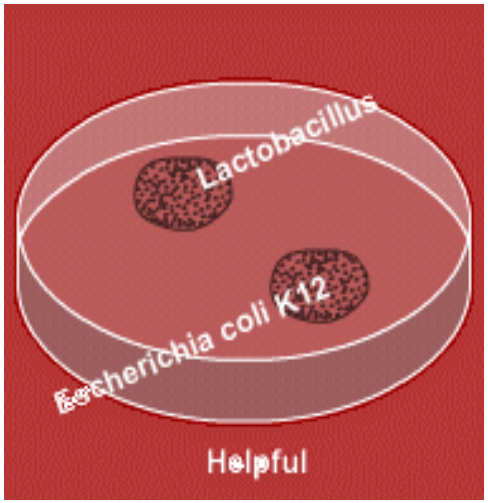
STRAIN DEVELOPMENT STRATEGIES



Dr. Bhavesh Patel
Principal
V P & R P T P Science College, Vallabh Vidyanagar

Introduction

- Use of wild type organism at industrial scale is a rarity
- An ideal microorganisms produces large quantities of desired commercial metabolite at a minimum cost.
- Thus, our basic need is : HIGH PRODUCTIVITY,
  REDUCED COST
- One way to reduce the cost is to develop variants of organism which produces greater amount of metabolite.
- The program designed for doing this is known as **Strain Improvement Techniques**
- The other way is to look for the strain that requires shorter fermentation time, have reduced oxygen need, exhibit less foaming, able to metabolise less expensive substrates etc.

Regulatory mechanism of microorganisms

- Microorganisms have certain regulatory mechanism that control the metabolite synthesis therefore they can not synthesis excess of metabolite over limiting the cells requirements.

- Suppression of these mechanism is necessary to developed the strains of higher yield of desired metabolite.

- There are several types of regulatory mechanism which will be discussed in detail later on in this unit

Methods used for strain improvement

- Mutation
- Recombination
- Protoplast fusion
- Site directed mutagenesis
- Gene technology

Mutation

- Any heritable change in the DNA sequence (quantitative or qualitative) of organism is known as Mutation.
- Mutations are of two types: Spontaneous & Induced Mutations.
- Strain improvement is accomplished by producing a mutant strain with the help of physical & chemical mutagens.
- For example A mutant strain of *Streptomyces aureofaciens* produces 6-methyl tetracycline in place of tetracycline , it is major commercial form of tetracycline.

Advantages of mutation

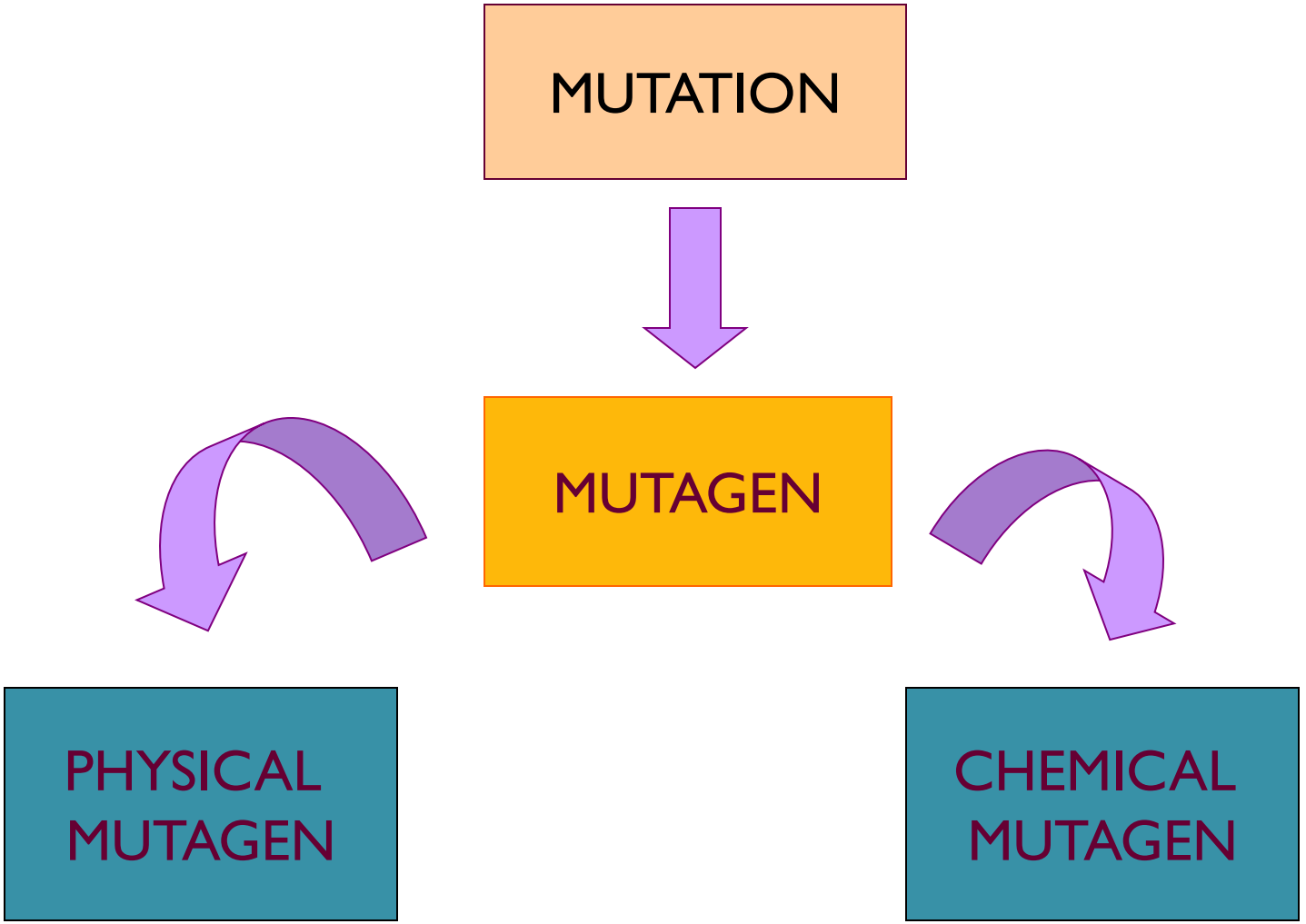
- It is very economic, easy process.
- It is simple mechanism in which no need to study about genome of particular organism.
- Results of strain improvement by mutation is quite promising. For eg. In the early day of Penicillin production the yield was less than 100 units/ml and with the strain improvement programme today it reaches to 85000 units/ml

Spontaneous and Induced mutations

- Mutation occurs spontaneously in nature. It can be induced in laboratory using certain physical or chemical agents called mutagenic agents.
- The rate of spontaneous mutation varies from 10^{-10} to 10^{-5} per generation and per gene. Because of its low frequency it is not advisable to go for spontaneous mutation for strain improvement at industrial scale.
- The frequency of mutation can be significantly increased by using mutagenic agents. This way the frequency can reach to 10^{-5} to 10^{-3}

Types of mutations

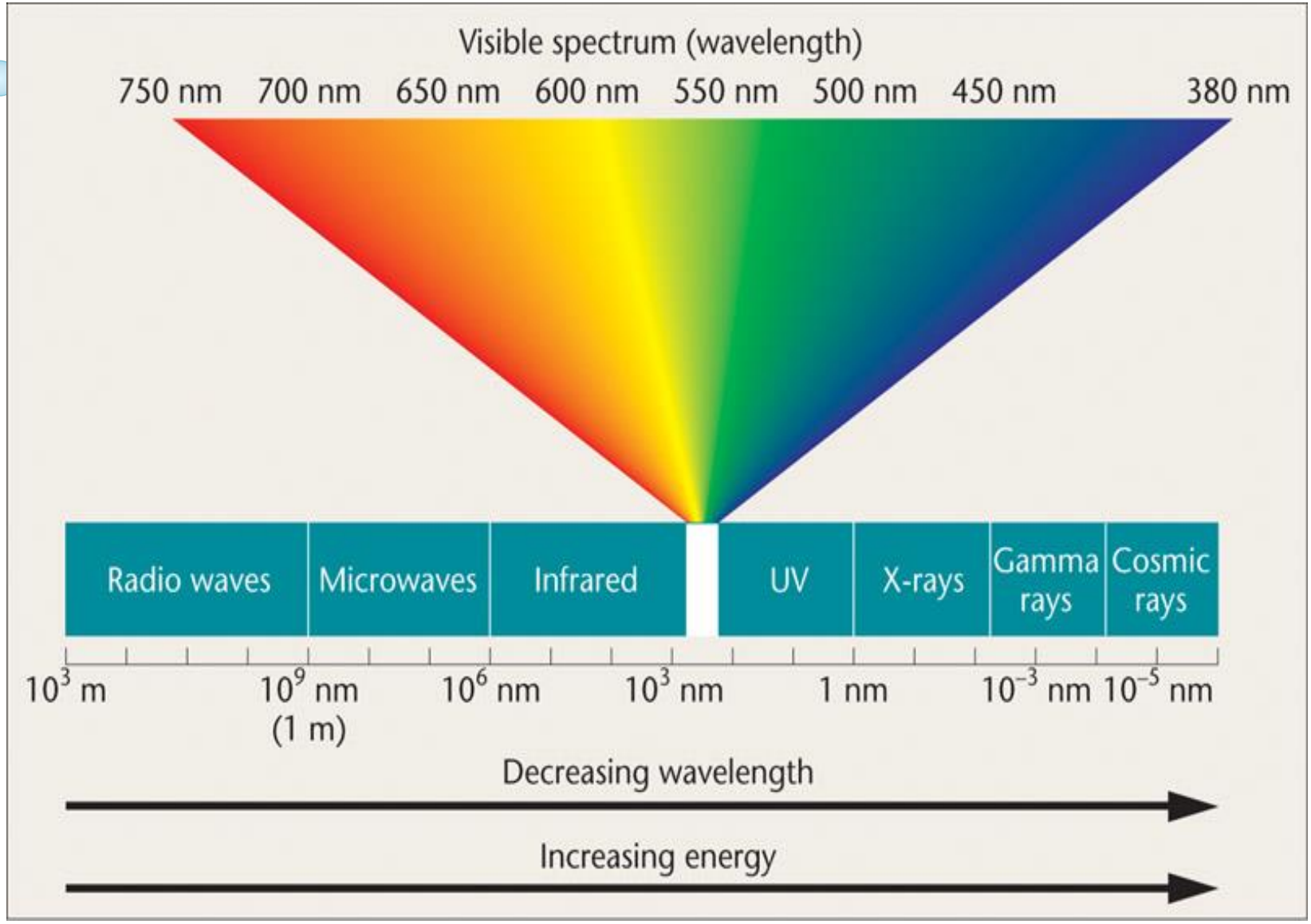
- Base pair substitution – Transition & Transversion
- Frame shift – Addition & Deletion
- Consequences of mutation – Missense mutation, Nonsense mutation, Silent mutation, Suppressor mutation, Forward mutation, Backward mutation, Conditionally expressed mutation etc.


MUTATION
MUTAGEN
PHYSICAL MUTAGEN
CHEMICAL MUTAGEN

Physical Mutagens

- Radiation
  - Ionizing radiations
    (X rays , gamma rays, cosmic rays)
  - Non ionizing radiations
    (UV radiations)

The electromagnetic spectrum



Radiation induced mutation

- The portion of the electromagnetic spectrum, containing wavelengths that are shorter and of higher energy than visible light includes-
  - Ionizing radiations (X rays , gamma rays, cosmic rays)
  - Non ionizing radiations (UV radiations)
- Energy content of a radiation depends on its wavelength
- Generally shorter wavelengths have higher energy
- High energy radiations can change the atomic structure of a substance by causing loss of an electron and formation of an ion. Such radiations are ionizing radiations.
- High energy ionizing radiations and UV radiations are important mutagens.

Genetic effect of radiation

- Break in one or both the strands of DNA (can lead to rearrangement, deletion, chromosomal loss, death if unrepaired)
- Damage to base / loss of base (mutation)
- There are two main ways radiation can damage DNA inside living cells.
  - Radiation can strike the DNA molecule directly, ionizing and damaging it.
  - Radiation can ionize water molecules, producing free radicals that react with and damage DNA molecules.

Nonionizing radiation: UV rays

- UV is the part of the electromagnetic spectrum between visible light and X-rays.

- UV light (200– 400 nanometers) has a unique property as a mutagen.
- Harmful effect of UV is from 240-300nm
- DNA absorbs UV strongly with maximum absorption at 260 nm
- Less energetic but its wavelength is preferentially absorbed by base of DNA and aromatic amino acid of protein

- UV (180-290nm) 'germicidal' most energetic and lethal. At these wavelengths UV kills microorganisms by penetrating cell membranes and damaging their DNA and other intracellular molecules, making them unable to reproduce and effectively killing them.

- In nature solar radiation are one cause of mutation but fortunately UV radiations are of very low radiation and do not penetrate tissue far enough to expose the germ cells of higher organisms.

Ultra Violet Radiations

- The major lethal lesion are pyrimidine dimer in DNA . Pyrimidines, especially thymine absorb strongly at 260 nm
- These are result of a covalent attachment between adjacent pyrimidines in one strands forming : Thymine-Thymine dimer
- This abnormal pairing disrupts the normal pairing of thymine with the corresponding adenine on the opposite strand, causes a bulge in the DNA strand and weakens the hydrogen bonds between T and A on opposite strands
- The damage if not repaired blocks DNA synthesis completely, resulting in cell death

Random photons of ultraviolet (UV) light induce aberrant bonding between neighbouring pyrimidines (thymine & cytosine) bases on the same strand of DNA. The will prevent the replication machine from duplicating the DNA.

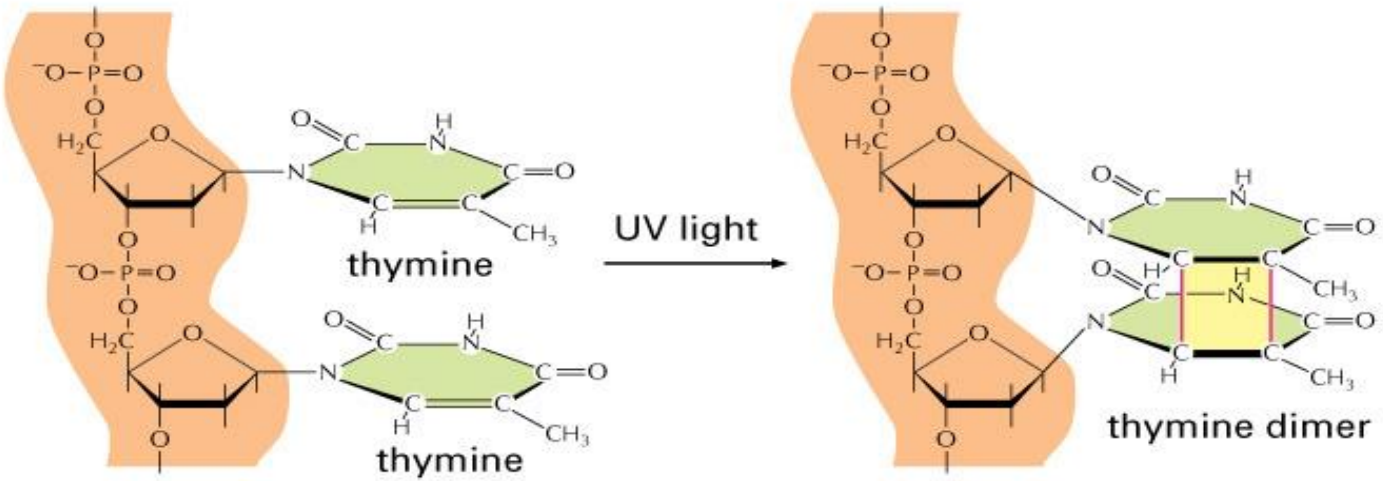
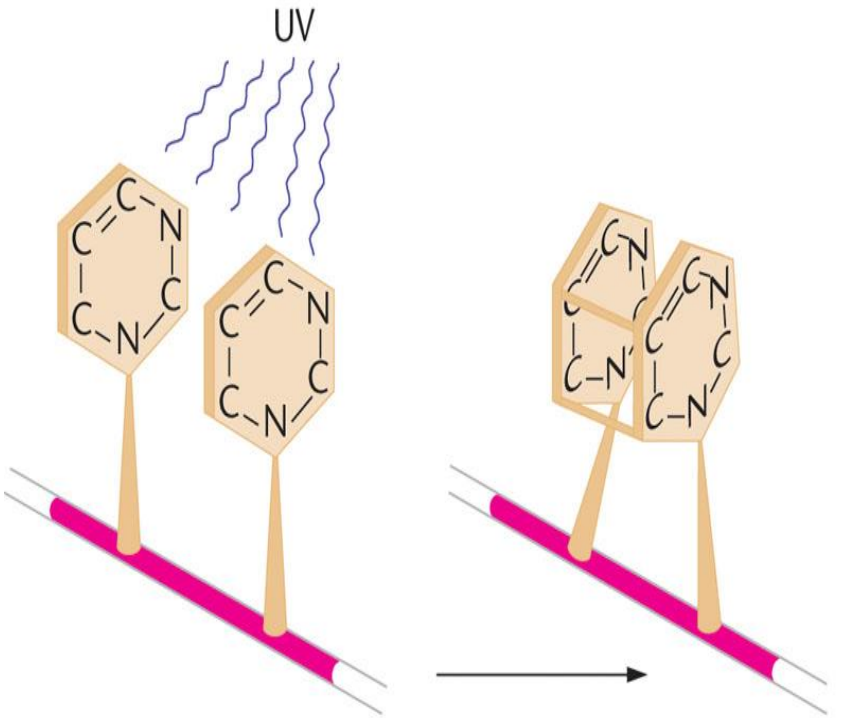
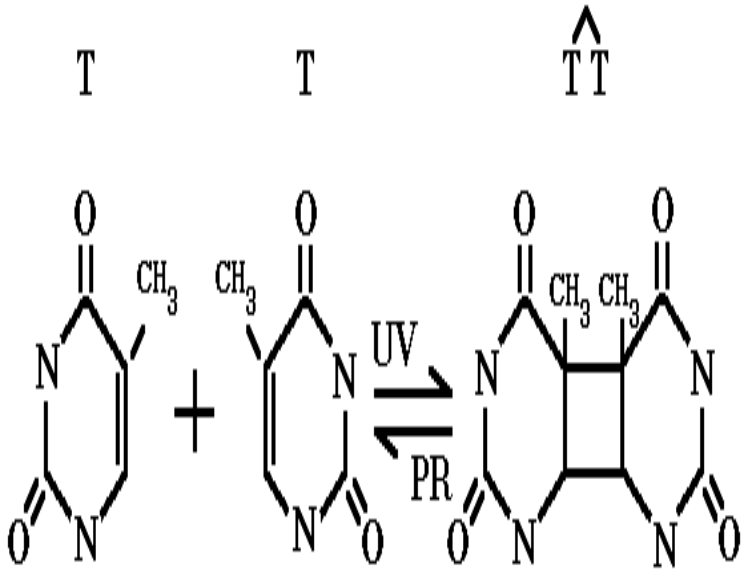


Figure 6-24 Essential Cell Biology, 2/e. (© 2004 Garland Science)

Thymine – Thymine Dimer



Dimer formed between adjacent thymidine residues along a DNA strand



Chemical Mutagens

- The chemical mutagens can be classified in to three based on their mode of action:

- Mutagens that affect non-replicating DNA

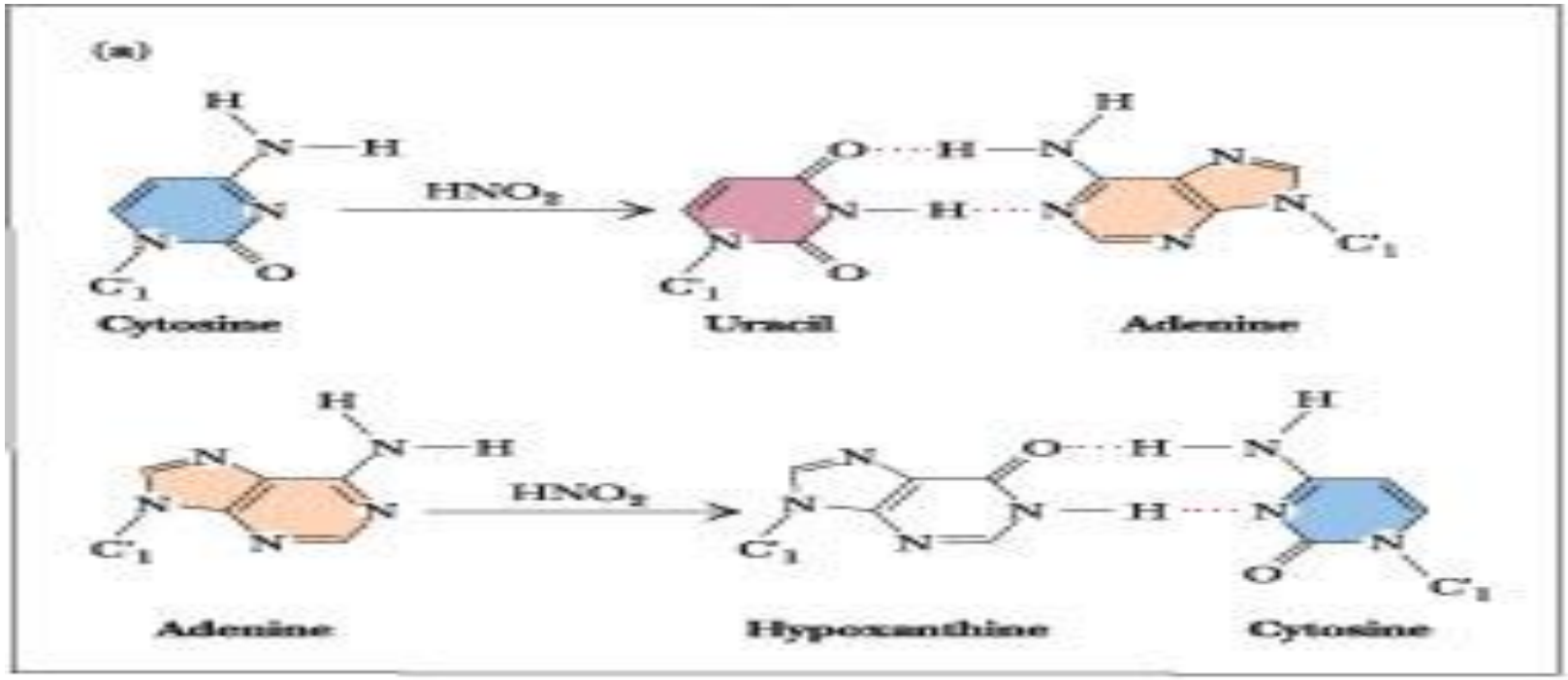
- Base analogues, which are incorporated into replicating DNA due to their structural similarity with one of the naturally occurring bases.

- Frame shift mutagens, which enters in to DNA during replication or repair and through this intercalation cause insertion or deletion of one or more nucleotide pairs.

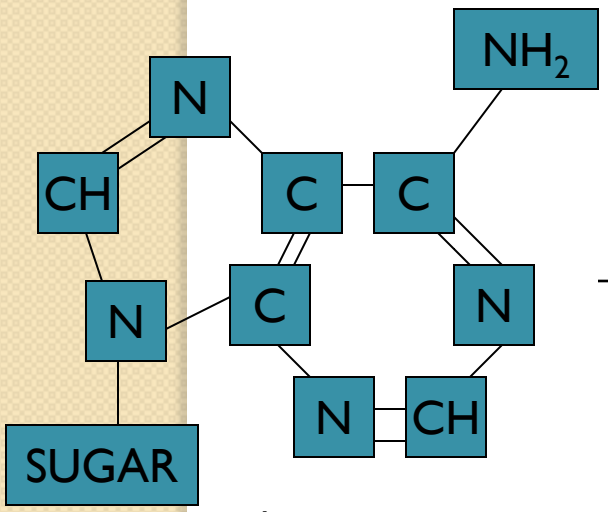
Nitrous acid

- Nitrous acid acts directly on both either replicating or non replicating DNA by oxidative de-amination of base that have amino group.

- The order of frequency of de-amination is adenine > cytosine > guanine.

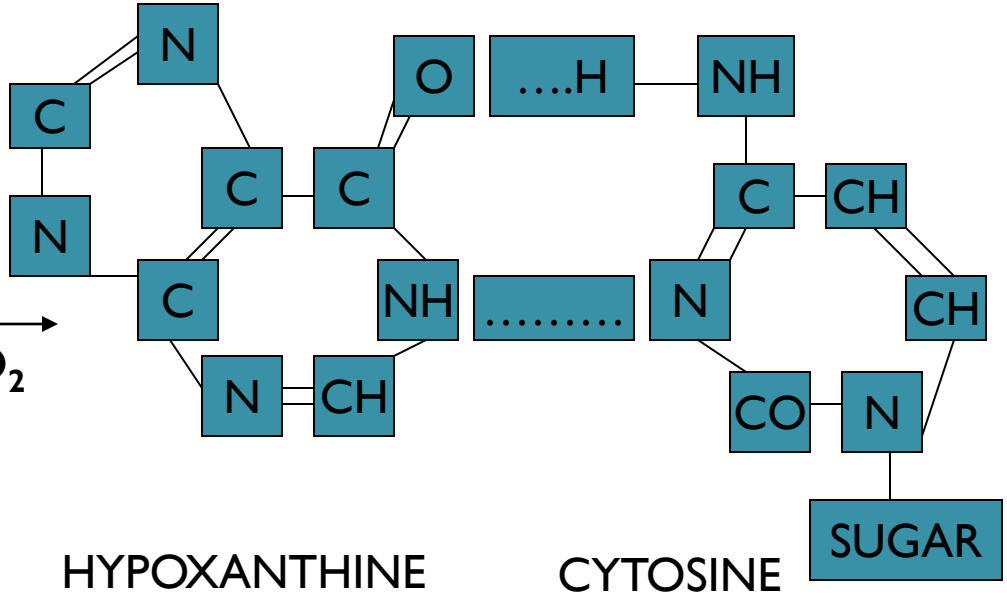


1



ADENINE

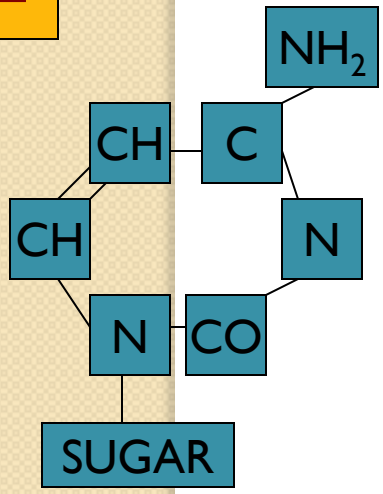
HNO_2



HYPOXANTHINE

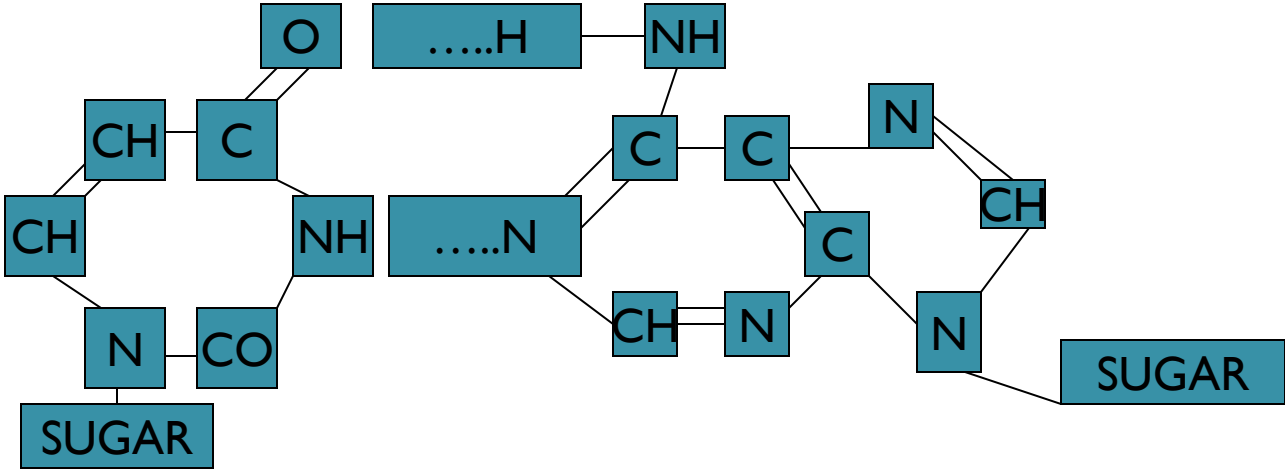
CYTOSINE

2



CYTOSINE

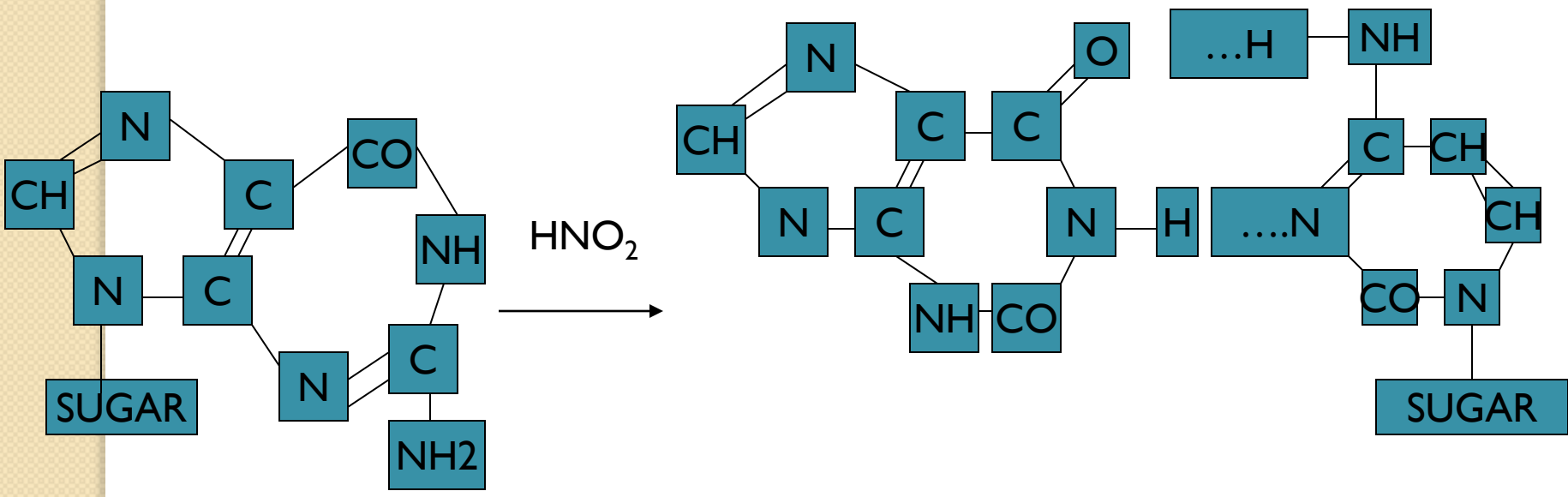
HNO_2



URACIL

ADENINE

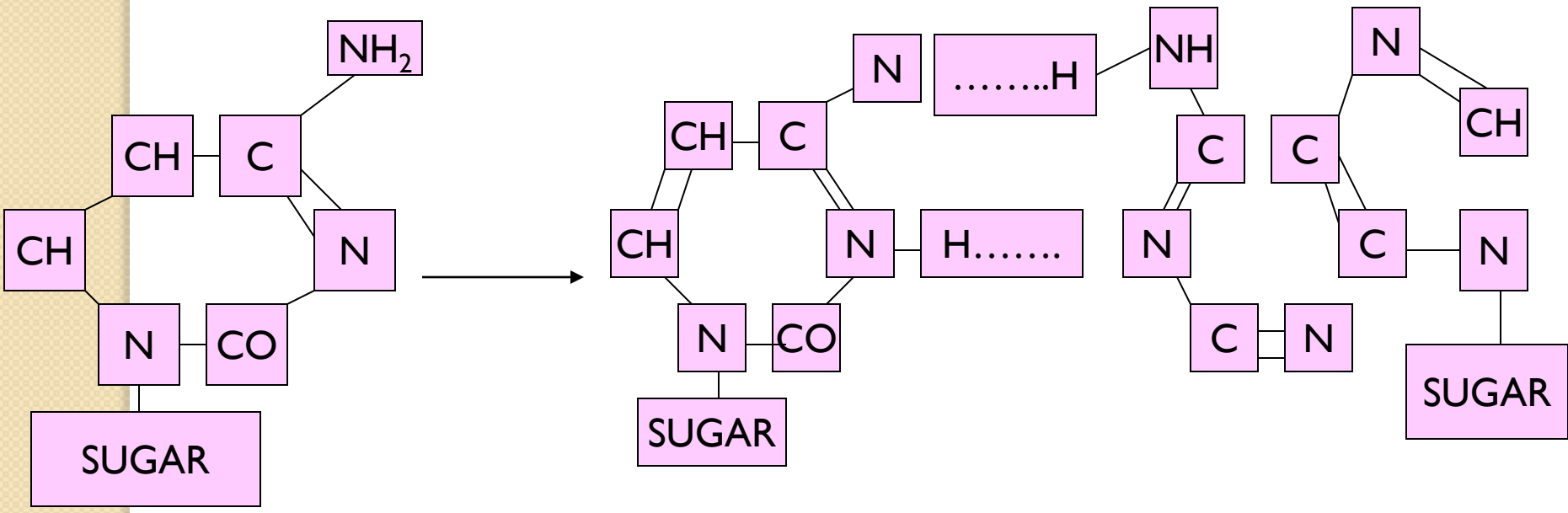
3



GUANINE XANTHINE CYTOSINE

Hydroxylating agent

- Hydroxylamine (NH_2OH) has a very specific mutagenic effect on DNA.
- It induce only GC-AT transition.
- It acts by hydroxylating amino ($-NH_2$) group of cytosine & resulting hydroxylamine cytosine can pairs with adenine to produce GC-AT transition.



CYTOSINE

HYDROXYLAMINE CYTOSINE

ADENINE

Effect of Hydroxylamine on Cytosine

Alkylating agents

- Alkylating agents also affects non-replicating DNA, which is a most potent mutagenic system for practical application.
- Frequently used agents are Ethyl Methane Sulfonate (EMS), Methyl Methane Sulfonate (MMS), Diethylsulfate (DES), Diepoxybutane (DEB), N-methyl N-nitro N-nitrosoguanidine (NTG) etc.
- Transition, Transversion, Deletion and frame shift mutations occur as a result of action of alkylating agents.

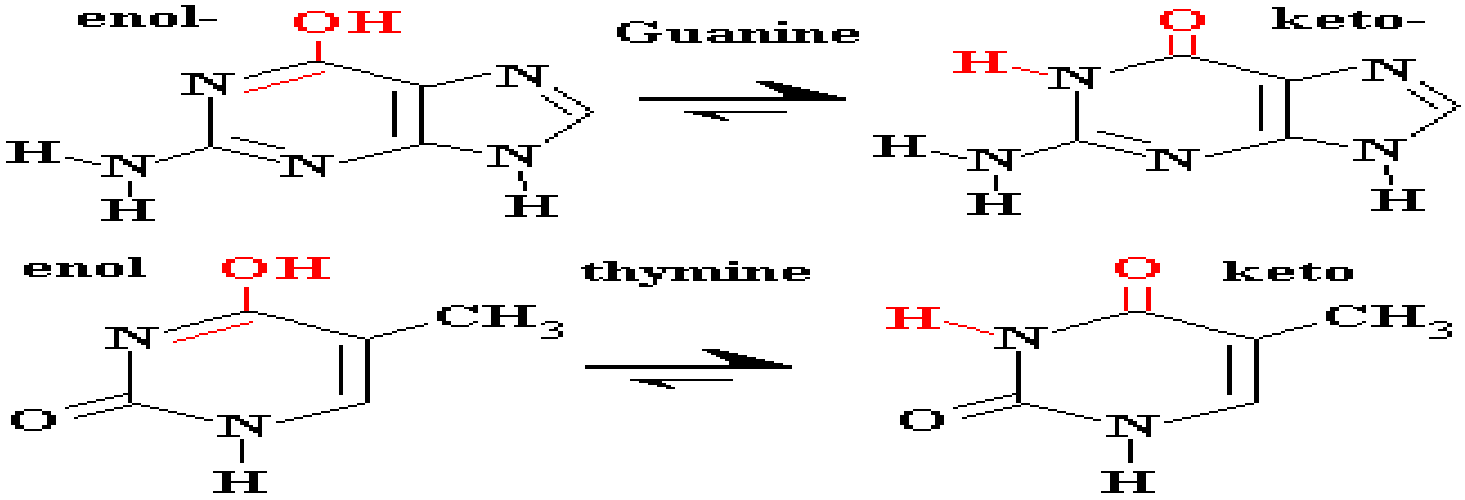
Base Analogues

- Base analogue is a chemical compound similar to one of the standard bases of DNA.
- They have base pairing properties different from the bases.
- They replace the standard bases & cause stable mutations.

  - 5-Bromouracil
  - 2 Amino Purine

Tautomers

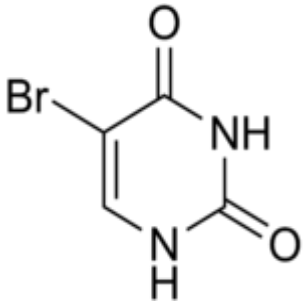
- Rearrangement in distribution of electrons on purine and pyrimidine bases result in formation of tautomers.
- They differ in arrangement of H atoms
- E.g. Standard *amino* and rare *imino* forms of C & A differ by a H atom shift between the adjacent C and N molecules.
- The molecular formulae do not change, only the configuration of bonds among atoms: the *amino* and *imino* forms are therefore tautomers of each other.
- Also enol and keto forms



5-Bromouracil

- It is a thymine analogue.
- Br at 5th position instead of CH3 group
- (Difference between U &T – CH3 present in T, as Br is present in 5th position of uracil, hence 5 BU)
- The presence of bromine, however increases the probability of tautomeric shifts

5BU



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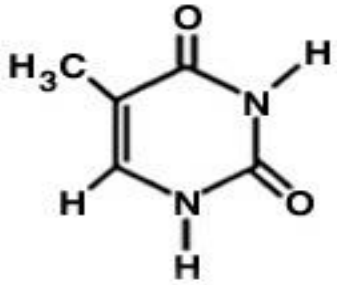

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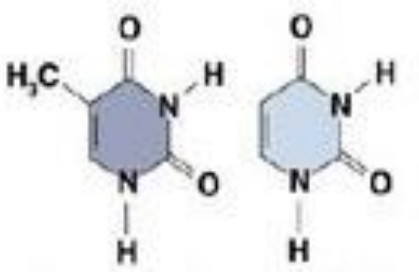
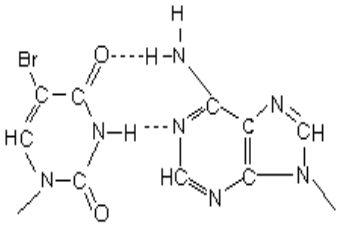
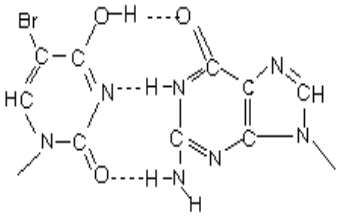


5BU



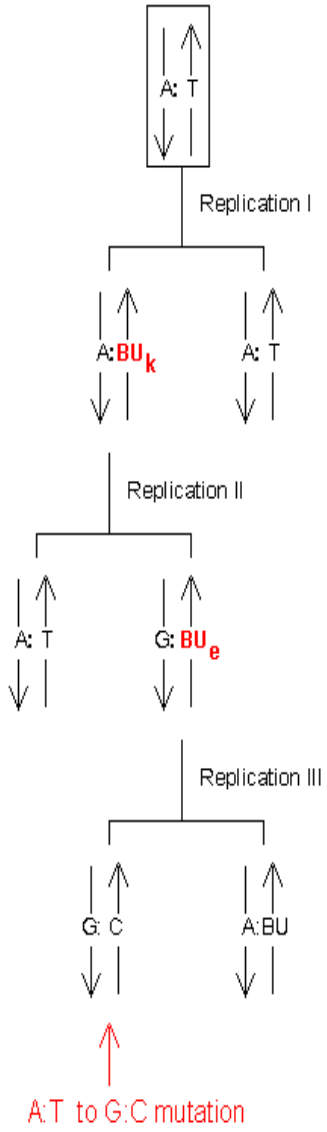
5-Bromouracil Adenine

keto form (BU_k)



5-Bromouracil Guanine

enol form (BU_e)



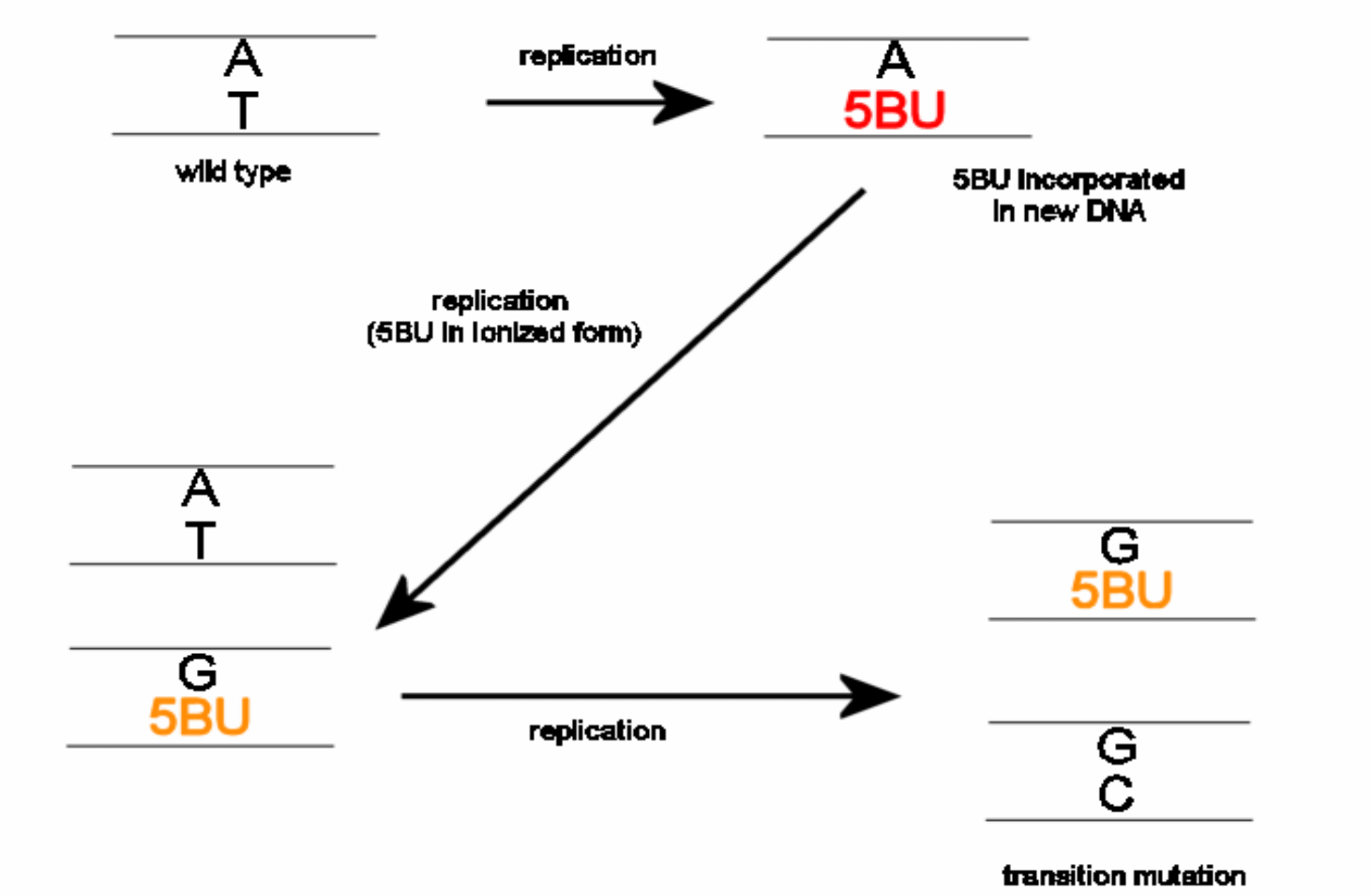
In the more stable keto form (C=O), 5BU pairs with adenine

After tautomeric shift to enol (C-OH) form, it pairs with guanine

If 5BU is present in its less frequent enol form at the time of incorporation in DNA, it will pair with G causing GC to AT transition

If it is incorporated in the frequent keto form, it pairs with A, and undergoes tautomeric shift to enol form during replication

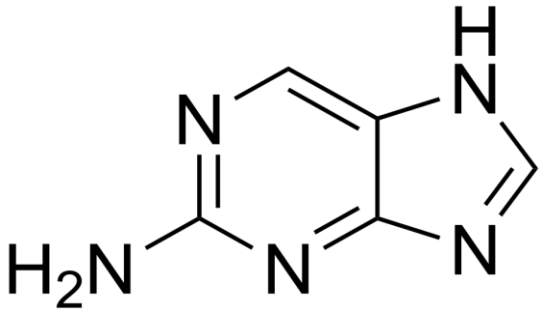
5BU- Transition



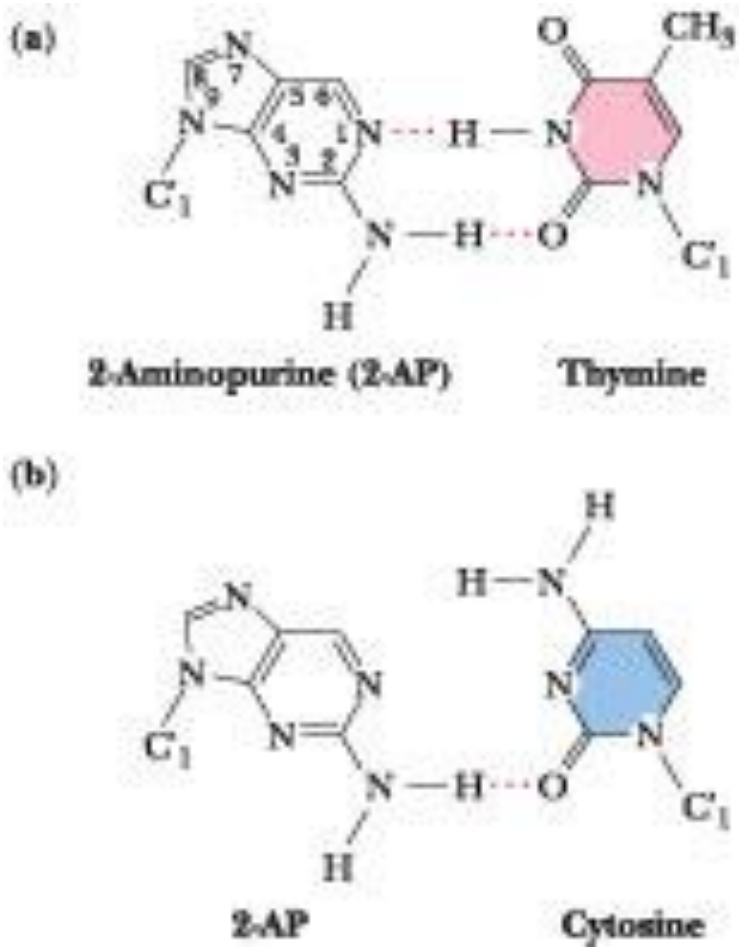
2 Aminopurine

- It is a purine analogue (adenine)
- The adenine analog, 2-aminopurine normally behaves like A and base-pairs with T.

- 2AP



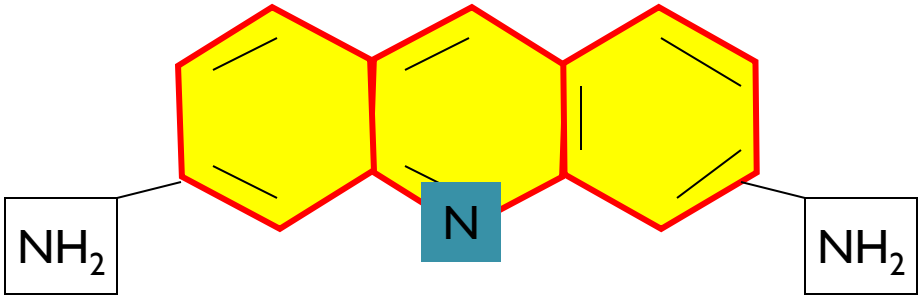
2AP



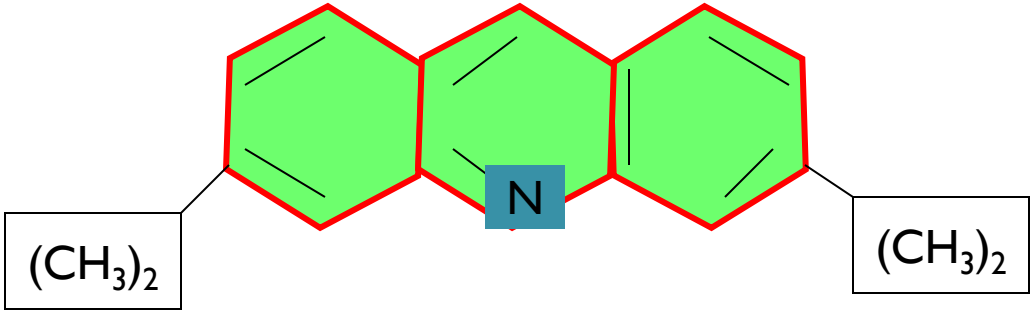
- It pairs with thymine by two H-bonds, when in amino form
- Pairs with cytosine, when in imino form.
- It induces transition mutation

Intercalating agents

- Those agents which can intercalate themselves in DNA between bases in adjacent pairs by a process of intercalation called Intercalating agents.
- Example: Ethidium bromide, acridine, acriflavin & proflavin dyes.
- These are flat molecules which intercalate between base pair of DNA, distort the DNA & result in deletion & insertion.
- Acriflavin is mutagenic for bacteria & higher plants.
- Proflavin is mutagenic for phages.
- Acridine attaches to phosphate backbone & cause deletion & insertion of single base pair.



PROFLAVIN



ACRIDINE ORANGE

Recombination

- Genetic recombination is the method that brings two genotype together to form a new genotype.
- Effective strain improvement must involve the use of sister strain, divergent strain and ancestral crosses at specific intervals, besides the use of careful mutagenesis to ensure the maintenance of genetic variability

Sexual & Parasexual cycle in fungi

Sexual recombination:

- Many fungi like Saccharomyces, Claviceps, Aspergillus shows complete sexual cycle.
- Here plasmogamy is followed by karyogamy after the fusion of hyphe. This leads to the formation of heterokaryotic mycelium.
- After diploid formation recombination occurs during meiosis process.
- A new genotype thus results either through crossing over or from combination of parent chromosome.

Sexual & Parasexual cycle in fungi

Parasexual recombination:

- Some of the fungi like Penicillium, Cephalosporium do not exhibit sexual cycle.
- In Parasexual cycle two hyphe of same or different polarity forms mycelium with nuclei of both parent strains
- Such heterokaryon is stable without merging of nuclei
- In rare cases nuclear fusion occurs and a diploid nucleus is formed. In such cases mitotic crossover between homologous chromosome may occur, resulting in genetic recombination

Sexual & Parasexual cycle in fungi

Parasexual recombination:

- In order to obtain recombinant, formation of haploid cell or spores must occur.
- Spontaneous haplodization is rare but can be induced by chemicals like p-fluorophenyl alanine. Here haploid forms not through meiosis but through random distribution of chromosome

Recombination in Bacteria

- Three types of recombination mechanisms are observed among bacteria:
- Transformation

  (Transfer of a piece of cell free DNA from surrounding)
- Transduction

  (Process mediated by bacteriophage: Generalized & Specialized transduction)
- Conjugation

  (Cell to cell contact, F+ &F-, HFR)
- In all the above methods only a part of DNA transfer from donor to recipient and thus form a pseudo zygote i.e. merozygote.

Protoplast Fusion

- Protoplast are the cell without cell wall. The cell wall can be removed using enzymes like Lysozyme in case of bacteria, chitinase and cellulase for fungi.
- Such protoplast are very fragile and to be protected. This can be done by using media containing an osmotic stabilizing agent such as sucrose.
- Recombination by protoplast fusion or protoplast transformation is one of the most important development in recent years.
- Fusion of protoplast is not easy as it has a strong negative charge on its surface.
- This can be manage by the use of PEG or electric shock (electro-fusion)
- After successful fusion cell wall is allowed to regenerate.

Protoplast Fusion

- Protoplast fusion is useful for the following-

1. Intraspecific recombination: For the strains which lack sexual or perasexual cycle or whose frequency of recombination is very low
2. Interspecific hybridization: To obtain completely new organisms capable of synthesizing new metabolites

Advantages –

- Here exchange of genetic material does not require any fertility factor.
- The entire genome can be transferred and not just a fragment
- The frequency of recombination increases significantly

Gene Technology

- Genetic engineering is most successful technology in production of high yielding strain.
- Additional genetic information can be brought in to cell by means of vector such as plasmid.
- Gene technology includes in vitro recombination & gene manipulation.
- These technique permit the introduction of specific DNAs sequence in to prokaryote or eukaryote organism & replicates in this organism.

Gene Technology

- Following steps are involved in Gene Technology-

1. The DNA sequence to be cloned must be available
2. The sequence must be incorporated into a vector
3. The vector – DNA to be introduce in host cell (transformation)
4. The selection and propagation of clone.

Regulatory mechanism of microorganisms

- Microorganisms have certain regulatory mechanism that control the metabolite synthesis therefore they can not synthesis excess of metabolite over limiting the cells requirements.
- Suppression of these mechanism is necessary to developed the strains of higher yield of desired metabolite.
- There are two regulatory mechanism:

  Feed back inhibition and Feed back repression

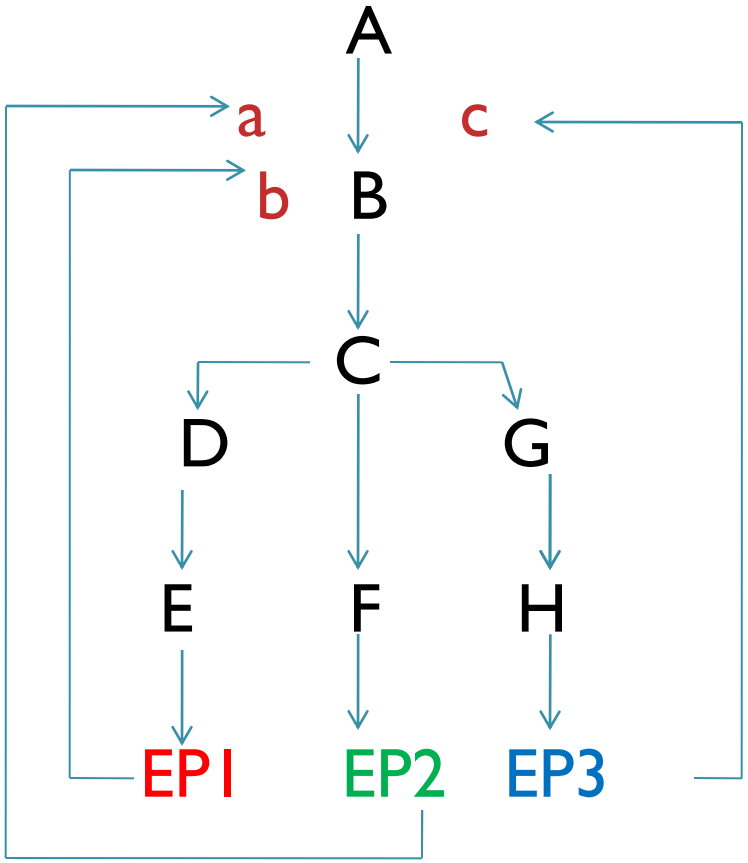
Regulatory mechanism of microorganisms

- Feed back inhibition is the situation where the end product of biochemical pathway inhibits the activity of enzyme catalyzing one of the reaction (usually the first reaction)
- Here the end product binds with enzyme at allosteric site
- Feed back repression is the situation where the end product prevents the synthesis of enzyme catalyzing the reaction of a pathway. This occurs at gene level.

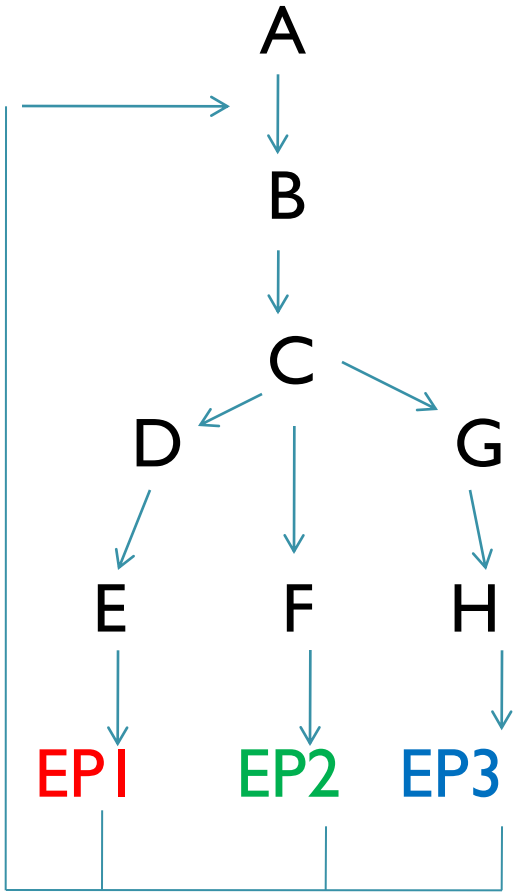
Types of feed back inhibition

- **Concerted or multivalent feed back control** – This is observed when more then one end product of a pathway combines and inhibit the first enzyme.
- **Cumulative feed back control** – Each of the end product of the pathway inhibits independently to the first reaction by certain percentage (50% if two end product is there) resulting in total inhibition.
- **Sequential feed back control** – End product here inhibits intermediate reaction and the accumulation of the intermediate further inhibits first reaction.

Cumulative



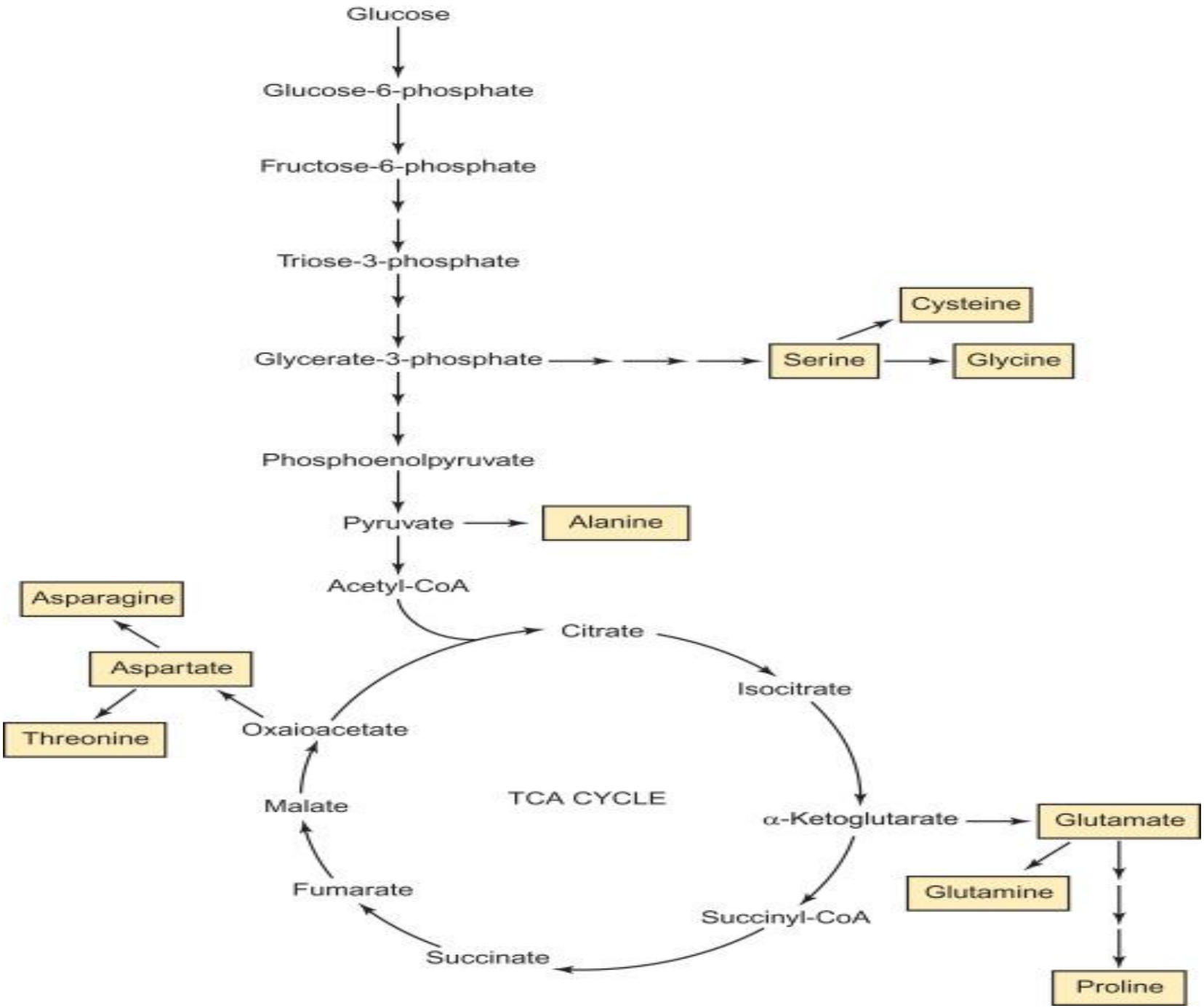
Multivalent

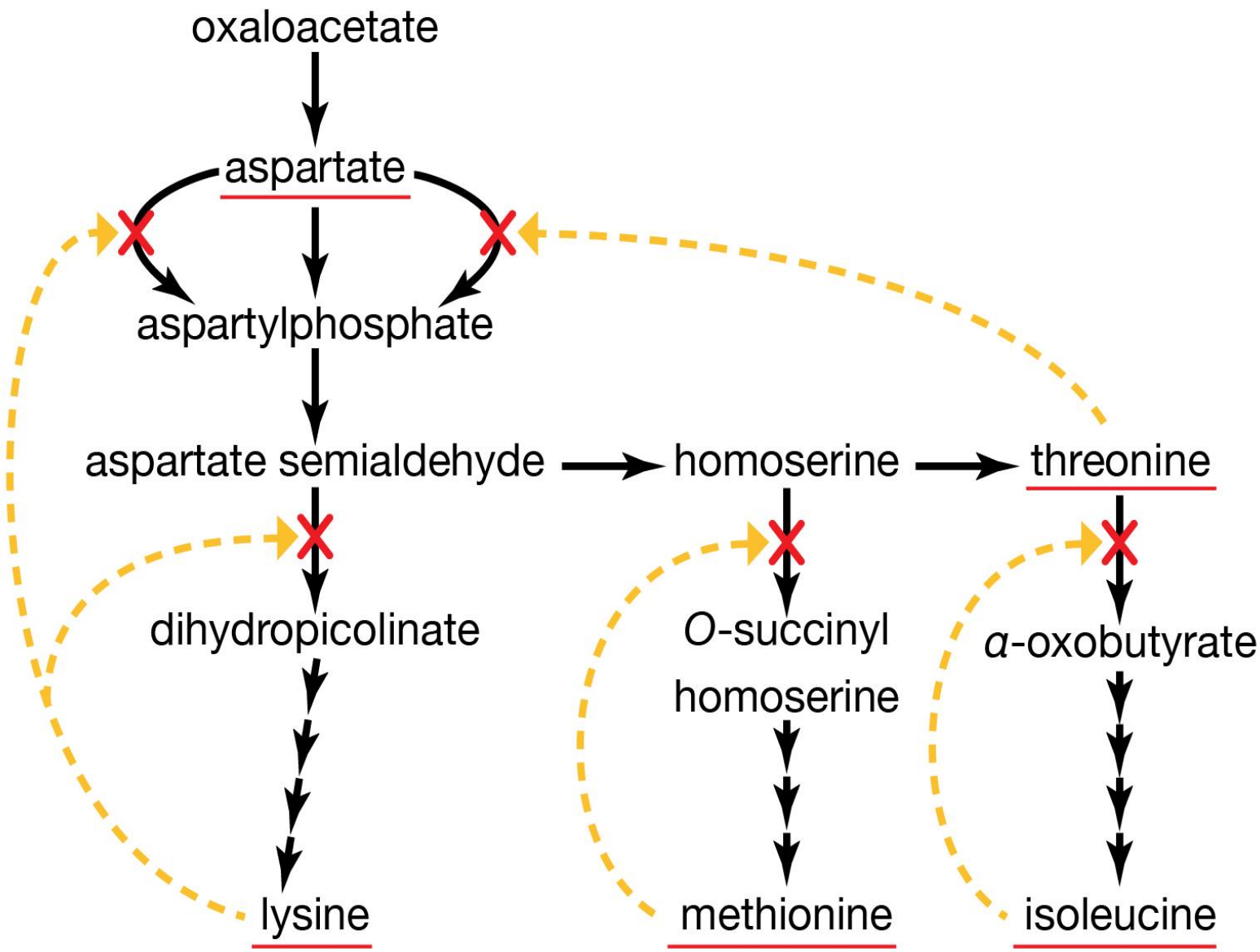


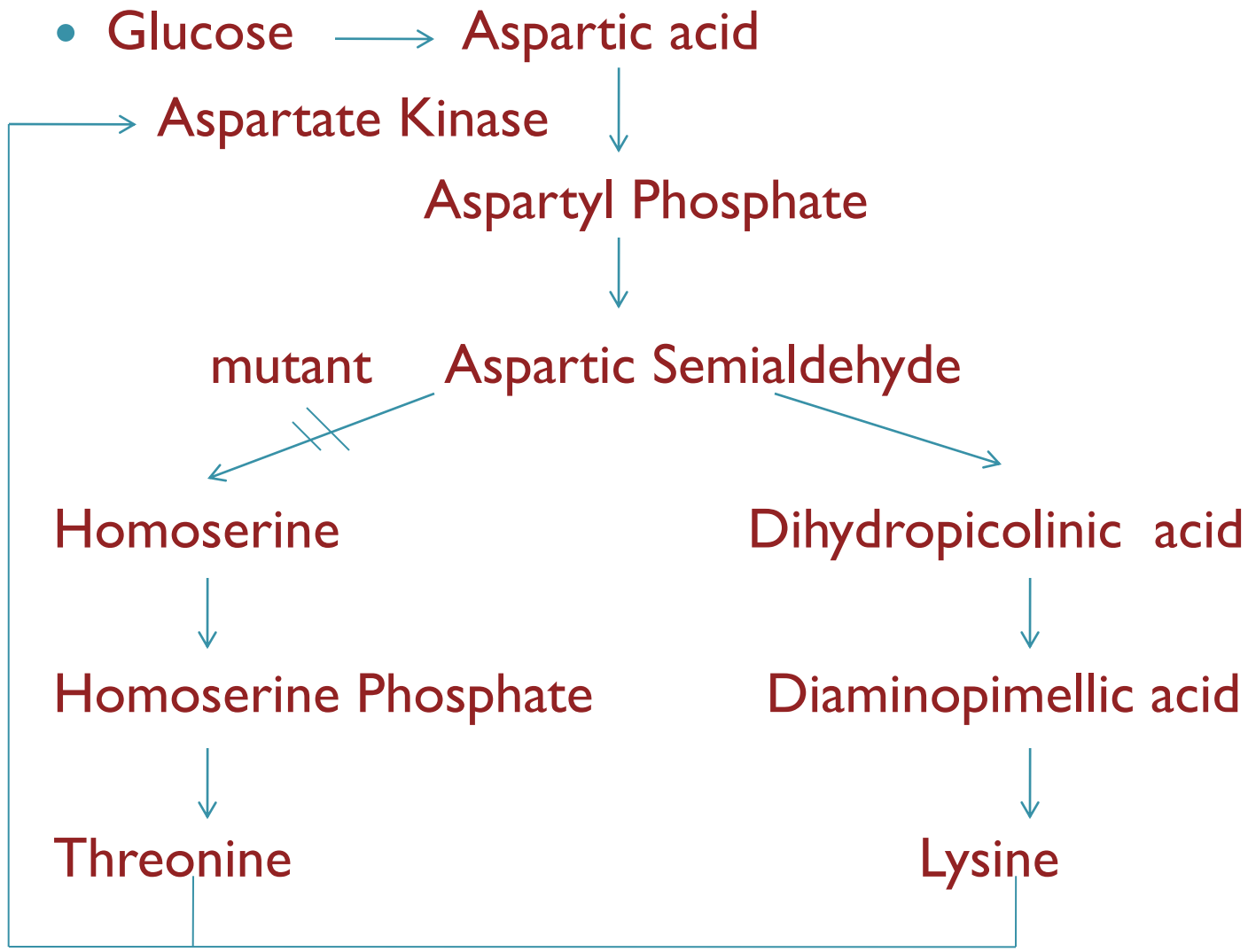
Overcoming of feed back inhibition

- In order to overcome from the effect of feed back control for the overproduction at industrial scale mutants can be modified in three ways –

1. The organism may be modified such that the end products that inhibits the key enzyme are lost from the cell due to abnormality in permeability of the cell membrane.
2. The organism can be modified such that it does not produce the end products that control the key enzyme.
3. The organism can be modified such that it does not recognize the presence of inhibiting levels of normal control metabolites.


Glucose
Glucose-6-phosphate
Fructose-6-phosphate
Triose-3-phosphate
Glycerate-3-phosphate
Cysteine
Serine
Glycine
Phosphoenolpyruvate
Pyruvate
Alanine
Acetyl-CoA
Asparagine
Aspartate
Threonine
Oxaioacetate
Citrate
Isocitrate
Malate
TCA CYCLE
α-Ketoglutarate
Glutamate
Glutamine
Fumarate
Succinyl-CoA
Succinate
Proline




Glucose
Aspartic acid
Aspartate Kinase
Aspartyl Phosphate
mutant
Aspartic Semialdehyde
Homoserine
Dihydropicolinic acid
Homoserine Phosphate
Diaminopimellic acid
Threonine
Lysine