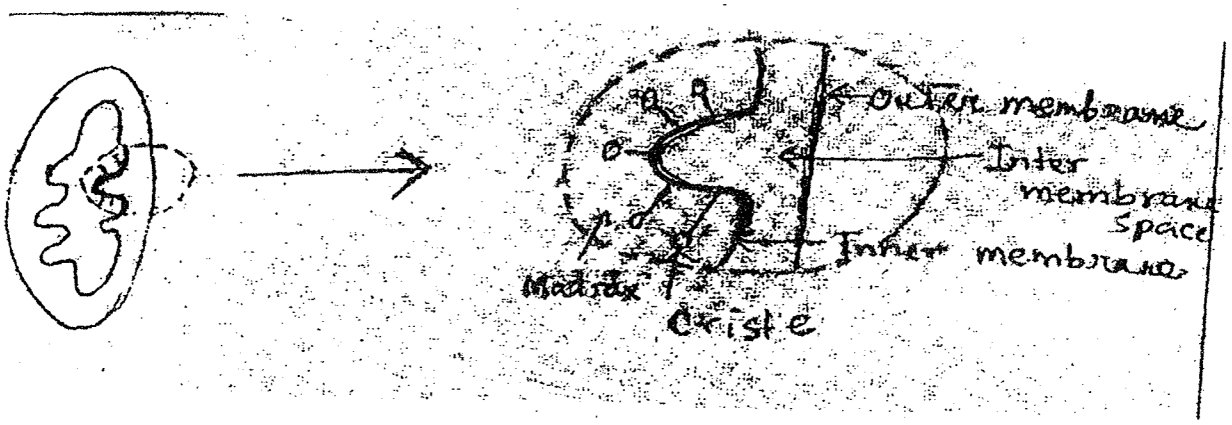


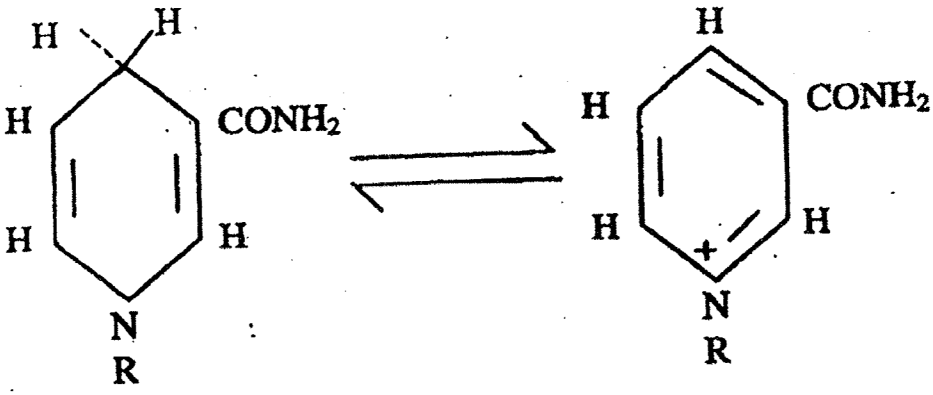
Fig.2 : Mitochondria.

2.2 Components of electron transfport chain

The inner membrane of mitochondria contains the dehydrogenase enzymes, cytochrome systems and enzymes involves in coupling of electron transport to phosphorylation. Inner membrane contains 60 differnt biologically active protein. Oxidation-reduction enzymes (dehydrogenase enzyme and cytochrome) in electron transport chains are embedded in inner membrane in a fixed orientation bound to the membrane and work together hand in hand as a multienzyme complex. The multitude of enzyme that catalyze cellular oxidation, channel electrons from the substrates [arise from the citric acid cycle and glycolysis) into just a few types of universal electron carriers. These are co-enzymes of various dehydraogenase enzymes like NAD^+, $NADP^+$, FMN, FAD, that undergo reversible oxidation and reduction.

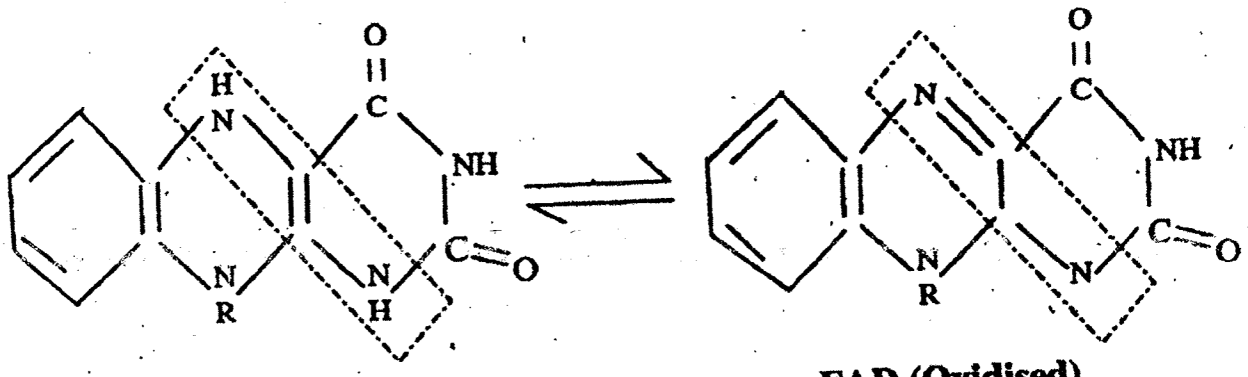
I. (Water soluble) - Two examples :

1. NAD – Nicotonamide Adminine Dinucleotide



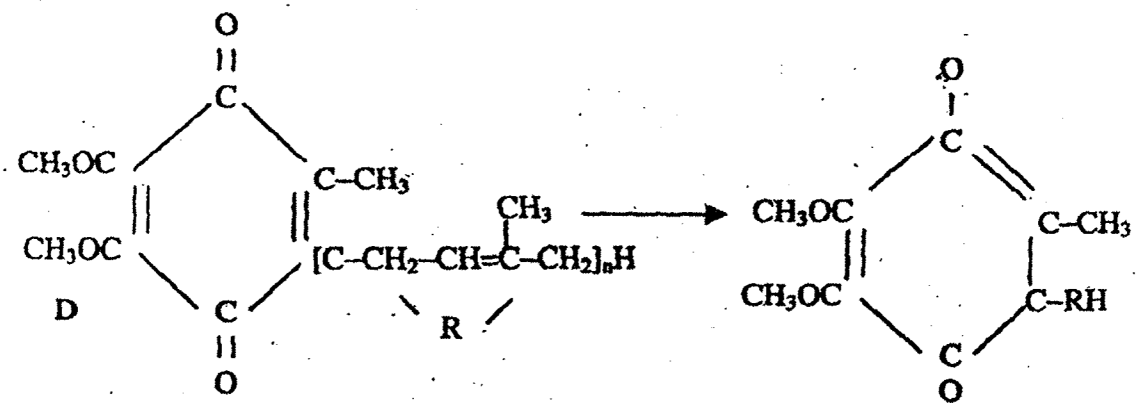
$NADH_2H^+$(Reduced form) **NAD^+ (Oxidised form)**

2. FAD – Flavin Adenine Dinucleotide



$FADH_2$ (Reduced) **FAD (Oxidised)**

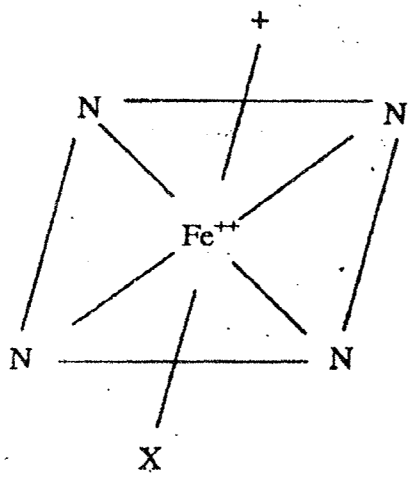
II. Lipid soluble quinones such as ubiquinone act as electron carries in the non-aquous environment of the membrane. This is designated as co-enzyme Q.



Co-enzyme Q (Oxidised form) **QH_2 (Reduced Form)**

III. Cytochromes which have tightly bound prosthetic groups that undergo reversible oxidation-reduction also serve as electron carriers. Some of these proteins are water soluble, but others are peripheral or integral membrane protein. These are an iron-containing protein. They all contain iron porphyrin prosthetic groups resembling hemoglobin and myoglobin in this respect. The cytochrome, undergo reversible

$$Fe^{++} \xrightarrow{e^-} Fe^{+++}$$ valence change during their catalytic cycle.



(Oxidised Form) $\quad Cyt + Fe^{++} \rightleftharpoons Cyt + Fe^{+++}$

IV Irion-Sulfer Protein

2.3 The Pathways of Electron Transport – The Rspiratory Chain (in mitochondria)

The sequence of electron-transfer reaction in the rspiratory chain from subsrate molecule to molecular oxygen is now fairly well established. NADH is the form in which electrons are collected from many different substrate through the NAD-linked dehydrogenase. These electrons funnel into the chain via flavoprotein-linked dehydrogenase. On the otherhand, other respiratory substrate (e.g., succinate) is dehydrogenated directly by flavin-linked dehydrogenase which funnels electron via ubiquinone or coenzyme Q.

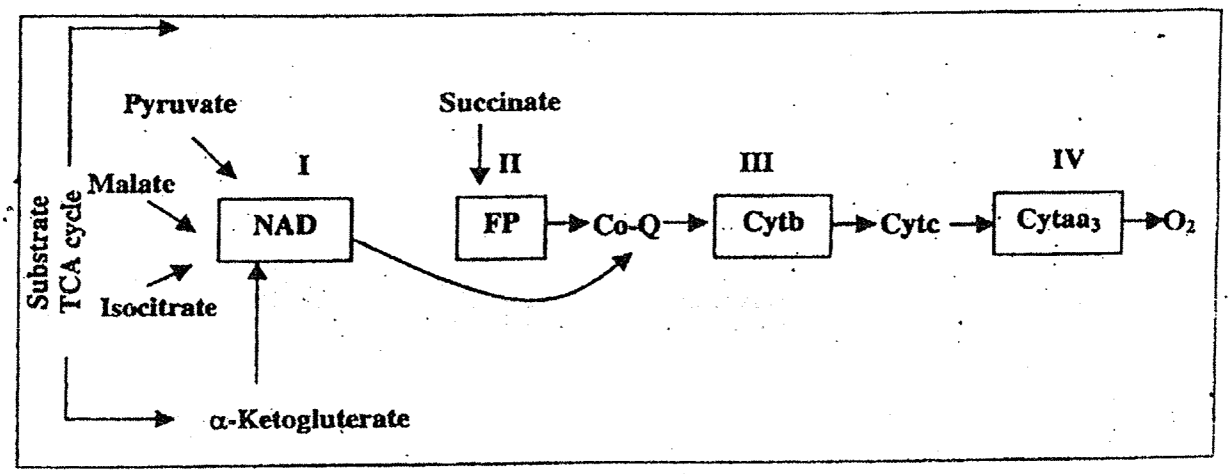
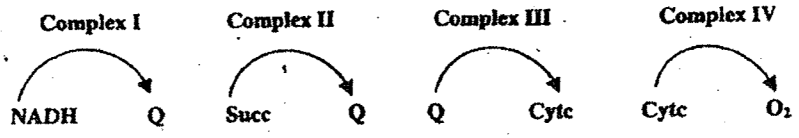


Fig. – 3 The Respiratory Chain

Electrons then flow through cytochromes ($a \rightarrow b \rightarrow c \rightarrow aa_3$) and finally to molecular oxygen. There is increasing evidence that a non-heme iron protein participate in the electron transfer from cytochrome b to cytochrome c (Fig. 3).

2.4 Four electron carrier complexes and electron transport

The electron carriers of the respiratory chain are organized into membrane-embeded supramolecular complexes that can be physically separated. Gentle treatment of the inner membrane of mitochondira with detergents allows resolution of four unique electron carrier complexes each capable of catalyzing electron transfer through a portion of the chain. Complex I and Complex II catalyse transfer to ubiquinone from two different electron donors; complex III carries electrons from ubiquinone to cytochrome c and Complex IV completes the sequence by transferring electrons from cytochrome c to oxygen.



Electrons flow from NADH to oxygen through these complexes (Fig. 4). Each complex contains several electron carriers that work sequentially to carry electron down the chain. Two small electron carriers are also needed to link these large complexes –ubiquin one or co-enzyme Q and cytochrome C.

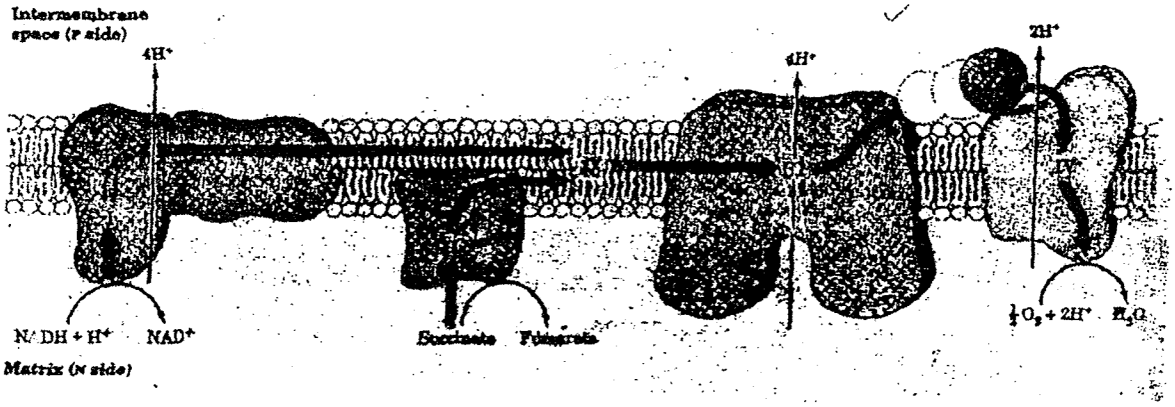


Fig. 4 : Electron Transport Chains in the inner membrane of Mitochondria.

Various Steps in Electron Transport :

Step 1 : NADH to NADH dehydrogenase or Complex I

Complex I binds with NADH and reoxidases it to NAD^+ passing the two electrons to a prosthetic group called FMN to produce $FMNH_2$. The electron then pass to FeS clusters within complex I which changes from Fe^{+++} (ferric) to Fe^{++} (ferrous). As the electron is passed to next electron carrier the iron atom changes back to Fe^{+++} State.

Step-2 : NADH dehydrogenase (Complex I) to ubiquinone (9 Co-Q)

Electrons from FeS cluster are passed onto Co-Q a small lipid soluble molecular in the inner membrane. Thus molecule can act as an electron carrier by accepting upto two electrons and two H^+ ions. In so doing Co-Q is converted to $Co\text{-}QH_2$.

Step-3 : Succinate dehydroganase catalyze the oxidation of succinate to fumerate.

Succinate dehydrogenase contains bound FAD that is reduced to $FADH_2$. The reoxidation of $FADH_2$ occurs in Complex-II, an integral protein of inner membrane. Two electrons pass from $FADH_2$ to Co-Q and enter the main electron transport chain.

Step-4 : Co-Q to cytochrome bc_1 complex or Complex III

when $Co\text{-}HQ_2$ donates two electrons to the next carrier in the chain, the cytochrome bc_1 complex or Complex III, the H^+ are released. The complex bc_1 contains two types of cytochrome,

cytochrome b and cytochrome c_1 as well as FeS protein. Electrons passing from Cyt bc_1 component move to next electron carrier cytochrome C.

Step-5 : Cytochrome c is a peripheral membrane protein that is loosely bound to the outer surface of the inner membrane. Then it binds to complex IV, also known as cytochrome oxidase and donate the electron.

Step-6 : Complex IV contains two cyochrome a and a_3. This transfer electron from Cytc to molecular oxygen.

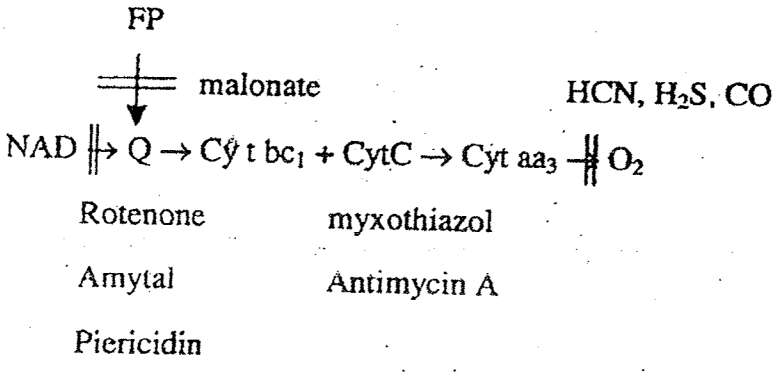
2.5 Evidence on the sequence of electron carrier

Electron Transport Inhibitors

Several inhibitors of specific electron carriers are known and were used in the original studies to determine the order of the components in the respiratory chain.

Three inhibitors have been found to block electron transport in the span between NADH and Co-Q : *rotenone, amytal* and *piericidine*. *Rotenone* is an extremely toxic plant substance used by South American Indian as a fish poison, now used as insecticide. *Amytal,* a barbiturate, *Piericidine* an antibiotic that resembles ubiquinone in strucure. These compounds are believed to act on NADH–dehydrogenase and so prevent NADH oxidation but the oxidation of $FADH_2$ can still occur since this feed electrons into the chain at Co-Q. *Malonate* blocks the path from $FADH_2$ to Co-Q.

Another characteristic inhibitor the antibiotic *antimycin* A (also *myxothiazol*) which blocks electron transport in the span from cytochrome bc_1 to chtochrome C. A third class of inhibitors block electron transport from cytochrome aa_3 to oxygen. It icnludes HCN, H_2S, co, etc.



Further evidence in support of the sequence of Electron transport chain.

All of the electron carriers in the electron Transport chain interact according to their redox potentials. Every time that an electron transfer occurs, the accepting carrier has a higher affinity for electrons than the donating carrier. Thus there is a net flow of electron from NADH (*most negative reodox potential, least affinity for electron*) to O_2 (*most positive redox potential,* highest affinity for electron). This ensures a unidirectional flow of electron in the innermembrane of mitochondria (Table 1).

Table-1

The standard redox potential of a biologically important redox couple in inner membrane

Reductant	Oxidant	Redox Potential E_0' volt
H_2	$2H^+$	–0.420
$NADH + H^+$	NAD^+	–0.320
$FADH_2$	FAD	–0.200
Co-OH_2	Co-Q	+0.100
Cyt.b Fe^{++}	Cyt b Fe^{+++}	+ 0.077
Cyt c_1 reduced	Cyt c_1 oxidised	+ 0.220
Cyt c red	Cyt c_{ox}	+ 0.254
Cyt a red	Cyt a_{oxd}	+ 0.280
Cyt a_3 red	Cyt a_{3oxd}	+ 0.550
H_2O	$^1/_2O_2+2H^+$	+ 0.816

2.6 The probable sites of the three energy delivery stages of the respiratory chain :

These three delivery stages have been predicted from calculation of free energy change cocurring during the transfer of a pair of electron equivalent in each ofthe successive electron transferring steps in the respiratory chain. There are three places in the chain in which relatively large decrease in free energy occurs, each suffcient to provide the energy for the formation of ATP (7.3 Kcal) (Fig. 5). These three sites are :

I. Span between NADH and Co-enzyme Q where 12.2 Kcal of energy is released.

II. Span between cytochrome bc_1 and cytochrome c where 9.4 Kcal of energy is released.

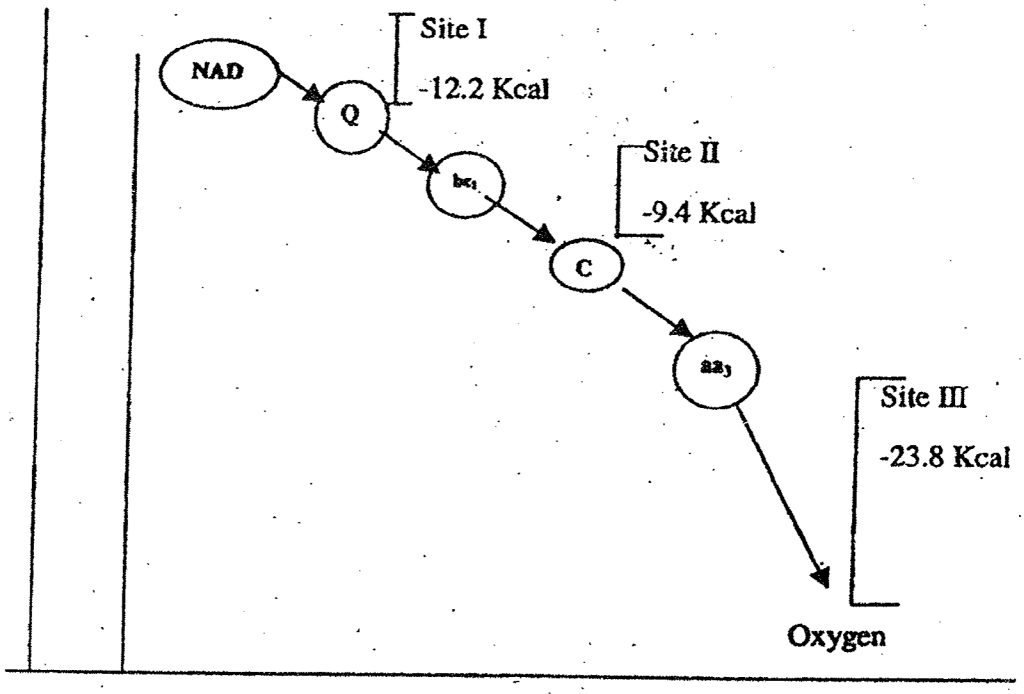


Fig. 5 : Sites of energy delivery.

As a pair of electron equivalents goes from NADH to molecular oxygen, i.e., the entire length of the respiratory chain, the standard free energy change calculated (theoretically) is –52,700 Calmole^{-1} as shown below:

$NADH + {}^1/_2O_2 + H^+ \rightarrow NAD^+ + H_2O$

and the two half reactions and their redox potentials

1) $NADH \rightarrow NAD^+ + 2H^+ + 2E$ $\quad E_0' = -0.32$

2) ${}^1/_2O_2 + 2H^+ + 2e^- \rightarrow H_2O$ $\quad E_0' = +0.82$

$\therefore \Delta E_0' = +0.82 - (-0.32) = +1.14$

$\therefore$ Overall $\Delta G^\circ = -nF\Delta E_0'$

$= -2 \times 23063 \times (+1.14)$

$= -52.6$ Kcalmole^{-1}

Theoretically this amount of ΔG° may produce $\frac{52,700}{7,300}$ moelcules of ATP [Recall AdP + $P_i \rightarrow$ ATP + H_2O, ΔG° = +7300 calmole^{-1}] as ATP synthesis from ADP + P_i needs + 7300 calmole^{-1}. Clearly this large decline in free energy (–52,700 calmole^{-1}) is large enough to make several molecules of ATP from ADP and P_i under standard condition, provided an energy coupling mechanism is available.

Experiment shows when pure NADH was incubated with water-treated mitochondria, P_i and ADP in the absnece of TCA cycle intermediate, **three** molecules of ATP were formed from ADP + P.. Redox energy is transformed into phosphate bond energy to make ATP.

2.7 **The P/O ratio** (i.e., the number of molecules of inorganic phosphate taken up to phosphorylate ADP per atom of oxygen consumed) was established to be 3 on the average for ech of the five oxidavtive steps involved in TAC cycle:

		P/O ratio
(1)	Pyruvate → Acetyl CoA	→ 3
(2)	Isocitrate → α-Ketoglutarate	→ 3
(3)	α-Ketoglutarate → Succinyl CoA	→ 3
(4)	Succinate → Fumerate	→ 2
(5)	Malate → Oxaloacetate	→ 3

For steps 1, 2, 3 and 5 NADH was rapidly oxidized to NAD^+ at the expense of O and formed 3 molecules of ATP from ADP and P_i (P/O ratio is 3); while for step 4. Succinate donates its H-atom (electron) to FAD and 2 molecules of ATP are formed (P/O ratio is 2).

Calculation of efficiency of the system

Theoretically 52.6 Kcal can be synthesized when NADH is oxidized by oxygen which coul produce 52.6 /7.3≈7 ATP molecules. In actually only 3 ATP_s were synthesized.

$$\text{Therefore \% of efficiency} = \left(\frac{3\times 7.3}{52.6}\right) = \frac{21.9}{52.6}\times 100 = 40\%$$

2.8 Oxidative Phosphorylation :

Oxidative phosphorylation is such a process of energy coupling in the respiratory chain which results in formation of ATP at the expense of energies yielded by electron transport to oxygen.

The Chemiosmotic model proposed by Peter Mitchell

Mitchell's theory postulates that the energy from oxidation of components in the respiratory chain is ***coupled*** to the translocation of hydrogen ions (Proton, H^+) from the inside (matrix) to the outside of the inner membrane of mitochondria. The electrochemical energy inherent in the difference in proton concentrtion and separation of charge across the inner membrane, the proton motive force, drives the synthesis of ATP as proton flow passively back into the matrix through a 'proton pore' associated with ATP synthase (Fig. 6).

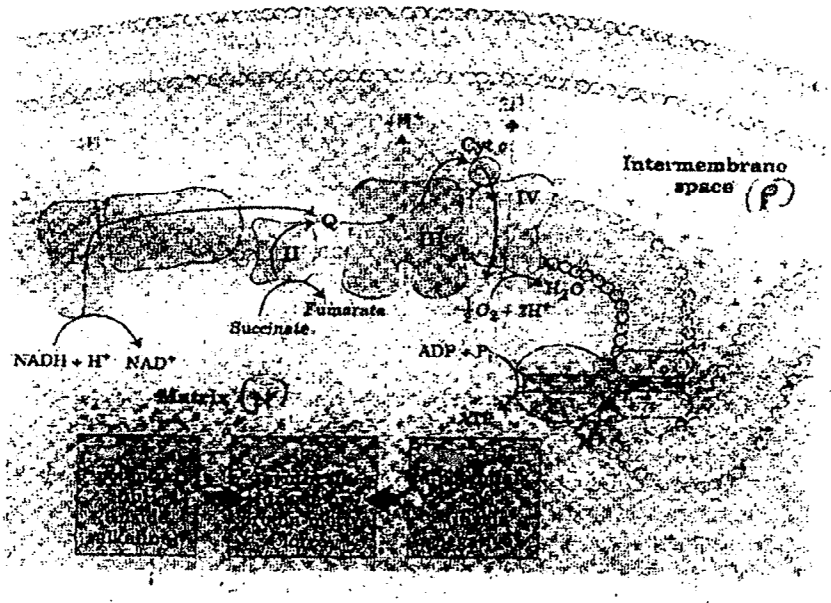


Fig.6 : Chemiosmotic model.

Each of the respiratory chain complexes (I, III, IV) acts as a proton pump. The inner membrane is impermeable to ions in general, and particularly to protons, which accumulate outside the membrane creating an electrochemical potential difference across the membrane, a chemical difference (ΔpH)

and an electrical potential difference ($\Delta\psi$). To emphasize this crucial role of the proton motive force the equation for ATP synthesis is written.

$$ADP + Pi + nH_p \rightarrow ATP + H_2O + NH_N^+$$

where H_p^+ and H_N^+ indicate H^+ on the intermembrane space and matrix side respectively.

Experimental Evidence :

When isolated nitochondria are suspended in a buffer containing ADP, P_i and oxidisable substrate succinate, three measurable processes occur 91) the succinate is oxidized to fumerate (2) Oxygen is consumed, (3) ATP is synthesized. With *Oligomycin* the flow of proton into the matrix through proton pore is blocked and so does ATP synthesis. DNP is an uncoupler, causes leakage of H^+ across the membrane, thus allows respiration (O_2-consumption) to occur without ATP synthesis. Moreover the inhibitors of electron transport (CN^-, CO, antimycin A) block both O_2-consumption and ATP synthesis.

2.9 ATP Synthease

ATP synthase has two functional domains F_0, F_1. It is an F-type ATPase. This large enzyme complex of inner mitochondrial membrane catalyses the formation of ATP from ADP and P_i accompanied by the flow of protons from P to N side of the membrane.

ATP synthase also called complex-V has two distinct components, F1, a peripheral membrane protein and F_0 which is integral to the membrane. The subscript 0 of F_0 stands for oligomycin – sensitive. F_1 is the first factor identified as essential for oxidative phosphosylation.

Experiment :

In the laboratory, small membrane vesicles formed from inner membrane iof mitochondria carry out ATP synthesis coupled to electron transport. When F_1 is gently extracted from these vesicles the 'stripped' vesicles still contain intact respiratory chains and the F portion of ATP synthase. The 'stripped' vesicle can catalyze electron transfer from NADH to O_2 but cannot produce a proton gradient. F_0 has a proton pore through which proton leak as fast as they are pumped by electron transfer and without a proton gradient the F_1 depleted 'stripped' veside cannot make ATP. Isolated F_1 on the otherhand, catalyses ATP hydrolysis and therefore originally was named as F_1ATPase. When F_1 is

added back to the depleted veside, it reassociate with F_0 plugging proton pore restoring its capacity to couple electron transport with ATP synthesis.

F_0F_1 ATPase

The crystalline structure of F_0F_1 ATPase from yeast was prepared in 1999.

Mitochondrial F_1 has nine subunits of five different types, α_3, β_3 $\gamma\delta\alpha\beta\chi\delta\varepsilon$. Each of β subunits has one catalytic site for ATP synthesis. The knob like portion of F_1 is a flattened sphere consiting of alternating α and β subunits arranged like section of an orange. The polypeptides that make up te stalk of the F_1 crystal structure are asymmetrically arranged, with one domain of single γ subunit making up a central shaft that passes through F_0 and another domains of γ associated primarily with one of the three β subunits (β-empty). Although the amino acid squence of β subunits are similar, their conformations differ in part because of the association of the γ-subunit with just one of the three. The conformational difference among β subunits extends to differences in their ATP/ADP binding sites. The corresponding β subunits are designed as β-ATP, β-ADP and β-empty. The axle also contain an ε-subunit. F_0 complex making up the proton pore is composed of three subunits a, b, c (ab_2C_{10-12}) subunit C is small, very hydrophobic consisting almost entirely of two transmembrane helixes, with a small loop extending from the matrix side of the membrane. The two b subunits of F_0 associate firmly with α and β subunits of F_1 holding them fixed relative to the membrane (Fig. 7).

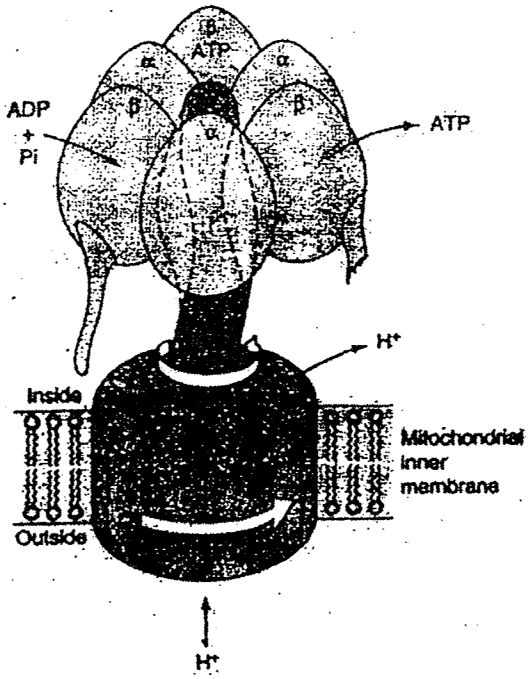


Fig. 7 : F_0F_1 ATP Synthase.

Mechanism of ATP production by ATP Synthase

In F_0 the membrane – embedeed cylinder of C subunits are attached t the shaft γ-subunits of F_1 in the form of 'bent axle'. As proton flows through the disk of 'C' units from P-side to N-side via F_0, the cylinder and the shaft rotate. The γ submit fits inside the F_1 subcomplex of three α and three β subunits which are fixed to the membrane and do not rotate. As γ subunit rotatses, the subunits change conformation.

Paul Boyer proposed a mechanism in which the three active sites of β takes turn catalyzing ATP-synthesis. A gives β subunits starts in the β-ADP conformation which binds with ADP and P_i from the surrounding media. The subunit now change conformation assuming the β-ATP form that tightly binds and stabilize ATP. Finally the subunit change to β-empty conformation, which has very low affinity for ATP and the newly synthesized ATP leaves the enzyme surface. Another round of catalysis begins.

2.9 Summary :

Principle of the chemiosmotic theory of oxidative phosphorylation (Fig. 8).

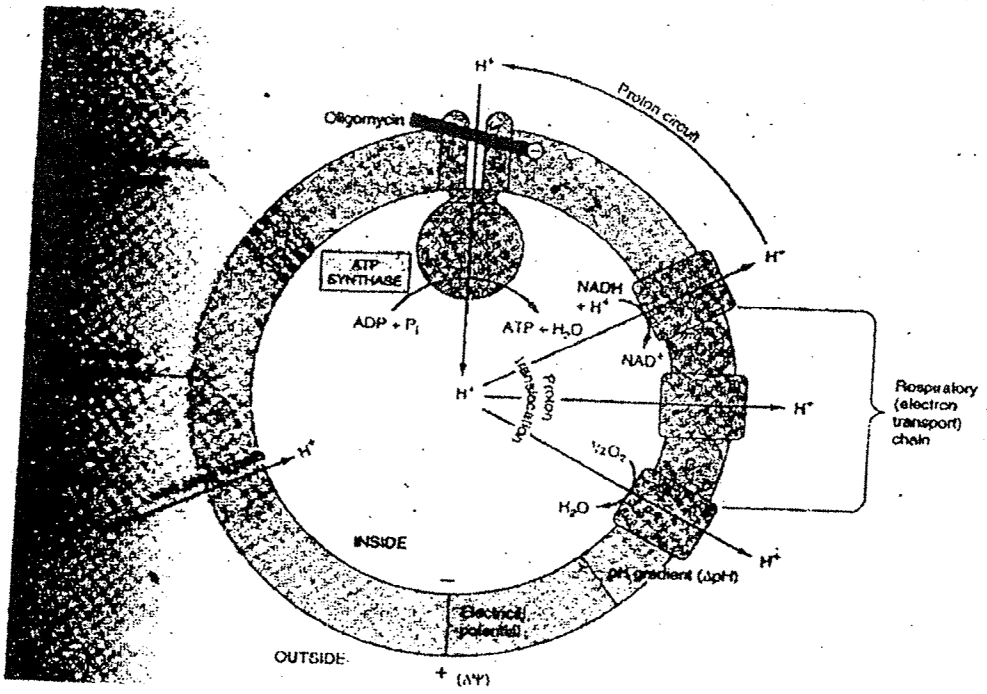


Fig. 8 : Summary.

The main proton circuit is created by the coupling of oxidation in the respiratory chain to proton translocation from the inside to the outside of the membrane driven by the respiratory chain complexes I, III, IV each of which acts as a proton pump. F_0F_1 protein subunits utilize energy from the proton gradient to promote phosphorylation.

Uncoupling agents such as DNP allows leakage of H^+ across the membrane, thus collapsing the electro chemical proton gradient. Oligomycin specially blocks conduction of H^+ through F_0.

Proton flows through the F_0 portion of F_0F_1. ATP synthase. The streaming proton through 'F_0-Pore' causing cylinder C subunit and γ-subunit to rotate about the logn axis. γ-subunit comes into contact with a β-subunit of F_1. One comple rotation of γ-subunit causes each β subunit to cycle through all three possible conformations and 3 ATP molecule are produced.

Suggested Questions

Q1. (a) How can the standard free energy change be calculated in an oxidation-reduction reaction?

(b) What is P/O ratio?

Q2. Calculation the percentage efficiency of energy conservation in ATP as succinate is oxidized to fumerate by molecular oxygen in intact mitochondria (P/O=2). Two half reactions and their E'o's are as follows:

(1) Succinate $\rightarrow$ Fumerate $E_0' = +0.03$ volt

(2) $^1/_2O_2 + 2H^+ + 2e \rightarrow H_2O$ $E_0' = +0.82$ volt

[Given F = 23063 cal.mole^{-1} volt^{-1}]

Q3. (a) Write briefly on the four electron carrier multienzyme complexes located in the inner membrane of mitochondria.

(b) What are the three possible energy delivery steps in the electron transport chain where ATP can be synthesized?

Q4. Discuss how the inhibitors in the various locations of electron transport chain and oxidative phosphorylation give valuable information about the sequence of various multienzyme complexes in the inner mitochondrial membrane and oxidative phosphorylation.

Q5. Write how a concentration gradient of proton across the inner membrane of mitochondria formed by electron transport dries the synthesis of ATP.

Q6. Write on the structure and functional aspect of F_0F_1 ATP synthase located in the inner membrane of mitochondria.

Key words :

Oxidation, Reduction, Redox potential, Gibbs free energy, Electron transport chain, Mitochondria, Electron carriers, NAD, FAD, Coenzyme Q, Cytochromes, Electron transport inhibitors, P/O ratio, Oxidative phosphorylation, Chemiosmotic hypothesis, F_0F_1 ATP synthease, Oligomycin, Uncoupling agents.

Select Readings :

(1) Lehninger's Principle of Biochemistry (3rd ed.)

(2) **Harper's illustrated Biochemistry** (26th ed.)

(3) **Biophysics – an introduction by Rodney Collerill.**

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"Learner's Feed-back"

After going through the Modules / Units please answer the following questionnaire.
Cut the portion and send the same to the Directorate.

To
The Director
Directorate of Distance Education,
Vidyasagar University
Midnapore - 721 102

1. The modules are : (give ✓ in appropriate box)
   ☐ easily understandable; ☐ very hard; ☐ partially understandable.

2. Write the number of the Modules/Units which are very difficult to understand :
   .....
   .....
   .....

3. Write the number of Modules / Units which according to you should be re-written :
   .....
   .....
   .....

4. Which portion / page is not understandable to you? (mention the page no. and portion)
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5. Write a short comment about the study material as a learner.
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(Full Signature of the Learner)

Date : .....

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