CHAPTER 2

Genetic Homogeneity in Colonel Populations

LONG ANSWER QUESTIONS

☛ **Q.1. Comment upon Symbiotic Nitrogen Fixation.**

Ans.

Symbiotic Nitrogen Fixation or Biological Nitrogen Fixation

Schematic representation of the nitrogen cycle. Abiotic nitrogen fixation has been omitted.

Biological nitrogen fixation was discovered by the German agronomist Hermann Hellriegel and Dutch microbiologist Martinus Beijerinck. Biological nitrogen fixation (BNF) occurs when atmospheric nitrogen is converted to ammonica by an enzyme called nitrogenase. The reaction for BNF is :

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

The process is coupled to the hydrolysis of 16 equivalents of ATP and is accompanied by the co-formation of one molecule of H. In free-living diazotrophs, the nitrogenase-generated ammonium is assimilated into glutamate through the glutamine sythetase/glutamate synthase pathway.

Enzymes responsible for nitrogenase action are very suceptible to destruction by oxygen. Many bacteria cease production of the enzyme in the presence of oxygen. Many nitrogen-fixing organisms exist only in anaerobic conditions, respiring to draw down oxygen levels, or binding the oxygen with a protein such as leghemoglobin.

☛ **Q.2. Comment upon UV Radiations and Sterilization.**

Ans.

Ultraviolet (UV) Radiation

UV radiation causes damage in the DNA duplex of the bacteria and phages. The ultraviolet rays are absorbed and results excitation of macromolecules. The absorption maxima of nucleic acid and protein are more or less similar. The DNA molecule is the target molecule for UV rays but not the proteins. While, absorption spectrum of RNA is quite similar to that of DNA. The excited DNA leads to cross-linking, single strand breaks and base damage like minor cohesion and generation of nucleotide dimer like a major one. Purines are generally more radio-resistant than the pyrimidine of the latter, thymine is more reactive than cytosine. Thus, the ratio of thymine-thymine, thymine-cytosine, cytosine-cytosine (CC) dimer as show

in Fig. is 10:3:3, respectively. A few dimers of TU and UU also appear. The initial step in pyrimidine dimerization is known to be hydration of their 4 : 5 bonds.

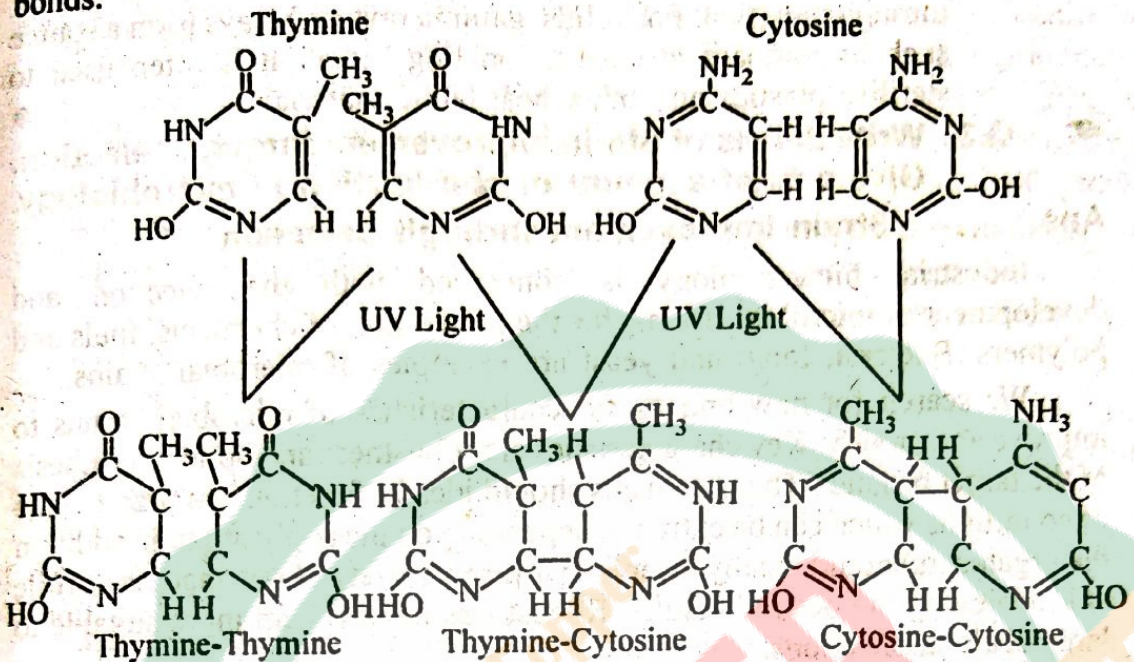


Fig. Formation of pyrimidine dimer induced by UV radiation.

Formation of thymine-thymine dimer results distortion of DNA helix because the thymines are pulled towards one another. The distortion causes in weakening of hydrogen-bonding to adenines in the opposing strand. This structural distortion inhibits the advance of replication fork.

Sterilisation is the entire destruction or removed of all living organisms from the object being sterilised. The methods used for sterilisation are as follows :

1. Filtration : The liquid or gas to be sterilised is passed by a filter with a porosity sufficient to remove any micro-organism in suspension. The cotton wool is used for gases. For liquids, a variety of filters are available, made of materials e.g. cellulose nitrate. This method is very useful for sterilisation of liquids containing heat labile components.

2. Heat : For general sterilisation, a time and temperature that kill all organisms including heat-resistant spores issued. The methods generally adopted are follows :

(a) **Dry heat :** Dry heating is done in a oven, where a temperature of 160°C for two hours is usually required.

(b) **Tyndallization :** Tyndallization includes a course of three periods of boiling at 100°C for 30 min daily.

(c) **Wet heat in an autoclave :** The usual method is a time of 30 minutes at a pressure of 1.05 kg/cm^3 that will give a temperature of 121°C. It is the best method of sterilisation.

3. **Chemicals :** Many chemicals are lethal to microbes. Hypochlorite solution and phenolic derivative are used as general laboratory disinfectants such as gaseous ethylene oxide.

4. **Radiation :** Ultraviolet light is very effective in sterilisation of air through radiation. For solids, gamma rays or X-rays form a source such as radioactive cobalt. Ionizing radiation is often used to sterilise plastics and other heat-labile materials.

☛ **Q.3. Write details of Strain improvement through selection. Give a brief account of sterilisation in microbiology.**

Ans.

Strain Improvement through Selection

Industrial biotechnology is concerned with the selection and development of microbial strains, for the production of chemicals, fuels and polymers. Bacteria, fungi and yeast are examples of microbial strains.

We search for new and useful characteristics of microbial strains to improve and modify. Key characteristics are robustness and optimal synthesis of the target product. The organisms should ideally function in a broad range of conditions which can be extreme, depending on the application. In addition the organisms should ideally be able to utilise a variety of raw materials while still achieving the desired results. Robustness makes them more suitable to large-scale applications.

We integrate all disciplines within the process :

- raw material selection
- strain selection
- strain improvement by genomic
- bioprocess development
- downstream processing (product isolation)

We are ultimately focused on translating our client's objectives into results.

☛ **Q.4. Write short notes on Microbial Assays.**

Ans.

Microbial Assays

Microbiological assay is applicable only to the B vitamins. The rate of growth of a species of microorganism that requires a vitamin is measured in growth media that contain various known quantities of a foodstuff preparation containing unknown amounts of the vitamin. The response (measures as rate of growth) to the unknown amounts of vitamin is compared with that obtained from a known quantity. A bacterial inhibition assay in an assay that uses bacterial colony growth as a semi quantitative indicator to measure the concentration of a product in an sample.

Example : Classical Guthrie Test

The Guthrie bacterial inhibition assay was a semiquantitative assay designed to detect elevated blood levels of the amino acid phenylalanine, using the ability of phenylalanine to facilitate bacterial growth in a culture medium with an inhibitor.

A drop of blood is usually obtained by pricking the heel of a newborn infant and is collected on a piece of filter paper. A small disk of the filter paper is punched out and placed on an agar gel plate containing Bacillus subtilis and B-2-thienylalanine inhibits bacterial growth. However, in the presence of extra phenylalanine leached from the impregnated filter paper disk, the inhibition is overcome and the bacteria grow. Within a day the bacterial growth surrounding the paper disk is visible. The amount of growth, measured as the diameter of the colony, is roughly proportional to the amount of phenylalanine in the serum. The result is read by comparing the diameter of each sample disk's colony to the colonies of a series of reference disks with standard phenylalanine content included on each large plate.

The Guthrie bacterial inhibition assay is sensitive enough to detect serum phenylalanine levels of 180-240 μmol/L (3–4 mg/dL). In healthy normal people, phenylalanine levels are usually under 120 μmol/L.

The test has been widely used throughout North America and Europe as one of the core newborn screening tests since the late 1960s. The classical Guthrie bacterial inhibition assay is gradually being replaced many areas by newer techniques such as tandem mass spectrometry that cand etect a wider variety of congenital diseases.

☛ **Q.5. How do you perform replicaplating ? Discuss different applications of the methodology.**

Ans. Mutation occurring in microorganisms can be detected and efficiently isolated from the parent organisms of other mutants. However studying mutation one must be aware of wild type characters of an organism so that mutants can easily be detected. As, mutations are rare about one per 10^7 to 10^{11} cells, it is very important to have a very sensitive detection system so that the rare mutant may not be missed from detection Thus, to rescue from these difficulties the probability of getting alterations and frequency of mutation are increased through inducing mutations.

In bacteria and other haploid microorganisms, the detection systems are straight forward since any new allele should be observed immediately. In albino mutation, the deletion is very simple. It needs only change in colour of bacterial colony. The other detection systems are rather complex. Some of the methods of detection have been described as follows :

(A) Resistance Selection Method

It is the approach for isolation of mutants. Generally the wild type cells are not resistant either to antibiotics or bacteriophages. Thus it is possible to grow the bacterium in the presence of the agent. This method is applied for isolation of mutants resistant to any chemical compounds which can be amended in agar, phage resistant mutants.

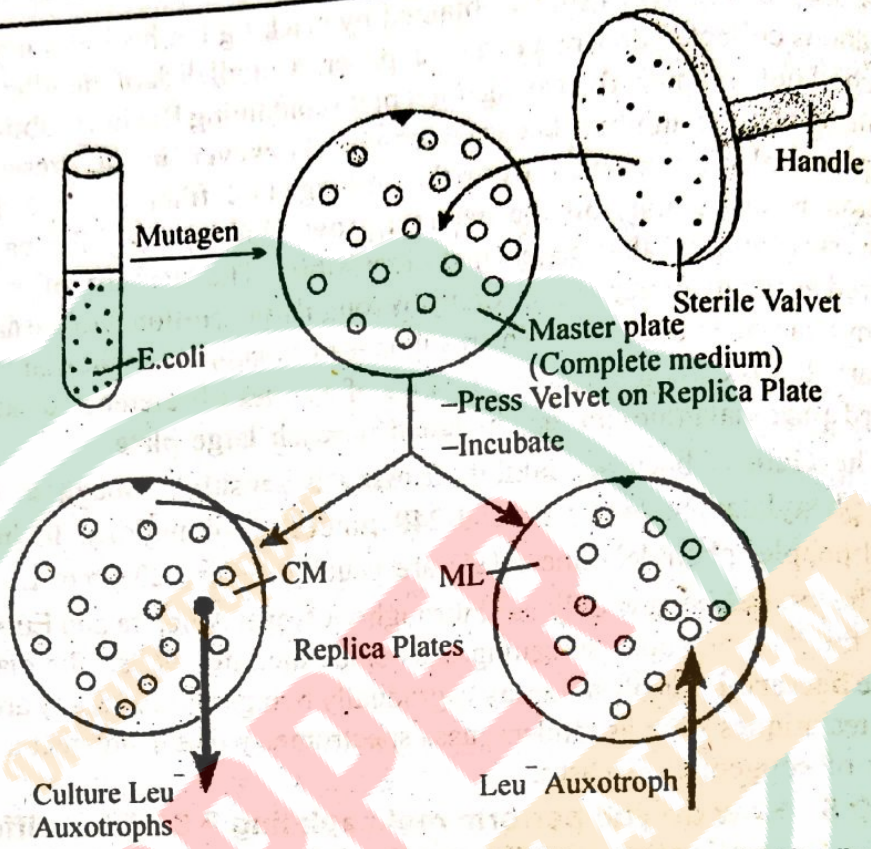


Fig. Replica plating technique for isolation of leucsine auxotrophs (leu⁻); CM, complete medium; ML, medium devoid of leucine.

(B) Replica Plating Technique

Lederberg and Lederberg (1952) have given replica plating technique. This technique is used to identify auxotrophic mutants which differentiates between mutants and wild type strains on the basis of ability to grow in the absence of an amino acid. Method of replica plating has been described earlier. While isolation of a leucine auxotroph through replica plating follows the following steps as shown in (Fig. A).

(a) Generate the mutants by treating a culture with a mutagen for example nitrosoguanidine.

(b) Inoculate a plate containing complete growth medium and incubate it at proper temperature. Both wild type and mutant survivors will from colonies on the complete medium. This plate containing complete medium is called master plate.

(c) Prepare a piece of sterile velvet and gently press on upper surface of master plate to pick up bacterial cells form each colony. Since pressed on master plate, again gently press the velvet on replica plates containing complete medium in one set and lacking only leucine in the other set. Hence, the bacterial cells are transferred in replica plates in the same position as in master plate.

(d) Incubate the plates and compare the replica plates with master plate for the bacterial colony not growing on replica plate. The leucine auxotrophs will not grow on replica plates devoid of leucine. Isolate and culture leu-cells growing on entire medium.

Replica plating can also be used to isolate the temperature sensitive mutants. It involves through forming colonies at 30°C and then transferring these colonies on two plates, one incubated at 30°C and the other at 42°C. The colony that grow at 30°C and absent at 42°C certainly consist of temperature sensitive mutation.

(C) Substrate Utilization Method

This method is employed in the selection of bacteria. Various bacteria utilize mere a few primary carbon sources. The cultures are plated onto medium containing an alternate carbon sources. Any colony that grows on medium can use the substrate and are possibly mutants. These can be isolated.

Sugar utilization mutants are also isolated by means of colour indicator plates. A popular medium is used for this purpose. The EMB agar contains two dyes eosin and methylene blue in the medium. Colour of these dyes is sensitive to pH. This medium also contains lactose sugar as carbon source and complete mixture of amino acids. Hence, both lactose wild type and lactose mutant cells can grow and form colonies on EMB agar plates. The Lac^+ cells catabolize lactose and secrete acids. Thus, local pH of the medium decreases. This results in staining of colony to dark purple. On the other hand, Lac^- cells are unable to utilize lactose and use some of the amino acids like a carbon source. After utilization of amino acid, possibly ammonia is produced that increases the local pH and decolourizes the dye resulting in white colony.

(D) Carcinogenicity Test

An understanding has developed to identify the environmental carcinogenes that cause mutation and induce cancer in organisms. This method is based on detecting potential of carcionogenes and testing for mutagenicity in bacteria.

Ames developed a method for deletion of mutagenicity of carcinogens which is commonly called as **Ames test**. It is widely used to detect the carcinogens. The Ames test is a mutational reversion assay in which various special strains of *Salmonella typhimurium* are employed. Each strain contains a different mutation in the operon of histidine biosynthesis. The Ames test follows the following steps as shown in (Fig.) :

(a) Prepare the culture of *Salmonella* histidine auxotrophs.

(b) Mix the bacterial cells and test substance in dilute molten top agar with a small amount of histidine in one set, and control with entire medium plus large amount of histidine.

(c) Pour the molten mix on the top of minimal agar plates and incubate at 37°C for 2 to 3 days. Until histidine is depleted all the His⁻ cells will grow in the presence of test mutagens. If histidine is completely exhausted only the reverants will grow on agar plate. The number of spontaneous reverants is low, while the number of reverants induced through the test mutagen is quite high. In order to estimate the relative mutagenicity of the mutagenic substance the visible colonies are counted and compared with control. The high number of colonies represents the greater mutagenicity.

A mammalian liver extract is added to the formational molten top agar before plating. The extract converts the carcinogens into electrophilic derivatives which will soon react with DNA molecule. In natural way this process founds in mammalian system when foreign substances are metabolized in the liver. Bacteria do not possess the metabolizing capacity as liver does thus, the liver extract is added to this test, just to promote the transformation.

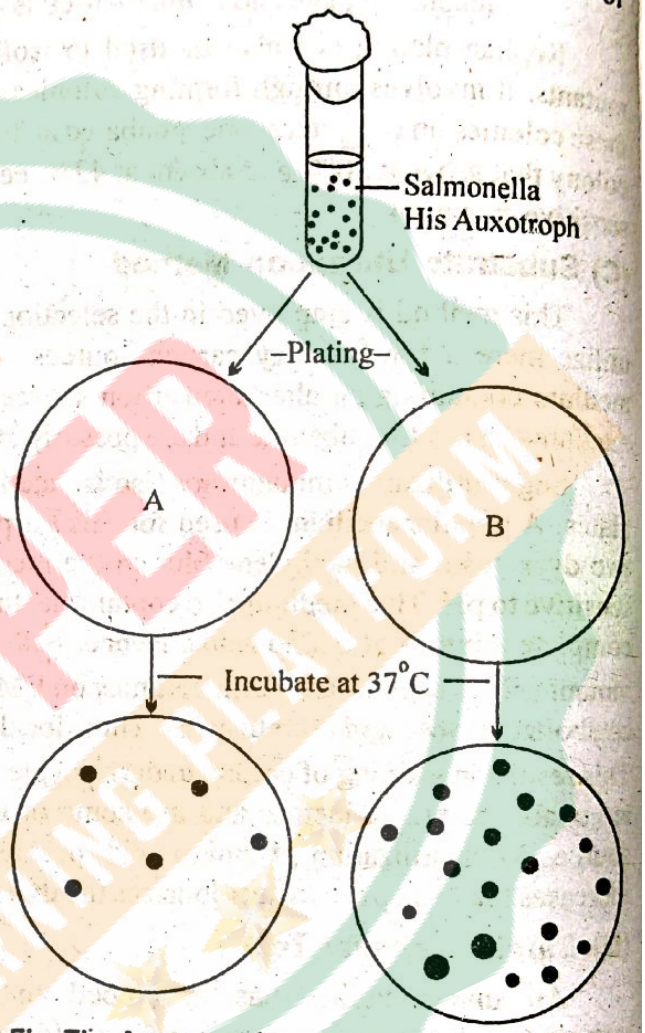


Fig. The Ames test for mutagenicity; A, complete medium containing a large amount of histidine; B. Medium containing test mutagen and a small amount of histidine.

Q.6. Describe the origin of spontaneous mutations.

Ans. The Origin of Spontaneous Mutations

Mutation involves changes in DNA. Different mechanisms are known that bring about alterations in DNA. These modifications may arise from error in DNA replication, damage to DNA from radiation. Errors found in replication through substitution of frame shift in DNA sequence.

1. Substitution

Substitution of base pairs is the most common mutation. In replication of DNA repair wrong base pairs are incorporated. Base pair substitutions is of two types, transition and transversion.

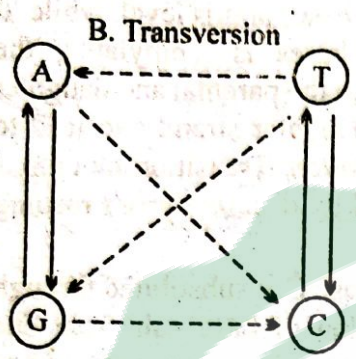


Fig. Transition (A) and Transversion (B) mutations.

(A) Transition Mutation : Transition is the replacement of one purine (for example A or G) by another purine (for example G or A) or one pyrimidine (for example T or C) through another pyrimidine (for example C or T). Four types of changes are possible in transition such as AT → GC, GC → AT, TA → CG, CG → TA as shown in (Fig.). In replication, errors in DNA arise with high frequency. Some of the bases exist in alternative forms having various base pairing properties. Tautomerism is the relationship between two structural isomers which are in chemical equilibrium and readily convert into one another. Generally the bases exist in keto form, but at a time they can take on either amino or enol form as shown in (Fig. B). A rare form of adenine can pair with cytosine and the end form of thymine can pair with guanine. Such tautomeric shifts alter the hydrogen bonding characteristics of the bases and allow for purine substitution or pyrimidine for pyrimidine substitution. They lead to stable modification in nucleotide sequences.

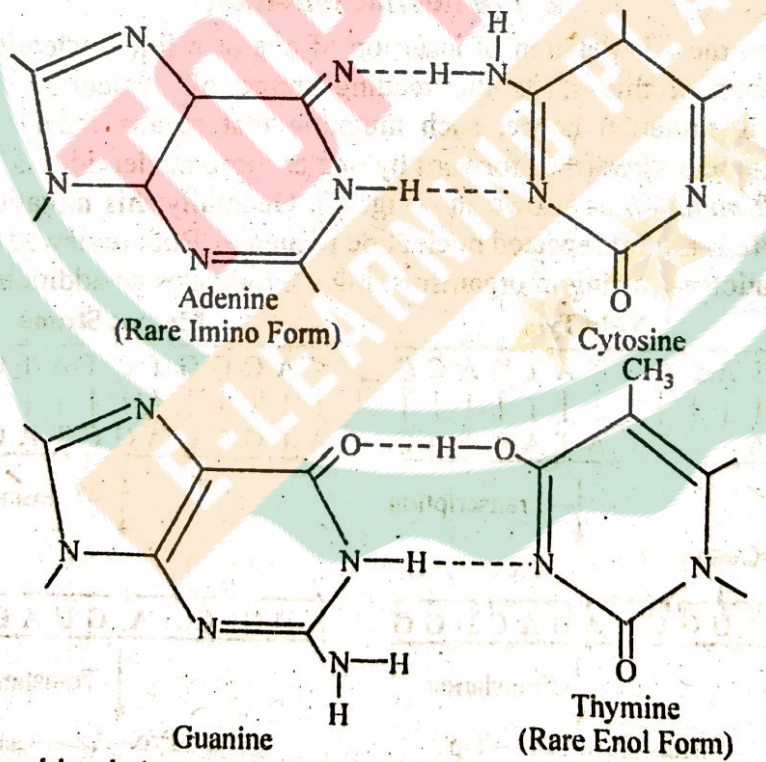


Fig. Base-pairing between rare imino form of adenine, cytosine and guanine and enol form of thymine.

In replication an incorrect base is correctly hydrogen bonded and incorporated to the template strand. Even the editing system does not identify it as incorrect. Later on when the base assumes its normal function, the mismatch repair system corrects the mismatching bases at this level. while, if the daughter strand containing the incorrect bases is methylated, the mismatch repair system fails to distinguish between the parental and daughter strands. Therefore, the incorrect bases exist in daughter strand and lead to mutation. Such mutation is called *transition mutation*. Transition mutations are common, although most of them are repaired by different proof reading function.

In a strand of DNA molecule a pyrimidine base C is substituted through another pyrimidine base T which is the transition of base pair from one pyrimidine to another. After replication the error is retained in one strand and inherited in the progeny. In the second generation after replication, purine base G is substituted by another purine base giving rise to mutant progeny.

(B) Transversion mutation : Transversion mutation involves the substitution of purine through a pyrimidine or a pyrimidine by a purine. This type of mutation is rare because of steric problems of pairing of purines with purines and pyrimidines with pyrimidines. Eight types of changes are possible in transversion such as AT → TA, AT → GC, GC → CG, GC → TA, AT → TA, TA → GC, CG → GC, CG → AT as shown in (Fig. A).

2. Frameshift Mutation

When there is deletion of insertion of one or a few nucleotides in the DNA molecule, this shifts the reading frame of nucleotide sequences resulting in mutation hence, such mutation that results form shifting in reading frame backward or forward by one or more nucleotides is known as *frameshift mutation* as shown in (Fig. C). Generally this mutation occurs where there is a short repeated nucleotide sequence. Both reviewed the frame shift mutation occurring in organisms. Fig. C. Indicates an addition of one T

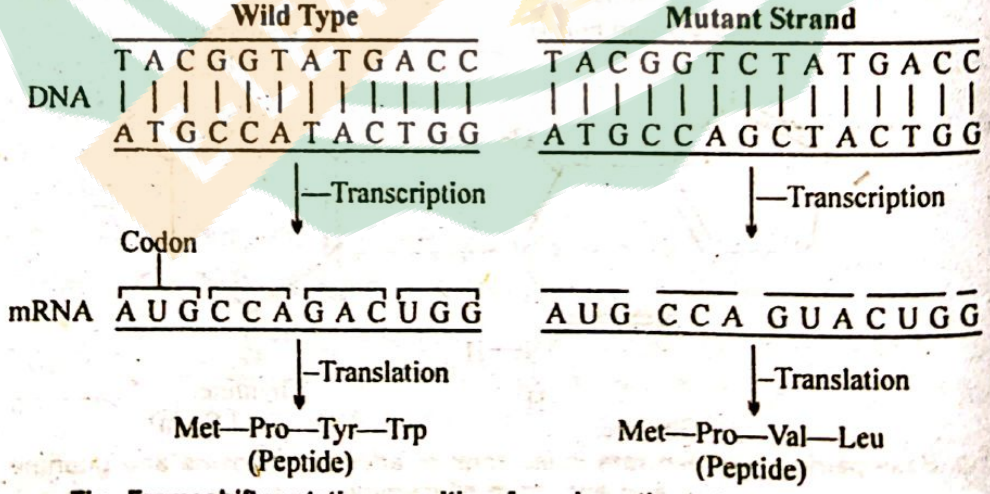


Fig. Frameshift mutation resulting from insertion of a GC base pair (see arrow) and sense strand T.

because of slippage of the new strand (A) and a deletion found as a result of slippage of the parental strand (B). Thus, there is shifting in sequence of genetic code.

The same can be explained through an analogy of a code of words. When a gene gives the message : THE DOG CAN EAT JAM, each word of three letters represents a codon. After addition of a letter. A after G, the new sentence becomes : THE DOG ACA NEA TJA M, that also is meaningless. Similarly, after removal of C the new sentences becomes THE DOG ANC ATJ AM, which also is meaningless.

3. Spontaneous Deamination of 5-Methylcytosine

One more source of spontaneous mutation is a change in 5-methylcytosine a methyl form of cytosine. Both MeC and C occasionally loose an amino group. The C is changed to U, and MeC to 5-MeU (thymine) as shown in (Fig. D). The C pairs with A but not G, thus, replication of a molecule containing GU base pair will finally causes in substitution of an AT pair for the original GC pair. The process in successive round of replication is GU → AU → AT. The uracil form DNA is removed through uracil glycosylase, thus the conversion of C → U rarely results in mutation. In addition 5-MeC loses an amino group and changes into 5-methyluracil which is actually the thymine. There is no removal mechanism, thus GMeC pair becomes a GT pair which may be corrected by mismatch repair system. This system does not recognise MeC, hence, it is present in a methylated strand. It

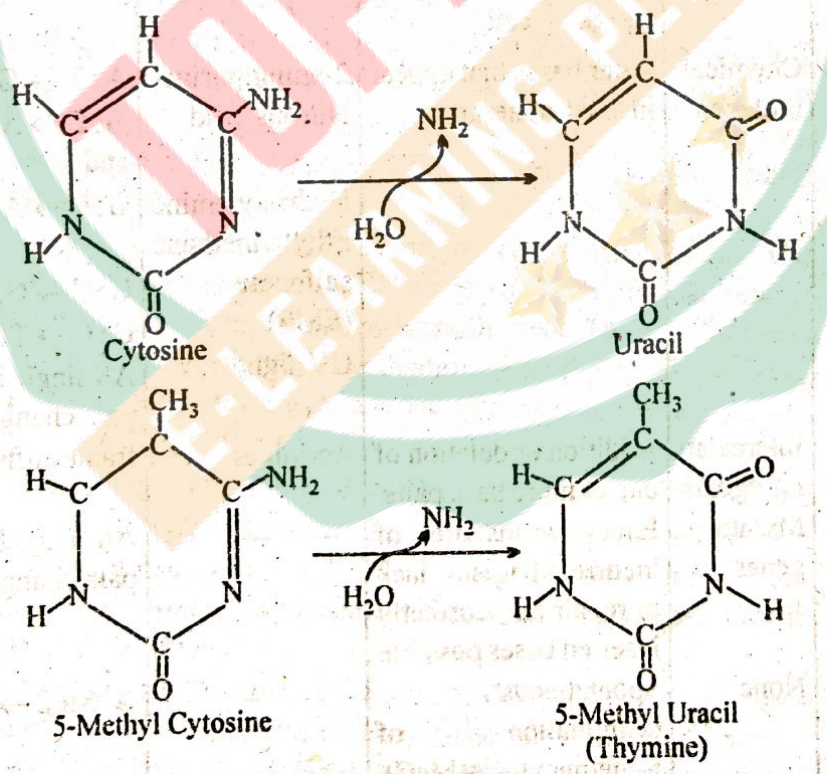


Fig. Deamination of cytosine and 5-methyl cytosine.

is changed randomly sometimes correct GC pairs and some times wrong AT pair. Thus, MeC acts as mutable site in a gene and this site is known as hot spot.

It should be noted that methylated bases are the normal constituents of DNA in some microorganism but not the mutagens. Some organisms contain both C and MeC. Methylation results in protection of DNA against enzymes synthesized by viruses. The bacteriophage T2 contains 5-hydroxylmethyl-cytosine instead of cytosine.

☛ **Q.7. Discuss the Induced Mutations.**

Ans. **Induced Mutations**

Any agent which directly results damage to the DNA alters the base sequence or interferes with repair system will certainly induce mutations in DNA of organisms. The agents causing damage to DNA are known as mutagens such as chemicals and radiation.

Type of Mutagens (after Friefelder, 1987)

S.No.	Mutagen	Mode of action	Example	Consequence
1.	Base analogue	Substitutes for a standard base during replication, a new base pair appears in daughter cell.	5-Bromouracil	A : R → G : C and G : C → A : T
2.	Chemical mutagen	After bases that appear in next generation	2-aminopurine Nitrous acid Hydroxylamine Ethyl methane sulfonate (EMS) UV light	A : T → G : C G : C → A : T and G : C → A : T G : C → C : G G : C → TA All single base pair changes
3.	Intercalating agents	Addition or deletion of one or more base pairs	Acridines	frameshifts
4.	Mutator genes	Excessive insertion of incorrect bases or lack of repair of incorrectly inserted bases possible	—	All single base pair change
5.	None	Spontaneous deamination of 5-methycytosin(MeC)	—	G : MeC → AT

I. Chemical Mutagens

Singer and Kusmierek (1982) have published an excellent review on chemical mutagenesis.

Different types of Chemical Mutagens

S. No.	Class of Chemical	Chemical Mutagens
1.	Alkyl sulphonates	Diethylsulphonate (DES)
		Methylmethanesulphonate (MMS)
		Ethylmethanesulphonate (EMS)
2.	Acridines	Ethyleneimine (EI)
3.	Epoxide	Ethyleneoxide (EO)
		Diepoxybutane (DEB)
4.	Mustard	Nitrogen mustard
		Sulphur mustard
5.	Nitrosamines	Diethylnitrosamine (DMN)
		Diethylsulphonate (DES)
		Nitrosomethylurea (NMU)
6.	Others	Nitrous acid
		Maleic hydrazide
		Hydroxylamine

1. Base Analogues

A base analogue is a chemical compound identical to one of the four bases of DNA. It can be incorporated into a growing polynucleotide chain when normal process of replication occurs. These compounds have base pairing properties different from the bases. They replace the bases and results stable mutation. A very common and widely used base analogue is 5-bromouracil which is an analogue of thymine. The 5-BU functions like thymine and pairs with adenine (Fig. A(a)). The 5-BU undergoes tautomeric shift from keto form to enol form caused by bromine atom. The enol form can found for a long time for 5-BU than for thymine (Fig. A(b)). When 5-BU replaces a thymine, it generates a guanine during replication which in turn specifies cytosine causing G:C pair.

In the replication, keto form of 5-BU substitutes for T and the replication of an initial AT pair becomes an A:BU pair (Fig. B(a)). The rare enol form of 5-BH that pairs with G is the first mutagenic step of replication. In the other round of replication G pairs with C. Hence, the transition is completed form AT → GC pair.

The 5-BU can also induce the conversion of GC to AT. The *enol* form infrequently works as an analogue of cytosine rather than thymine. Because of error, GC pair is converted into a G:BU pair which in turn becomes an AT

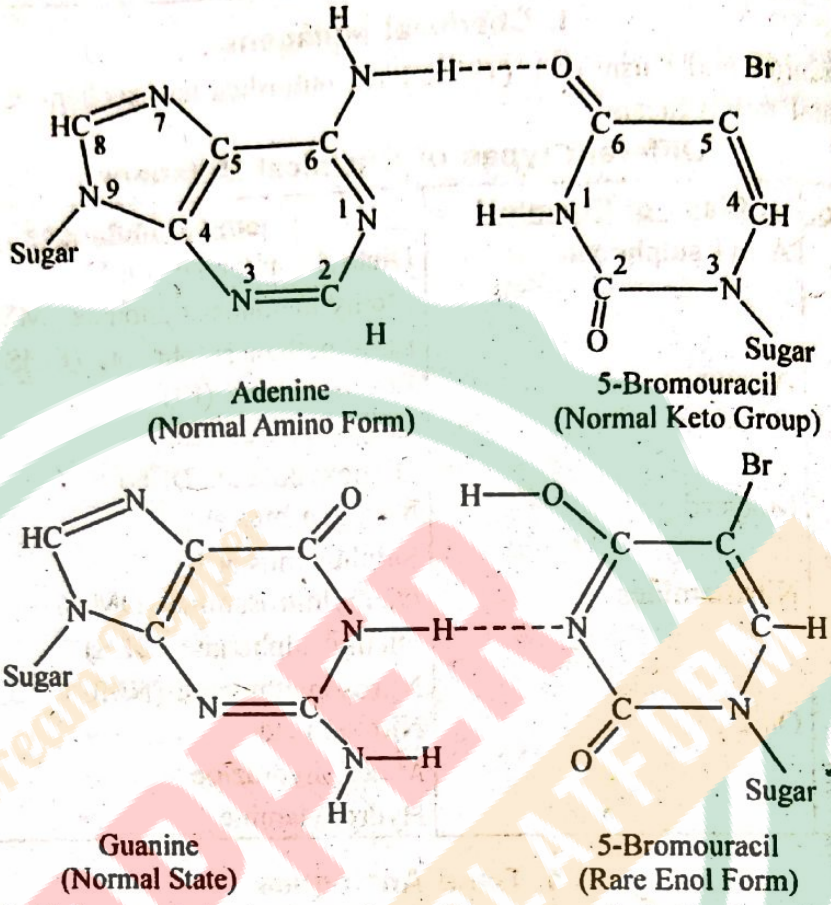


Fig. A. Mutagenesis by base analogue 5-bromouracil. A. the keto form of 5-BU pairs with adenine; B. 5-BU is tautomarised to enol form and pairs with guanine rather than adenine.

pair (Fig. B(b)). Because of such pairing properties 5-BU is used i chemotherapy of viruses and cancer. Due to pairing with guanine it disturb the normal replication process in microorganisms.

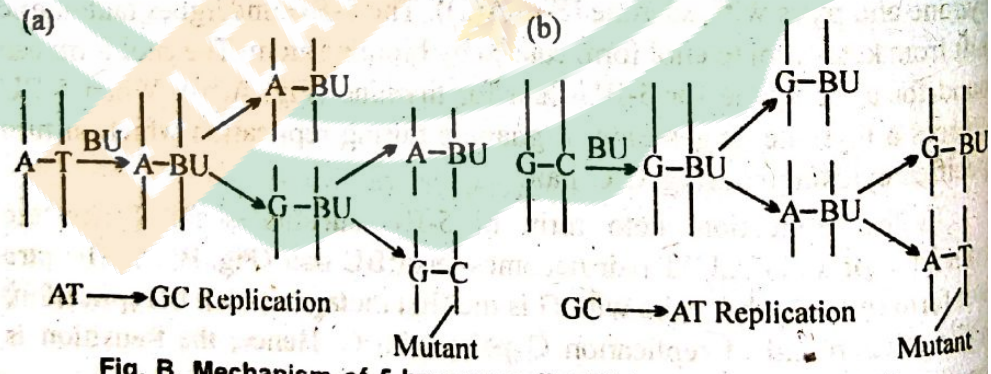


Fig. B. Mechanism of 5-bromouracil (BU)-induced mutagenesis. (a) AT→GC replication; (b) GC → AT replication.

The 5-bromodeoxyuridine can replace thymidine in DNA molecule The 2-aminopurine with thymine but it is able to form a single hydrogen bon

with cytosine resulting in transition of AT to GC. The 2-AP and 2,6-DAP are not as effective as 5-BU and 5-BDU EMS has the specifity to remove guanine and cytosine from the chain and formed gap. Any base (A, T, G, C) inserted in the gap. During replication chain without gap will result in normal DNA. In the second round of replication gap is filled by favourable base. When the correct base is inserted, normal DNA sequence will be produced. Insertion of incorrect bases result in transversion of transition mutation.

2. Chemicals Changing the Specificity of Hydrogen Bonding

There are many chemicals which after incorporation into DNA change the specificity of hydrogen-bonding. Those which are used as mutagens are nitrous oxide, hydroxylamine and ethyl methane sulphonate (EMS).

(i) **Nitrous Oxide :** Nitrous oxide changes the amino group of bases into keto group through oxidative deamination. The order of frequency of deamination > cytosine > guanine.

(ii) **Deamination of Adenine :** Deamination of adenine causes in formation of hypoxanthine, the pairing behaviour of which is like guanine. Thus, it pairs with cytosine instead of thymine replacing AT pairing by GC pairing as shown in Fig. C(a).

(iii) **Deamination of Cytosine :** Deamination of cytosine results in formation of uracil through replacing —NH_2 group with —OH group. The affinity for hydrogen bonding of uracil is like thymine, therefore, C-G pairing is replaced by U-A pairing as shown in (Fig. C(b)).

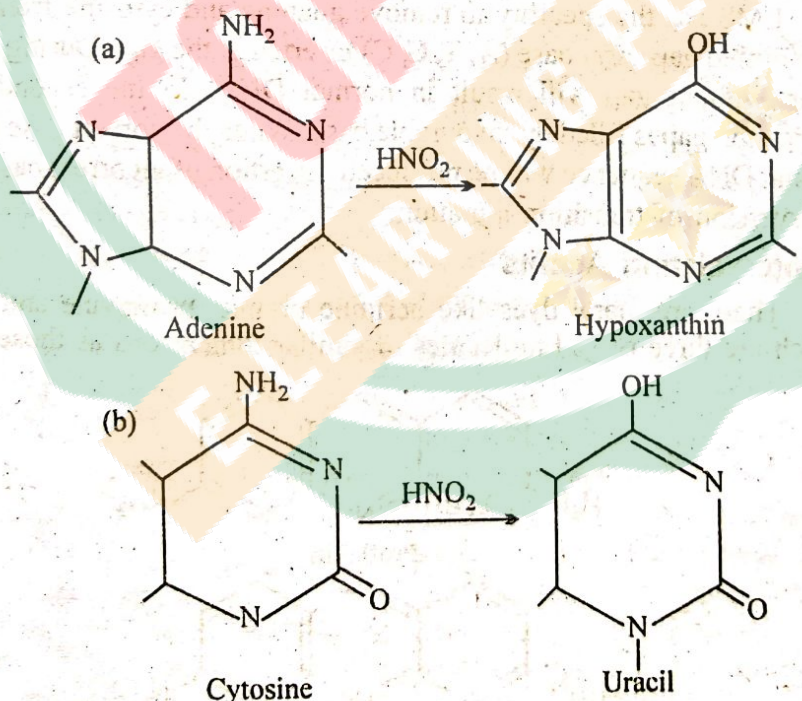


Fig. C. Deamination by nitrous oxide of adenine into hypoxanthin (a) and cytosine into uracil (b).

(iv) **Hydroxylamine :** It hydroxylates the C_4 nitrogen of cytosine and changes into a modified base via deamination which causes to base pairs like thyamine. Thus, GC pairs are changed into AT pairs.

(v) **Deamination of Guanine :** Deamination of guanine causes in formation of xanthine, the later is not mutagenic. Xanthine behaves like guanine because there is no change in pairing behaviour. Xanthine pairs with cytosine. Therefore, G-C pairing is replaced through X-C pairing.

3. Alkylating Agents

Addition of an alkyl group to the hydrogen bonding oxygen of guanine and adenine residues of DNA is done by alkylating agents. As a result of alkylation, possibility of ionization is increased with the introduction of pairing errors. Hydrolysis of linkage of base-sugar founds resulting in gap in one chain. This phenomenon of loss of alkylated base from the DNA molecule is known as **depurination.** Depurination is not always mutagenic. The gap created by loss of a purine can effectively be repaired. Following are some of the important widely used alkylating agents :

(i) Ethyl ethane sulphonate (EES)—$CH_3CH_2SO_3CH_2CH_3$

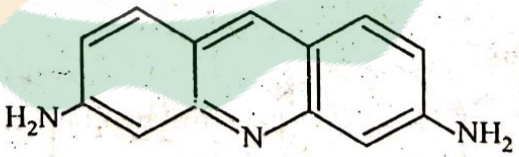
(ii) Ethyl methane sulphonate (EMS)—$CH_3CH_2SO_3CH_3$

(iii) Dimethyl sulphate (DMS)

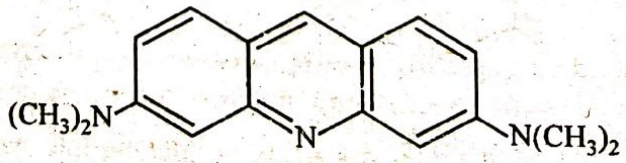
EMS has the specifity to remove guanine and cytosine from the chain and formed gap. Any base (A, T, G, C) inserted in the gap. During replication chain without gap will result in normal DNA. In the second round of replication gap is filled by favourable base. When the correct base is inserted, normal DNA sequence will be produced. Insertion of incorrect bases result in transversion of transition mutation.

4. Intercalating Agents

There are many dyes like acridine orange, proflavine and acriflavin which are three ringed molecules of similar dimensions as those of purine



Proflavin



Acridine Orange

Fig. D. Chemical structure of two mutagenic acridine derivatives.

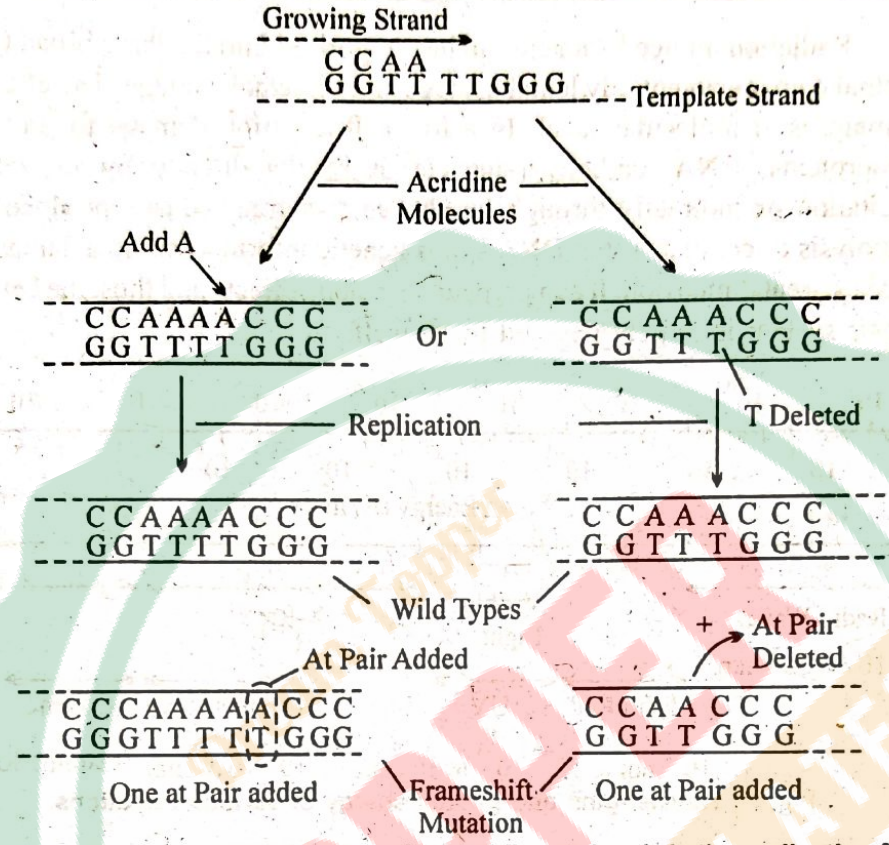


Fig. E. Mechanism of intercalation of an acridine molecule in the replication fork.

pyrimidine pairs. In aqueous solution these dyes can insert themselves in DNA (i.e., intercalate the DNA) between the bases in adjacent pairs by a process known as **intercalation.** Thus the dyes are known as intercalating agents. The acridines are planer (flat) molecules which can be intercalated between the base pairs of DNA, distort the DNA and results deletion or insertion after replication of DNA molecule. Due to deletion or insertion of intercalating agents, there found frameshift mutations. ●

☛ **Q.8. Write an essay on radiation's as mutagens.**

Ans. **Radiations as Mutagens**

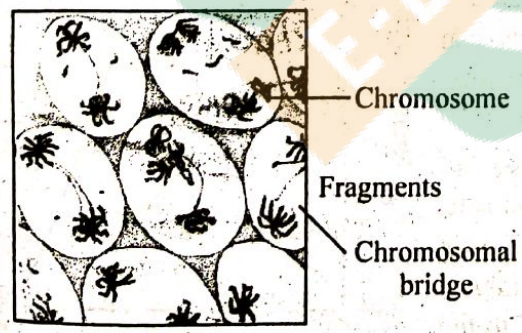


Fig. Radiation-induced chromosomal bridges and fragments in cells of X-rayed anthers of *Trillium*.

Radiation is the most important phenomenon among the physical mutagens. Radiations damaging the DNA molecules exists in the wave length range below 340 nm and photon energy above 1 electrovolt (eV). The destructive radiation X-rays, γ-rays ultraviolet (UV) rays, alpha (α) rays, beta (β) rays, cosmic rays, neutrons etc.

Radiation induced damage can be categorised into the three broad types : lethal damage potentially lethal damage and sublethal damage. The effect of damage is at molecular level. In a live cell radiation damage to proteins, lipoproteins, DNA, carbohydrates etc. is results directly by ionization/ excitation or indirectly through highly reactive free radicals produced by radiolysis of cellular water. DNA stores genetic informations so a damage to it takes great dimension. It can perpetuate genetic effects and thus, the cellular repair system is largely devoted to its welfare.

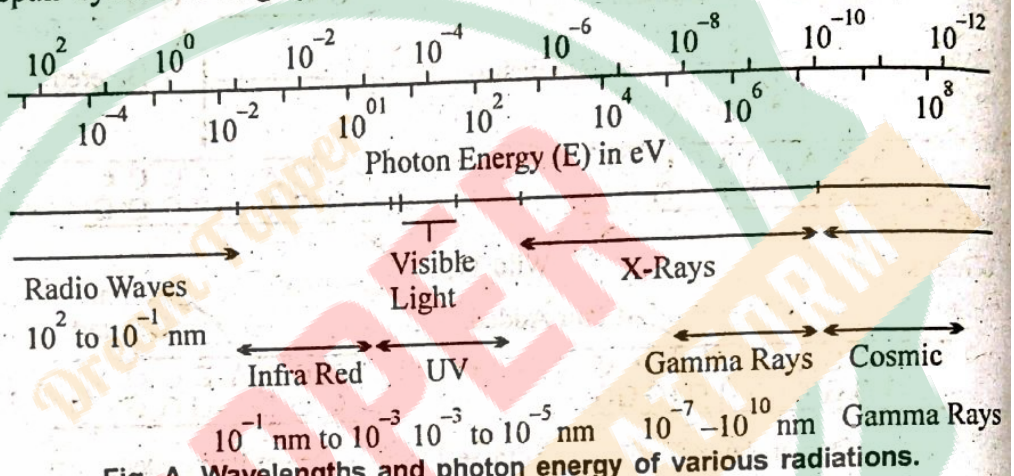


Fig. A. Wavelengths and photon energy of various radiations.

If the bacteria are exposed to radiation they gradually lose the ability to develop colonies. This gradual loss of viability can be expressed graphically through plotting the surviving colonies against the gradually increasing exposure time. This dose-response graph is known as **survival curve.** The survival curve is analysed through a simple mathematical theory called hit theory.

Hit Theory : Each organism possesses at least one sensitive site which is called target site. Radiation photons damage or hit the target site and inactivate the organisms. One can derive the equation based on this theory. The equations help to determine the survival curve for many kinds of populations of N identical organisms exposed to dose D of radiation causing damage. The number dN damaged through a dose dD is proportional to the initial population that has not received radiation; thus, $dN = KN$

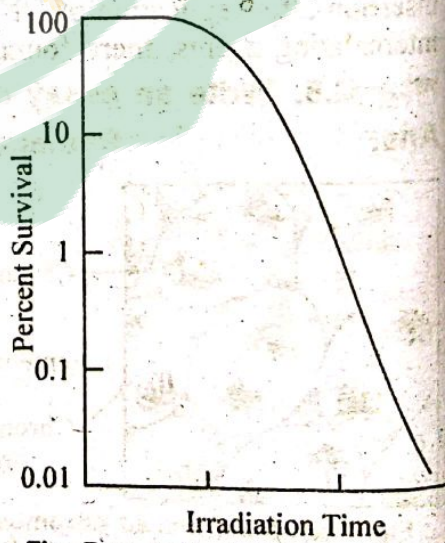


Fig. B. A typical ultra violet light survival curve for a bacterium.

where,

K is the constant which determines the effectiveness of dose.

Integrating this equation from N = No at D = O, we have

$$N = No^{e-KD} \quad ...(1)$$

The surviving fraction S = N/ No is

$$S = N/NO = e^{-KD} \quad ...(2)$$

A plot of S verus D gives a straight line with a slope of - K as shown in (Fig. B). This type of curves are known as exponential or **single hit curve.** The exponential curve is obtained if the phages are irradiated with X-rays.

When there is a population of different organisms and each organism consists of atleast *n* sites, each site must be hit to inactivate an organism. Thus, each organism is hit through n times. The probability of one unit being hit by a dose D is, $P = 1 - e^{-KD}$ thus, the probability of Pn will be

$$Pn = (1 - e^{-KD})^n$$

The surviving fraction S of the population is 1-pn or

$$S = 1 - (-e^{-KD})n \quad ...(3)$$

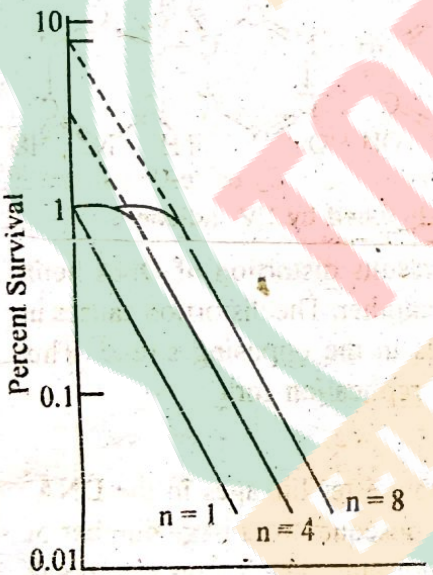


Fig. C. Survival curve for different values of n (hits time).

This equation can be expanded as follows :

$$S = 1 - (1 - ne^{-KD} + e^{-nKD})$$

At the large value of d, the higher order terms become negligible as compared to ne^{-KD}. Thus, at high dose D, $S = ne^{-KD}$ or

$$\text{In} \quad S = \text{In } n - KD \quad ..(4)$$

If the equation 3 is plotted for K = 1, various values of n reveals that for small values of D, In S gradually changes as shown in (Fig. C). At large value of D, equation 4 dominates and curve becomes linear. Freifelder has discussed the hit theory.

(A) Ultraviolet (UV) Radiation

UV radiation causes damage in the DNA duplex of the bacteria and phages. The ultraviolet rays are absorbed and results excitation of macromolecules. The absorption maxima of nucleic acid and protein are more or less similar. The DNA molecule is the target molecule for UV rays

but not the proteins. While, absorption spectrum of RNA is quite similar to that of DNA. The excited DNA leads to cross-linking, single strand breaks and base damage like minor cohesion and generation of nucleotide dimer like a major one. Purines are generally more radio-resistant than the pyrimidine of the latter, thymine is more reactive than cytosine. Thus, the ratio of thymine-thymine, thymine-cytosine, cytosine-cytosine (CC) dimer as show in Fig. (D) is 10:3:3, respectively. A few dimers of TU and UU also appear. The initial step in pyrimidine dimerization is known to be hydration of their 4 : 5 bonds.

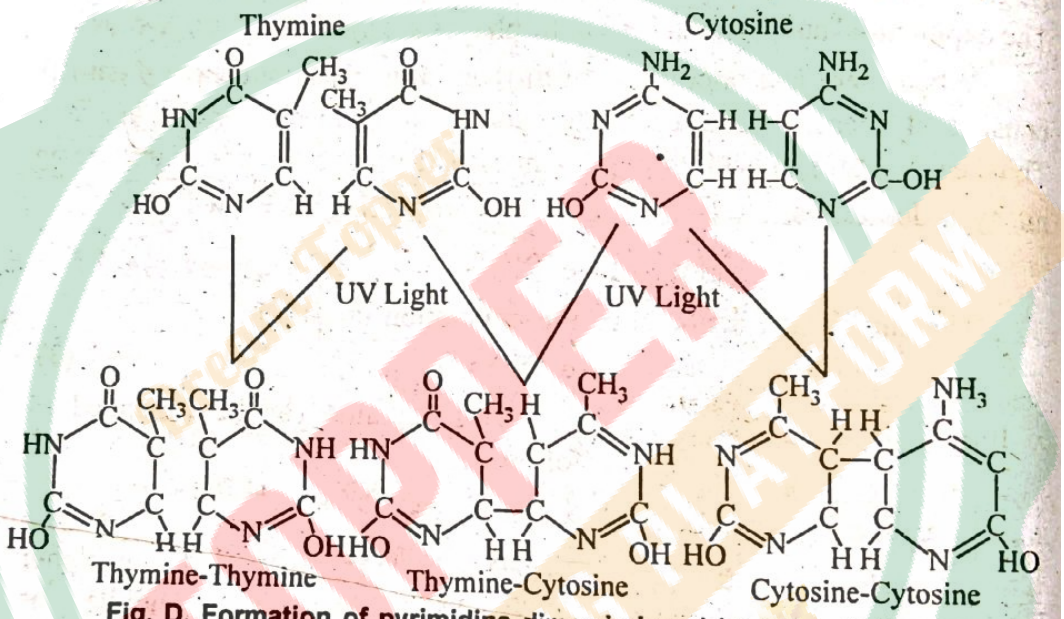


Fig. D. Formation of pyrimidine dimer induced by UV radiation.

Formation of thymine-thymine dimer results distortion of DNA helix because the thymines are pulled towards one another. The distortion causes in weakening of hydrogen-bonding to adenines in the opposing strand. This structural distortion inhibits the advance of replication fork.

(B) The X-Rays

The X-rays results breaking of phosphate ester linkages in the DNA. This breakage found at one or more points. Consequently, a large number of bases are deleted or rearranged in the DNA molecule. The X-rays may break the DNA either in one or both strands. Then breaks occur in both strands, it become lethal. The DNA segment between the two breaks is removed resulting in deletion. As both the X-rays and UV rays bring about damage in DNA molecule, they are used in sterilization of bacteria and viruses.

DNA Repairs Systems

The main objectives of biological system is to maintain base sequences of DNA from one generation to the other. Changes in DNA sequence arise

during replication of DNA damage. Through chemical mutagens and radiation. In replication when incorrect nucleotides have been added, they are corrected through editing system by DNA Pol I and DNA Pol III. The other systems also exist for correcting the errors missed by editing function. It is known as **mismatch repair** system. Mismatch repair system edits the errors left by DNA Pol I and DNA III and removes the wrong nucleotides. Proof reading by Pol I and III has earlier been described.

DNA is always damaged and mutated by various chemicals and radiation as discussed earlier. Only a few errors accumulate in DNA sequence. The stable errors cause mutation and the rest are eliminated. When errors in DNA sequence are corrected before cell division, no mutation occurs. While, there are some DNA damages which cannot be mutated because the damages are not replicated. Thus, such damages cause cell death.

There are several types of damages that occur in DNA : (a) modification of one or more bases by highly reactive chemicals such as alkylating agents like nitrosoamine and nitrosoguanidine, (b) loss of purine bases due to lcoal

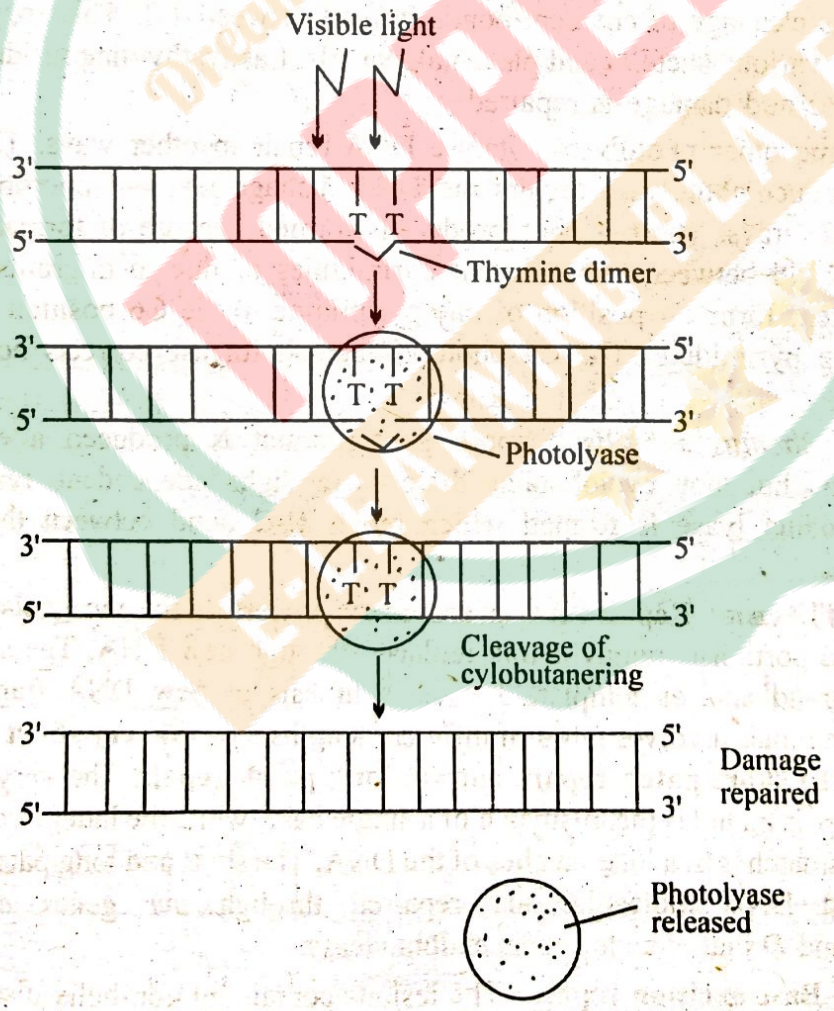


Fig. A: Photo reactivation for repair of thymine dimer.

pH change, (c) single strand or double strand break because of bending or shear forces, (d) dimer formation between two adjacent pyrimidine molecules due to ultraviolet and X-ray radiation because of dimerisation no hydrogen bond with opposing purine shall found. This causes in distortion of helix. Most of the spontaneous erros are temporary because they are soon corrected through a process known as DNA repair.

Mechanisms of DNA Repair

There are four major ways through which thymine-thymine dimer in DNA is repaired : *light induced repair* and *light-independent repair.*

(i) Photoreactivation : The UV damages results in cells are repaired after exposure of cells in visible light. This is known photoreactivation. In this mechanism an enzyme DNA *photolyase* cleaves T-T dimer and reverse to monomeric stage as shown in (Fig. (A). This enzyme is activated only when exposed to visible light. The mutant cells lack photolyase. This enzyme absorbs energy, binds of cyclobutane ring to defective sites of DNA and promotes cleavage of covalent bonds formed between T-T. This enzyme is found in various bacteria and placental mamals. Lastly, thymine residues are made free and damage is repaired.

Some other photolyses catalyse DNA repair in other ways. The 6-4 photoproduct photolyase repairs the DNA damage i.e., 6-4 photoproduct results UV rays. The 6-4 photoproduct is formed because of formation of C4-C6 bond between two adjacent pyrimidines or due to migration of a substitutent form C4 position of one pyrimidine to the C6 position of the adjoining pyrimidine. The C4 photoproduct photolyase corrects both the errors.

In *Bacillus subitilis* a spore photoproduct is produced after UV radiation, but not cyclobutane dimers. In light-independent reaction, photoproduct lyase is formed which repair C-C bond between the two thymines.

(ii) Excision Repair : It is an enzymatic process. In this mechanism, the damaged portion is removed and replaced through new DNA. The second DNA strand acts as template for the synthesis of new DNA fragment. Excision repair involves DNA of different lengths e.g., (a) **very short patch repair (b) short patch repair,** and (c) **long patch repair.** The very short patch repair includes the mismatch of a single base, while the latter two deals with mismatches in a long patches of the DNA. The short and long patches of damaged DNA molecules are repaired through *uvr* genes e.g., *uvr A, B C* and *D* that encode repair endonuclease.

(a) Base excision repair : The lesions containing non-helix distortion for example alkylating bases are repaired through base excision repair. It involves at least six enzymes known as DNA glycosylases.

Every enzyme recognises at least bases and removes from DNA strand. The enzymes remove deaminated cytosine, deaminated adenine, alkylated or oxidised base. Base excision repair pathway starts with a DNA glycosylation. e.g., the enzyme uracyl DNA glycosylase removes the uracyl that has wrongly joined with G which is really demainated cytosine (Fig. as shown in Fig. B(a). The AP-endonuclease and phosphodiesterase removes sugar-phosphate. AP-sites arise due to loss of a purine or a pyrimidine. A gap of single nucleotide develops on DNA which acts as template-primer for DNA polymerase to synthesise DNA and fill the gap by DNA lysase.

(b) Nucleotide excision repair : Any type of damage having a large change in DNA helix causing helical changes in DNA structure is repaired through this pathway. Such damage may arise due to pyrimidine dimers results due to sun light and covalently joins large hydrocarbon. In *E. coli* a repair endonuclease recognises the distortion produced by T-T dimer and makes two cuts in the sugar phosphate backbone on each side of the damage. The enzyme DNA helicases removes oligonucleotide from the double helix containing damage. DNA polymerase III and DNA ligase repair the gap produced in DNA helix as shown in Fig. B(b).

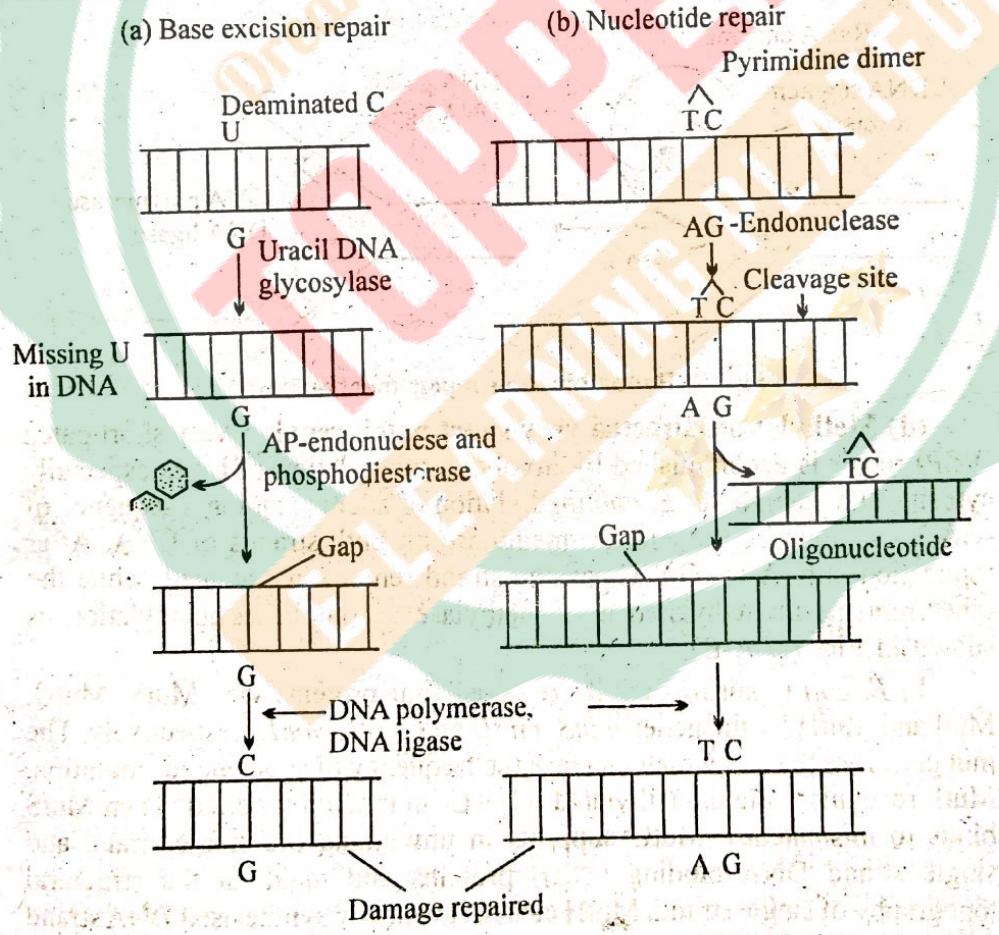


Fig. B. Excision repair pathways; (a) base excision repair (b) nucleotide excision repair.

(c) Recombination repair (daughter-strand gap repair) : If excision repair mechanisms fails, this mechanism, is needed to repair errors. This mechanisms, operates in the viral chromosome in host cell whose DNA is damaged. This mechanism operates mere after replication; therefore, it is also called *post-replication repair.*

Probably Rec A protein in *E. coli* catalyses DNA strand for sister-strand exchange. Therefore a single stranded DNA segment without any defect is excised from a strand on the homologous DNA segment at the replication fork. It is inserted into the gap created by excision of thymine dimer as shown in Fig. (C). As a results the combined action of DNA Pol I and DNA ligase joins the inserted piece. The gap formed in donor DNA molecule is also filled through DNA Pol I and ligase enzymes.

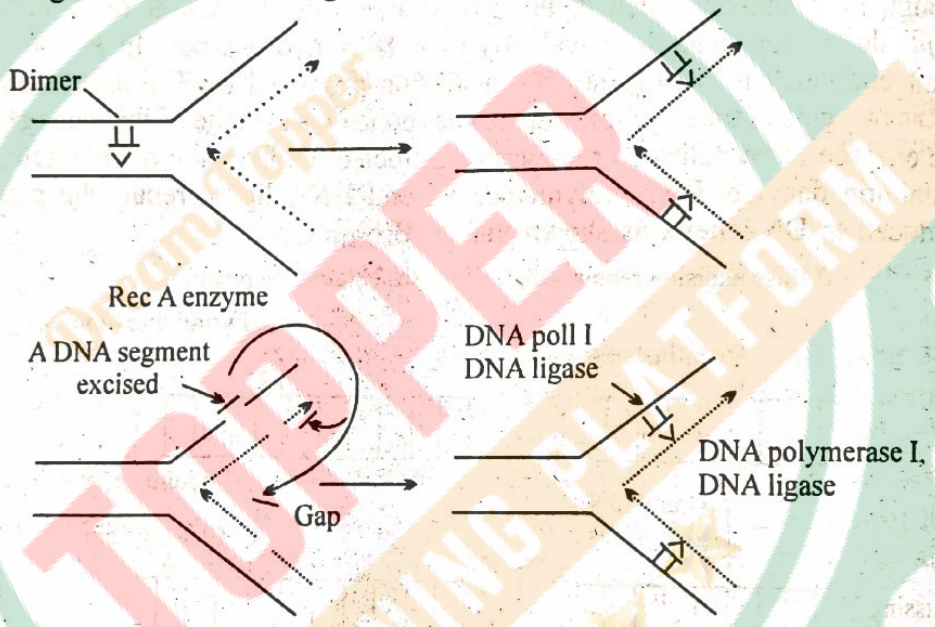


Fig. C. Recombination repair mechanism.

(d) Methylation-directed very short patch repair : Very short patch (VSP) repair is accomplished by involving methylation of bases especially cytosine and adenine. In *E. coli* methylation of adenine and in a sequence of -GATC- is done by the enzyme methylase on both strands of DNA. After replication only A of -GATC- of one strand remains methylated, while the other remains unmethylated until methylase accomplishes methylation as shown in Fig. (D A-B).

In *E. coli* repairing activity required four proteins viz., MutS, MutU, Mutl and MutH by the genes *mutS, mutU* and *mutL, mutH,* respectively. The mut genes are the loci which increase the frequency of spontaneous mutation. MutL recognises the unmethylated -GATC- in transition period. Then MutS binds to mismatches. MutU supports in unwinding the single strand and single strand DNA binding (SSB) proteins and maintain the structural topography of single strand. MutH cleaves the newly synthesised DNA strand and the protein MutU separates the mismatch strand (A).

While, there is a gradient of methylation along the newly synthesised strand. Least methylationoccurs at the replication fork. The parental strand is uniformly methylated. The methylated bases direct the excision mechanisms of the newly synthesised strand containing the incorrect uncleotides (B). In this transition period, the repair system works and distinguishes the old and new strands and repairs only the enw strands.

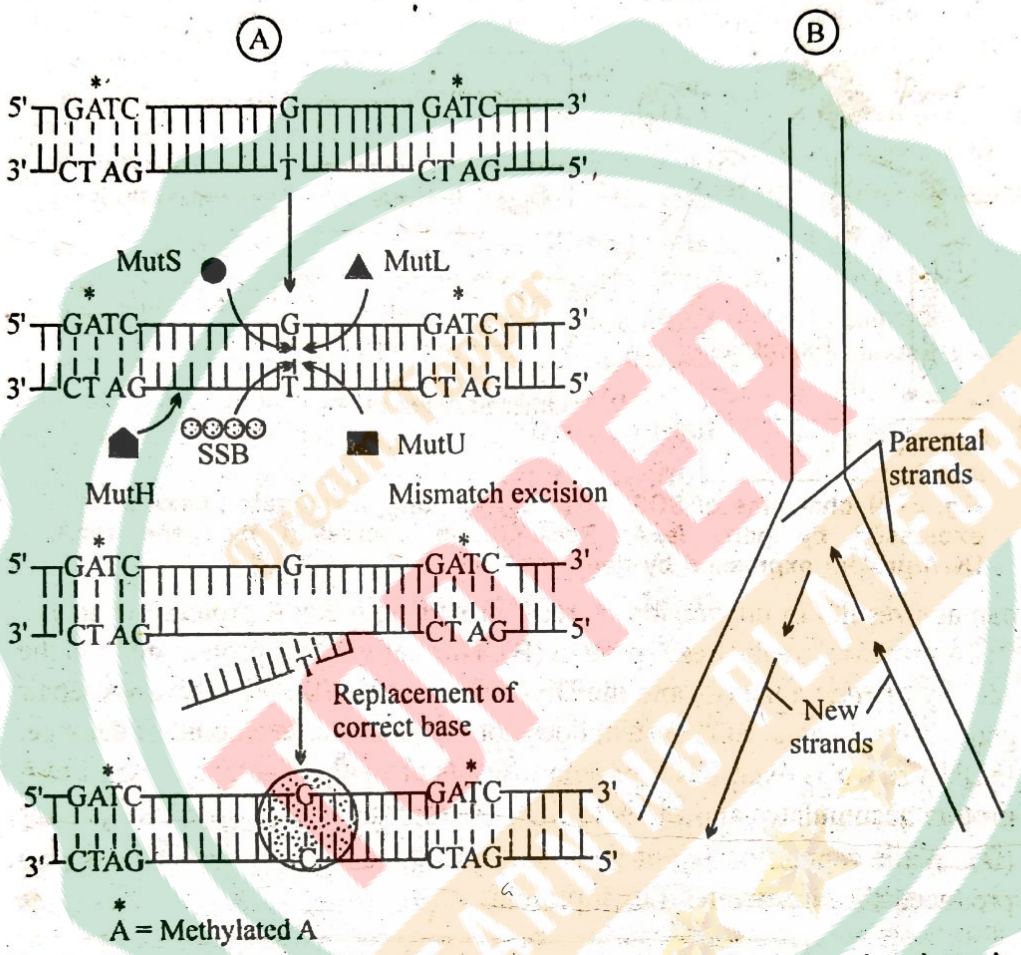


Fig. D. Mismatch repair. (A) excision of a newly synthesised strand and repair system; (B) arrows shows the region where methylation is not complete and dark region line shows the region where methylation is complete.

(e) SOS Repair : SOS repair is by-pass repair system. It is also known as emergency repair. The damage in DNA itself induces the SOS regulatory system which is a complex cellular mechanisms. SOS works where photodimers are formed which lead to cell death SOS is the last attempt to minimise mutation for survival. It induces a number of DNA repair processes. SOS system acts in the absence of a DNA template. Thus, many errors arise leading to mutation.

Generally genes of SOS system remains in repressed condition caused through a protein LexA. Repression of SOS is inactivated by RecA protease. It is formed after the conversion of RecA protein by DNA damage. DNA

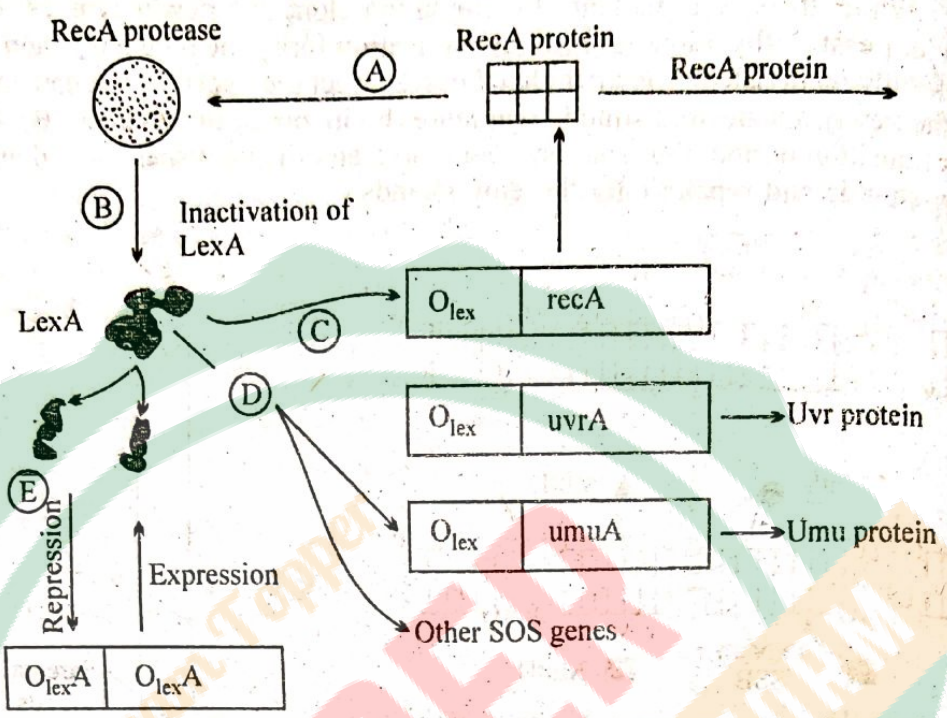


Fig. E. Mechanisms of SOS repair system O_{lex} lexoperator, LexA-protein expressed by gene *lexA;* RecA-protein expressed by gene *recA;* UvrA-protein expressed by gene *uvrA.*

damage results in conversion of recA protein into RecA protease (Fig. A). RecA protease breaks LexA protein (B). Normally, LexA protein inhibits the activity or *recA* gene (C) and the DNA repair genes (D). Finally, dNA repair genes are activated. SOS system does not repair the large amount of damage. If DNA repair is over, RecA protein loses its proteolytic activity. Then LexA protein accumulates and binds to SOS operator and turns off SOS operator (D). While repression is not complete. Beside, some RecA protein is also produced that inactivates LexA protein.

SHORT ANSWER QUESTIONS

Q.1. Write short note on silent mutation.

Ans. **Silent mutation**

Silent mutation is another type of point mutation that could not be detected until the nucleic acid sequencing is done. Any change in gene does not affect the phenotypic expression as the code is degenerate *i.e.*, more than one code specify an amino acid. For example, when the codon CGU is changed to CGC, still it would code for arginine. Similarly, both AAG and AAA specify alanine. When the codon AAG is changed to AAA, the latter codon will still code for lysine even after change in base sequence of DNA. This mutation is of silent type as even after change in base sequence of DNA, there is no change in the amino acid sequence and expression of phenotype characters.

☛ **Q.2. Explain the second site mutation.**

Ans. Second Site Mutation or Suppressor Mutation

Spontaneous reversion rarely results in a restoration of the wild type base sequences. Mere Leu^- mutats observed in a population of about 10^9 Leu^+ cells and mutation causing mutants would be distributed over 100 different sites. After the growth of these cells, in a second population, there may also be 100 mutants some having mutation at sites present in the first population but most of these having mutation on other sites. In various population there will be about 500 mutation uses when a reversion occurs at one particular site, it yields wild type base sequence. On average mere $100/500 \times 10^9$ would be mutated at that site. Hence, the phenomenon of reversion into wild type phenotype by a second mutation in a different gene is known as second site mutation or suppressor mutation.

The second site mutation overcomes the effect of first mutation. If the second mutation is within the same gene, the mutation is known as a second site reversion or intragenic suppression. While the reverant phenotypes are wild type, the original sequences of DNA will not be found.

☛ **Q.3. Write a short note on following :**

(i) Non sense Mutations.

(ii) Missense Mutations.

Ans. (i) Non-sense Mutations

Most of the mutations affect only one base pair in a given location, therefore, these mutations are known as point mutation or **gene mutation.** There are different types of point mutations. Nonsense mutation is one type of point mutation. There are 64 codons that code for amino acid out of which three codons are called termination codons that do not encode for any amino acid. If any change occurs in any codon, it brings about changes in amino acids which specify an amino acid to termination codon. This process is known as **non-sense mutation.** For example, UAC codes for tyrosine. When undergoes base substitution (C-G), it becomes UAG i.e., a termination codon. This results in synthesis of incomplete polynucleotide chain which remains inactive mere a fragement of wild type protein is produced which has a little or no biological function unless the mutation is very near to the carboxyl terminus of the wild type protein. The non-sense mutations bring about drastic change in expression of phenotypic characters because in such mutation the structure and function of enzymes are changed.

(ii) Missense Mutations

Missense mutation is other type of point mutation. It one amino acid in a polypeptide chain is replaced by the other amino acid, this type of mutation is called missense mutation. e.g., when a protein valine has been mutated to aspartic acid due to loss of activity, it can be restored through the wild type phenotype by a missense suppressor that substitutes alanine for asparatic acid.

A missense mutation occurs by insertion, deletion or substitution of a single base into a code, e.g., the codon GAG specifying glutamic acid could be changed to GUG which codes for valine, Missense mutation which arises from substitution synthesises proteins which differ from the normal protein by a single amino acid. Substitution occurs in three different ways : (i) a mutant tRNA may recognise two codons perhaps by a change in anticodon loop, (ii) a mutant tRNA can be recognised by a wrong aminoacyl synthetase and be misacylated and (iii) a mutant synthetase can change a wrong tRNA molecule. While, when a supperssor that substitutes alanine for aspartic acid works with 20% efficiency, every protein to which a cell synthesises atleast one aspartic acid is replaced. In this situation a cell probably cannot survive.

Inspite of substitution of a single amino acid many proteins are still functional. It depends on type and location of amino acid. e.g., when a non-polar amino acid in the polypeptide chain is replaced through a polar amino acid, it will drastically change the three dimensional structure of the protein and also change the function. But when the polar amino acid is replaced by the another, there will be little or no effect on protein. Missense mutation plays an important role in providing new variability in organisms and driving the evolution as are not lethal and remain in the genome.

☛ **Q.4. What do you mean by reversion? Explain.**

Ans. **(I) Reversion**

Reversion is the phenomenon of reverting of a mutant organism to the original phenotype characteristics. Reversion may occur spontaneously through induction or mutagens, e.g.

Forward Reverse

$$\underset{\text{wild type}}{\text{AAA (Lys)}} \xrightarrow{\text{Forward}} \underset{\text{Mutant}}{\text{GAA (Glu)}} \xrightarrow{\text{Reverse}} \underset{\text{Wild type}}{\text{AAA (Lys)}}$$

$$\underset{\text{wild type}}{\text{UCC (Ser)}} \xrightarrow{\text{Forward}} \underset{\text{Mutant}}{\text{UGC (Cys)}} \xrightarrow{\text{Reverse}} \underset{\text{Wild type}}{\text{AGC (Ser)}}$$

The reversion frequency of an organism is a useful criterion for the identification of point mutation. While when if deletion has taken place, reversion will not found. In bacteria, reversion events can be detected by measuring the ability of formation of bacterial colonies on solid growth medium. e.g., when 10^7 Leu^- bacterial cells are plated on solid medium devoid of leucine about 60 colonies are formed. These cononies must be consisting of Leu^+ bacteria that would have reverted spontaneously form Lac^- to Lac^+cells. Thus, the reversion frequency can be calculated as follows :

No. of colonies formed = 60

No. of cells plated = 10^7

Reversion frequency = $60/10^7 = 6 \times 10^{-6}$

This value of frequency is a feature of the reversion of point mutants. As, the production of spontaneous reverants found randomly, the reversion of

a double mutants requires two independent events. Hence, the reversion frequency would b $(6 \times 10^{-6})^2 = 3 \times 10^{-11}$ ●

☛ **Q.5. What is spontaneous mutation? Explain the random nature of mutation.**

Ans. **Spontaneous Mutation**

There is no means to know if and which cell will undergo mutation. Any gene of a cell of microorganisms is vulnerable to mutation. It is not sure, while which gene will mutate. Every gene is subject to mutation, thus, mutation occurs in a gene spontaneously and there is possibility of mutating the genes in a cell and more probability of occurrence of mutant allele in a given population of a microorganism. In addition, it is not sure that mutation will be beneficial, it may be even deterimental too. Hence, the spontaneous mutation arises randomly in a population of organisms. Spontaenous mutations are rare ranging from 10^{-6} to 10^{-8} per generation depending on the gene and organism.

The Random Nature of Mutation

Before 1940's it was believed that mutation occurs in bacterial population in response to a given selective condition. It means a medium containing antibiotic substance. But Luria and Delbruck demonstrated the spontaneous and non-adaptive nature of mutation. These experimentations gave birth to microbial genetics. They found the origin of mutation in *E. coli* conferring resistance to phage T1 infection. The number of TI^{-r} mutant cells arising in different cultures of TI^{-s} cells was compared with the number found in repeated samples of the same size taken from a single culture. The result was analysed by a statistical test named as fluctuation test. The number of cells increased to 2.8×10^9 cells/ml. Each of small culture and ten of 0.2 ml of large culture were plated onto individual plate which were already uniformly spread with 10^{10} particles of T1 phage. After incubation the number of $T1^{-r}$ colonies were counted. The number of bacterial cells inoculated in each plate was the same but the number of Tl^{-r} colonies depended on whether the cells had grown in small individual culture or in large culture. Out of 20 cultures no $T1^{-r}$ cell was detected in 11 small cultures. In rest of 9 cultures the number of $T1^{-r}$ cells ranged from 1 to 10^7. On the other hand, each of 10 samples of large culture had the same number. This experiment indicates that $T1^{-r}$ cells arose by spontaneous mutation at different times in the growth of cultures in the absence of phage T1, thus, the number in different cultures varied greatly. While, when $T1^{-r}$ cells arise in response to phage, these should be about equal numbers in all population of the same size. ●

☛ **Q.6. Explain the evidences for spontaneous mutation.**

Ans. **Evidence for Spontaneous Mutation**

Lederberg and Lederberg (1952) gave the direct evidence for the origin of spontaneous mutation in $T1^{-r}$ cells of *E. coli* without exposing to phage.

This was presented through a procedure known as **replica plating** as shown in Fig. The steps of replica plating involve : (a) plating of bacteria on nutrient

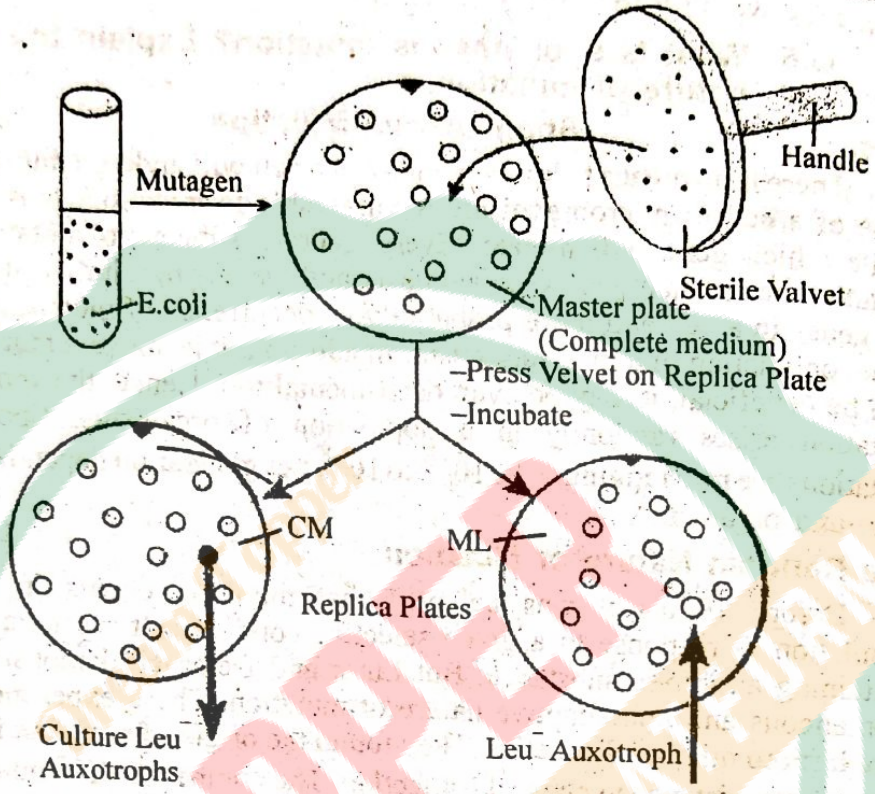


Fig. Replica plating technique for selection of $T1^{-r}$ E. *coli* cells.

medium and proper incubation of plates for growth of bacterial colony, (b) preparation of a solid support mounted with a piece of sterile velvet and gentle pressing it onto surface of plate supporting bacterial colonies, (c) again gentle press of velvet on a Pertiplate containing fresh medium that allowed to transfer the bacterial cells on fresh plate. Precaution was taken to have the identical position of both the plates and velvet also. The master plate contained 10^7 colonies of bacteria, growing on non-selective medium, while the replica plate contained T1 phage particles spread onto medium. After proper incubation a few $T1^{-r}$ cells formed colonies in the same position on each of replica plate as in master plate. The master plate did not contain T1 phage. Thus, appearance of $T1^{-r}$ cells on replica plate can be explained to occur due to the mutation for resistance that had taken place by chance in the master plate not exposed to T1 phage particles.

Q.7. Discuss the Mutation Rates.

Ans.

Mutation Rates

The probability of a gene undergoing mutation in a single generation is called mutation rate. For the study of population genetics, evolution and analysis of effect of environment mutagens and measurement of mutation rates are important. In bacteria, a mutation can found at any time during growth in culture. The mutant bacterial cell divides and increases the number

at the same rate as the normal cells divide. Thus, measuring the mutation rates is rather complicated.

Fluctuation test is an important method for estimation of mutation rates in bacteria. A culture may contain many mutants, some mutants or none. When mutation rate per generation is μ, the probability of getting *n* mutants in a culture of N cells is Po, through the Poisson distribution $e^{-\mu N}$.

The total number of divisions of individual cell needed to yield N cells from 1 cell is N–1, where N is the large number of bacterial cells.

Hence, $\mu N = \text{In Po}$

or $\mu = (1/N)\,\text{In Po}$ ...(1)

The mutation rate can be calculated by substituting the values in equation No. 1

$$\mu = (1/5.6 \times 10^8)\ \text{In}\ (11/20)$$

$$= 1.1 \times 10^{-9} \text{ per cell per round of replication.}$$

For the first time Benzer (1961) found that at a particular site mutation occurs with high frequency than the other site within a gene. He mapped several thousand independently isolated rII mutation in T4 phage. Hence, the favoured sites for mutation at high frequency were called **hot spots.** Coulondre *et al.* (1978) have studied the molecular basis of abse substitution hot spots in *E. coli.*

☛ **Q.8. Write a short note on uses of mutations in microorganisms.**

Ans. **Uses of Mutations in Microorganisms**

The most interesting and important advancement in microbial genetics in the use of mutation for detection of many more special features of biochemical interest and enhanced production of useful metabolic products.

Main example are given as follows :

(i) For Understanding the Metabolic Regulation

Various bacterial mutants have been isolated which show changes in amount of a particular protein of its responses to external signals. e.g., the enzyme synthesized by *gal*K, *gal*T and *gal*E genes are normally not present in bacteria. These are synthesized only if galactose is supplied in the growth medium. In addition, these mutants have also been isolated in which these enzymes are always present irrespective of presence or absence or galactose in the growth medium. Indicates that a regulatory gene must be associated for switching on or switching off the enzyme production.

(ii) Demonstration of Metabolic Pathways in Microorganisms

It has been demonstrated by isolating the three different classes of gal mutants that galactose is utilized by three distinct genes, *ga/K, ga/T* and *ga/*E. The Gal^+ cells are grown on medium containing radioactive galactose. Since the ^{14}C-Gal is metabolized, many differnt radioactive compounds can be found in the growth medium. In the beginning of addition of ^{14}C-Gal three radiolabelled compounds viz., ^{14}C-galactose 1-phosphate, ^{14}C-uridine

diphosphogalactose (UDP-Glu) and ^{14}C-uridine diphosphoglucose are detected. When mutation occurs in three different genes, it will block the specific step of metabolic pathway. e.g., the mutant cells containing a *gal*K⁻ mutation would not be able to metabolize ^{14}C-Gal, therefore, ^{14}C galactose remains unutilized because *gal*K gene product converts into the first metabolic product. The *gal*T-mutant cannot convert the first metabolic product into the second product, but accumulates Gal-1-P. Indicates that the first step of galactose metabolism is the conversion of galactose by *gal*K gene product to gal-1-P. However, if *gal*E mutant is used the UDP-Gal is found in the culture medium. Indicates that *gal*E gene is associated with conversion of UDP-Gal into a product X. On the basis of conversion of these products the biochemical pathway must be as follows :

$$\text{Gal} \xrightarrow{\text{gal K}} \text{Gal-1-P} \xrightarrow{\text{gal T}} \text{UDP-Gal} \xrightarrow{\text{gal E}} X$$

(iii) Determination of Function

A mutation defines a function. For example, a wild type *E. coli* cells can uptake lactose from 10^{-5} M solution by a passive diffusion through the cell membrane. But the mutants cannot uptake lactose even at the concentration higher than 10^{-5} M. It indicates that the genetically determined process is involved in lactose uptake.

(iv) For Locating the Site of Action of External Agents

An antibiotic, rifamycin is known to inhibit RNA synthesis. In the begining it was unknown about the precise activity of rifamycin whether it works through checking the synthesis of precursor molecule by binding to DNA and in turn through inhibiting the transcription of DNA into RNA, or by binding to RNA polymerase. Two types of rifamycin resistant mutants were isolated. First with altered cell wall in which rifamycin could not enter and the second with altered RNA polymerase. These findings prove that the antibiotic rifamycin acts through binding with the enzyme RNA polymerase.

(v) For Matching a Biochemical Entity with a Biological Function

E. coli synthesizes an enzyme, DNA polymerase that polymerizes the DNA. It was thought that DNA polymerase I is also synthesizes the bacterial DNA. In Pol A-mutant of *E. coli* the activity of polymerase I has been observed reduced by 50 time. After biochemical analysis of cell extracts of Pol A-mutants of *E. coli* two other enzymes, DNA polymerase II and DNA polymerase III were isolated. The purified enzymes synthesized the DNA molecule.

In another study, a temperature sensitive mutation in *dna*E gene was detected which was not able to synthesize DNA at 42°C, but synthesized the DNA normally at 30°C.Form the culture of DNAE⁻ (Ts) mutant the enzyme DNA polymerase I, II and III were isolated and assayed separately. It was observed that DNA polymerase I, II and III were active at 30°C and 42°C and polymerase III was active only at 30°C but not 42°C.