Vivekanad College , Kolhapur (Empowered autonomous)

Department of Microbiology

PPT Bank

(2018-2023)

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Sr.No.	Name of Topic	Class	Course
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4	Proteins	B.Sc.I	Basic Biochemistry
5	Gene transfer in bacteria	B.Sc.II	Microbial genetics and molecular biology
6	Basic concepts of microbial genome	B.Sc.II	Microbial genetics and molecular biology
7	Screening	B.Sc.II	Industrial and applid microbiology
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9	Monoclonal Antibodies	B.Sc.III	Immunology
10	Type II hypersensitivity	B.Sc.III	Immunology
11	Sanger’s method	B.Sc.III	Microbial Genetics
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What is culture medium

• The food material or substances liquid or gel designed to support the growth of microorganisms. in vitro (outside the body) is called **culture medium**.

important factors for bacterial growth

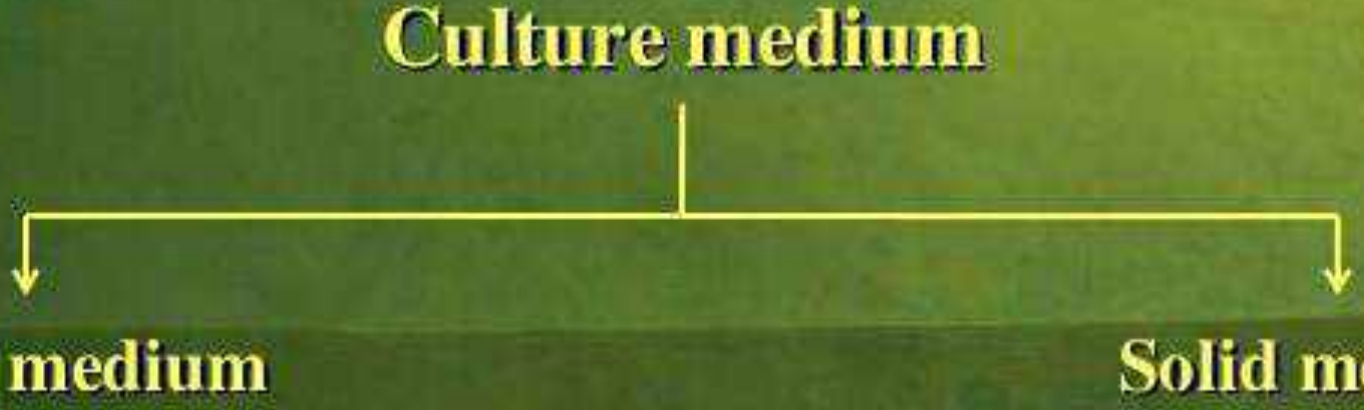
- Water
- Energy source
- Carbon source
- Nitrogen source
- Mineral salts
- Special growth factors

It is important to grow microorganisms outside the body for the following purposes:

1. to identify the cause of infection from the clinical sample, so that proper treatment can be given.
2. to study the characteristics or properties of microorganisms.
3. to prepare biological products like vaccines, toxoides, antigens...etc.

Need for Culture media:

- Bacteria: mixed population in nature
- By appropriate procedures they have to be grown separately (isolated) on culture media and obtained as pure culture for study
- Medium → Nutrients → support growth



Liquid medium:

- Diffused growth
- No characteristics for identification
- Difficult to isolate
- Earliest liquid medium: urine or meat broth used by Louis Pasteur

Solid medium:

- Distinct colony morphology
- Characteristics → easy to identify
- ***Colony*** – macroscopically visible collection of millions of bacteria originating from a single bacterial cell

- Earliest solid medium:
  Cooked cut potato by Robert Koch
- Gelatin - not satisfactory
  - liquefy at 24°C

Agar

- Frau Hesse
- Universally used for preparing solid medium
- Obtained from seaweed: *Gelidium* →
- No nutritive value
- Not affected by the growth of the bacteria.
- Melts at 98°C & sets at 42°C
- 2% agar is employed in solid medium

Types of culture media

I. Based on their consistency
   a) Solid medium
   b) Liquid medium
   c) Semi solid medium

II. Based on the constituents/ ingredients
   a) Simple medium
   b) Complex medium
   c) Synthetic or defined medium
   d) Special media

Solid media – contains 2% agar

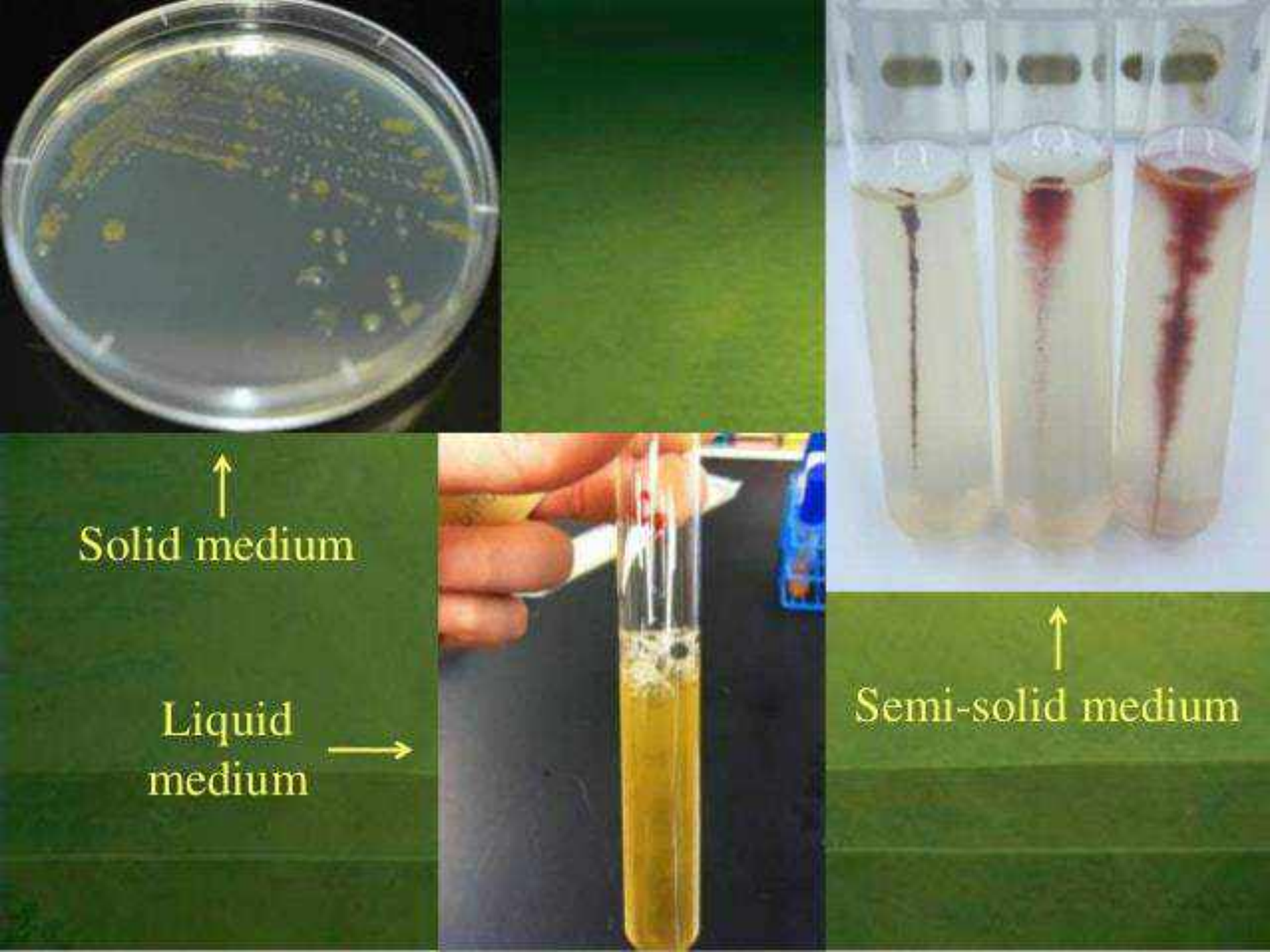
- Colony morphology, pigmentation, hemolysis can be appreciated.
- Eg: Nutrient agar, Blood agar

Liquid media – no agar.

- For inoculum preparation, Blood culture, continuous culture.
- Eg: Nutrient broth

Semi solid medium – 0.5% agar.

- Eg: Motility medium


Solid medium
Liquid medium
Semi-solid medium

Special media

- Enriched media
- Enrichment media
- Selective media
- Indicator media
- Differential media
- Sugar media
- Transport media
- Media for biochemical reactions

III. Based on Oxygen requirement

- Aerobic media
- Anaerobic media

Simple media / basal media:

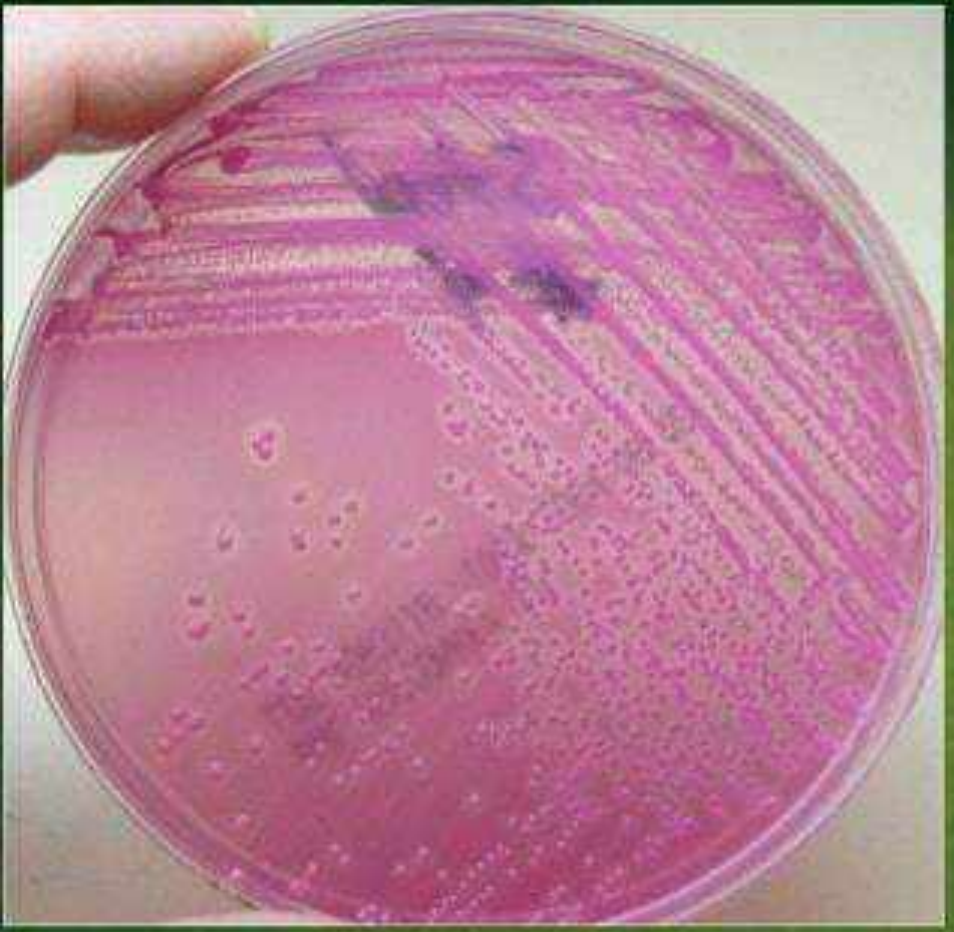
- Most common in routine diagnostic laboratories
  Eg: Nutrient Broth, Nutrient Agar
- NB consists of peptone, meat extract, NaCl, water
- NB + 0.5% Glucose = Glucose Broth
- NB + 2% agar = Nutrient agar
- Agar conc. Reduced (0.2 - 0.5%) = Semi-solid medium

Selective media

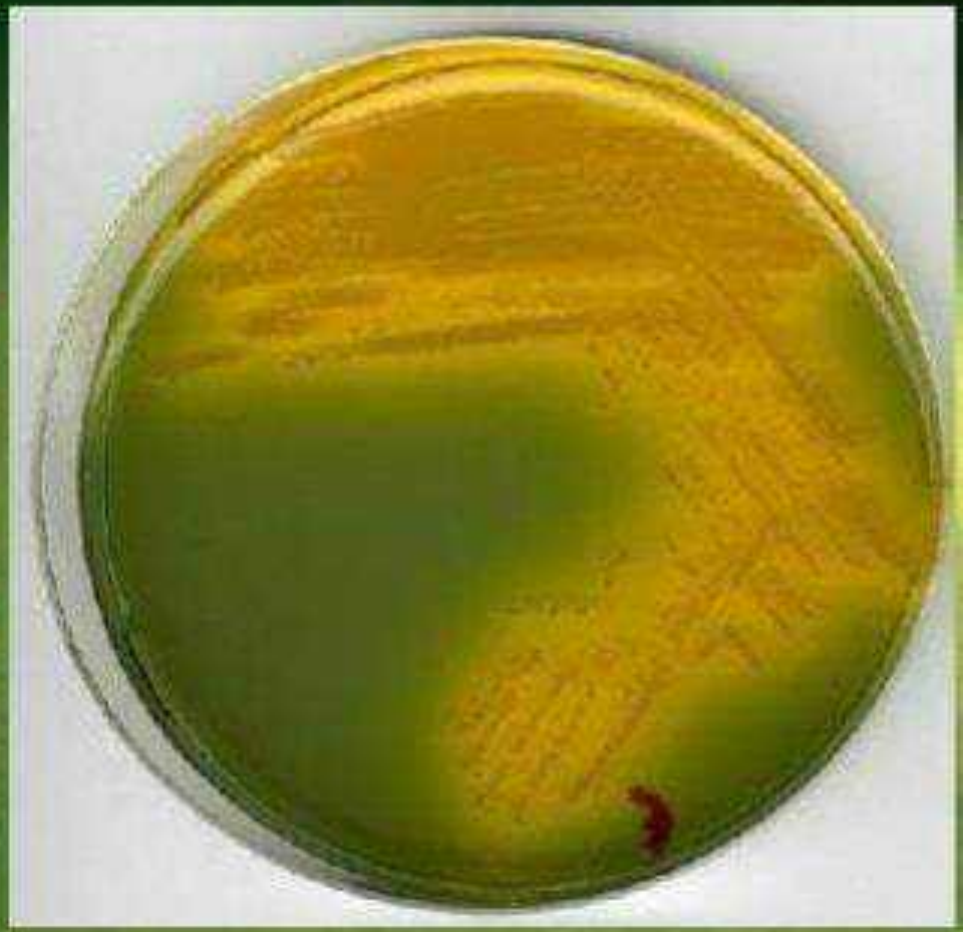
- The inhibitory substance is added to a solid media
- Increase in number of colonies of desired bacterium

Eg:

- Desoxycholate citrate medium for dysentery bacilli
- Mac Conkey's medium for gram negative bacteria
- TCBS – for *V. cholerae*
- LJ medium – *M. tuberculosis*



Mac Conkey's medium



Thiosulfate citrate bile salts sucrose (TCBS) agar

Differential media

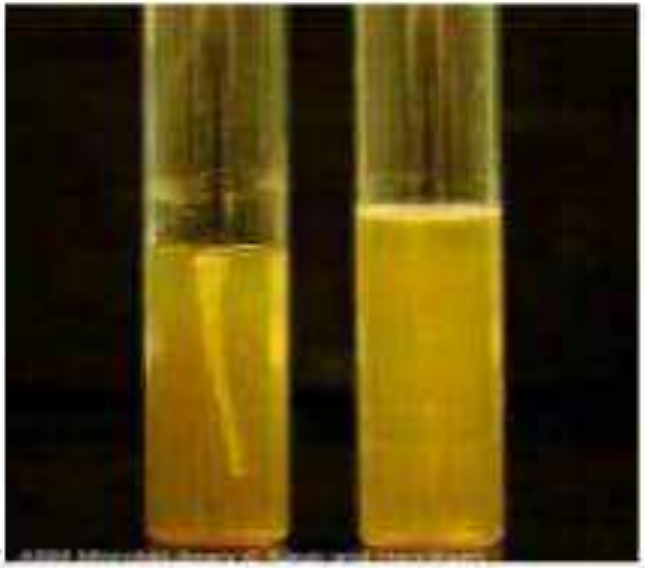
- Substances incorporated in it enabling it to distinguish between bacteria.
- Eg: Mac Conkey's medium
  - Peptone
  - Lactose
  - Agar
  - Neutral red
  - Taurocholate
- Distinguish between lactose fermenters & non lactose fermenters.

MacConkey agar:

- Lactose fermenters – **Pink** colonies
- Non lactose fermenters – colourless colonies

Semi-solid media

- Such media are soft and are useful in demonstrating bacterial motility and separating motile from nonmotile strains .



Liquid media

- are sometimes referred as **" broth "**.
- bacteria grow uniformly producing general turbidity

eg. Nutrient broth

Simple media

- eg: Nutrient broth, N. agar
- NB consists of peptone, meat extract, NaCl,
- NB + 2% agar = Nutrient agar

Synthetic or defined media

- specially prepared media from pure chemical substances for research purpose and composition of every component is well known
- eg: peptone water –
- 1% peptone + 0.5% NaCl in water.

Sabouraud Dextrose Agar M063

Intended Use:

Recommended for the cultivation of yeasts, moulds and aciduric bacteria from clinical and non clinical samples.

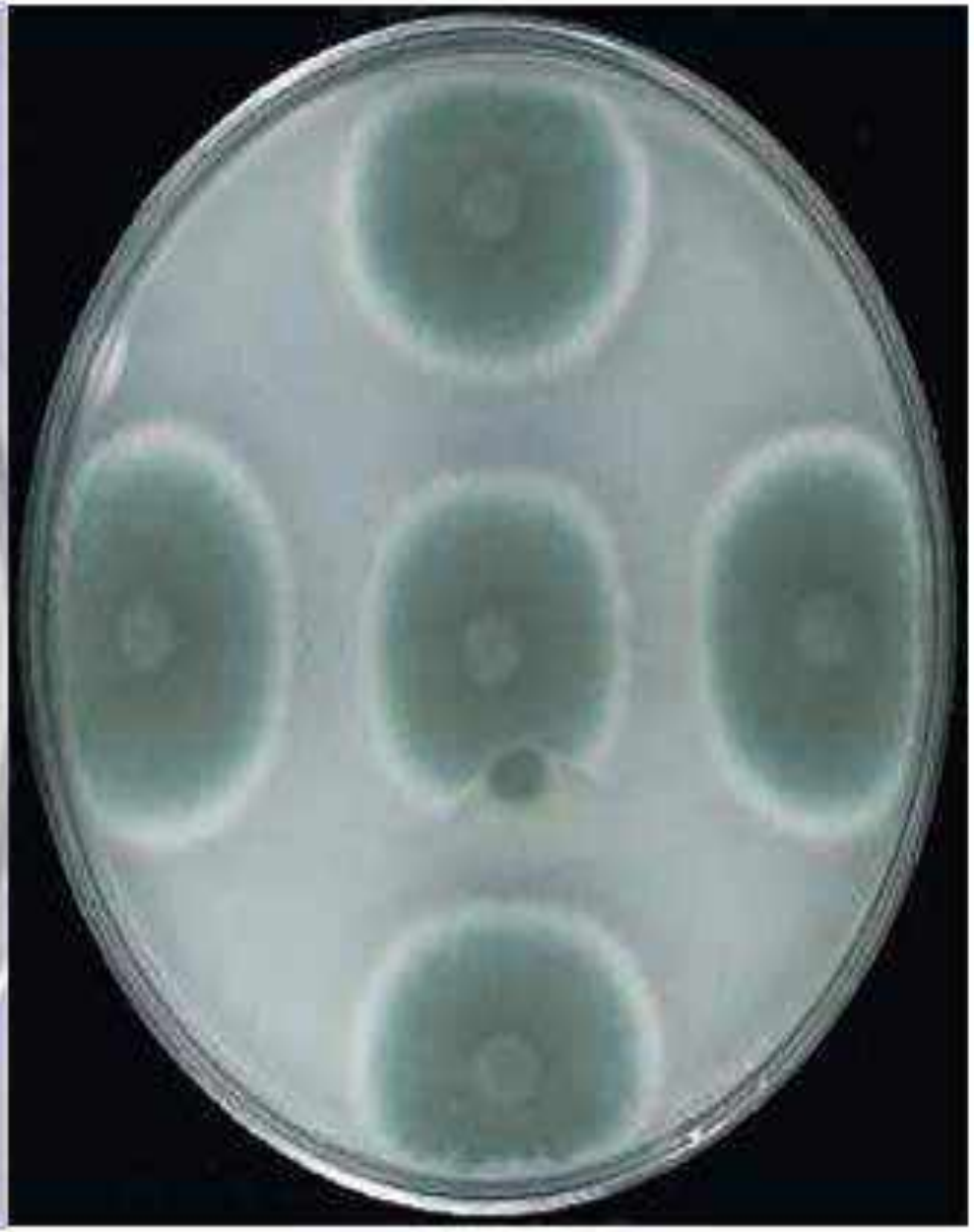
Composition**

Ingredients	Gms / Litre
Dextrose (Glucose)	40.000
Mycological, peptone	10.000
Agar	15.000
Final pH (at 25°C)	5.6±0.2

**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 65.0 grams in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.



Aspergillus flavus on PDA agar

Penicillium chrysogenum on PDA

Nutrients	Quantity (%)
$NaCl_2$ (Merck)	5%
Peptone (Oxoid)	3%
Agar (Oxoid)	2%
Skimmed Milk	1%
pH	7.0
Distilled water	100 ml

Submerged fermentation

The Submerged fermentation medium (Basal salt feather medium, table 2) was prepared as described by Hoq[10]. Conidia suspension of fungal spores containing 80,000 spores per ml (counted by Neubauer's chamber) was prepared in saline and

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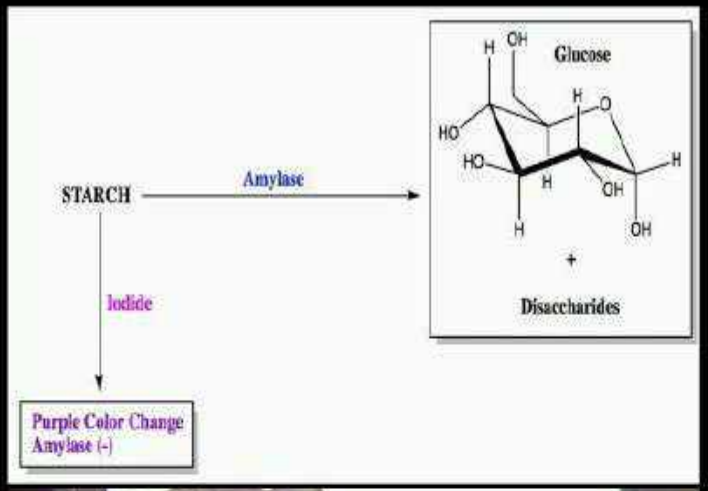
Composition of starch agar:

Peptone	5.0	g
Beef extract	3.0	g
Soluble starch	2.0	g
Agar	15.0	g
pH	7.0	

Starch Hydrolysis Test



- Some bacteria can perform **starch (amylose) hydrolysis** via an enzyme called **amylase**, which you may have seen in A&P.
- Amylase breaks starch down into **glucose (monosaccharides) and disaccharides** for catabolism in bacteria.
- If bacteria do not have amylase, starch is not broken down in plate, and starch therefore remains.
- **Iodide** causes remaining starch to turn **dark purple**.
  - **Purple** = Amylase (-)
  - Clearing = Amylase (+)


HIMEDIA
Media preparation & sterilization

Re-hydrate powder according to manufacturer's instructions

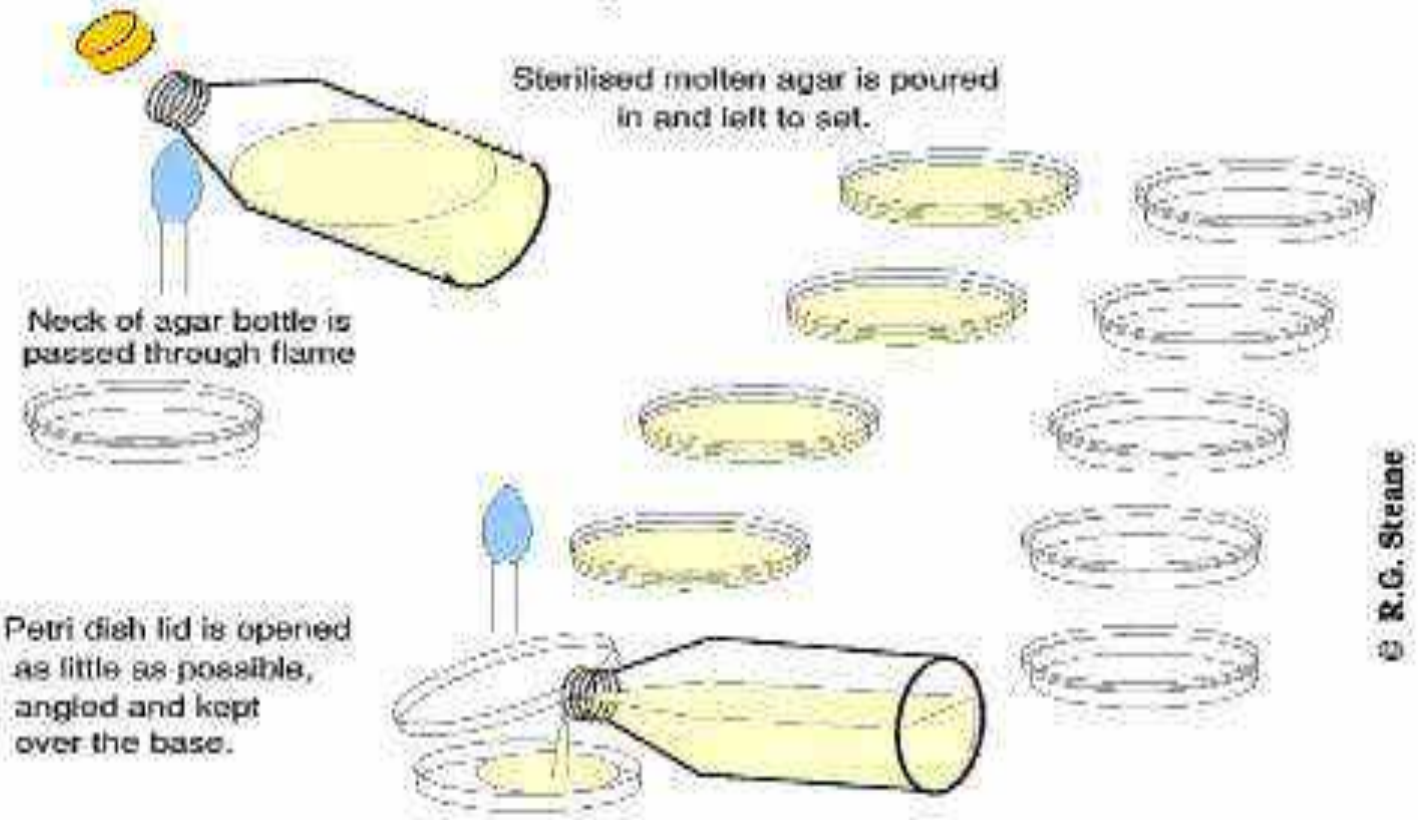
Before sterilization, ensure ingredients are completely dissolved, using if necessary.

A medium is sterilized

Pouring

1. Collect one bottle of sterile molten agar from the water bath.
2. Hold the bottle in the left hand; remove the lid with the little finger of the right hand.
3. Flame the neck of the bottle.
4. Lift the lid of the Petri dish slightly with the right hand and pour the sterile molten agar into the Petri dish and replace the lid.
5. Flame the neck of the bottle and replace the lid.
6. Gently rotate the dish to ensure that the medium covers the plate evenly.
7. Allow the plate to solidify.
8. Seal and incubate the plate in an inverted position.

"Pouring a Plate"



Each Petri dish hold about 20 ml, so 200ml will do for 10.


Agar Plate
Agar Deep Tube
Broth
Agar Slant

CARBOHYDRATES

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Carbohydrates

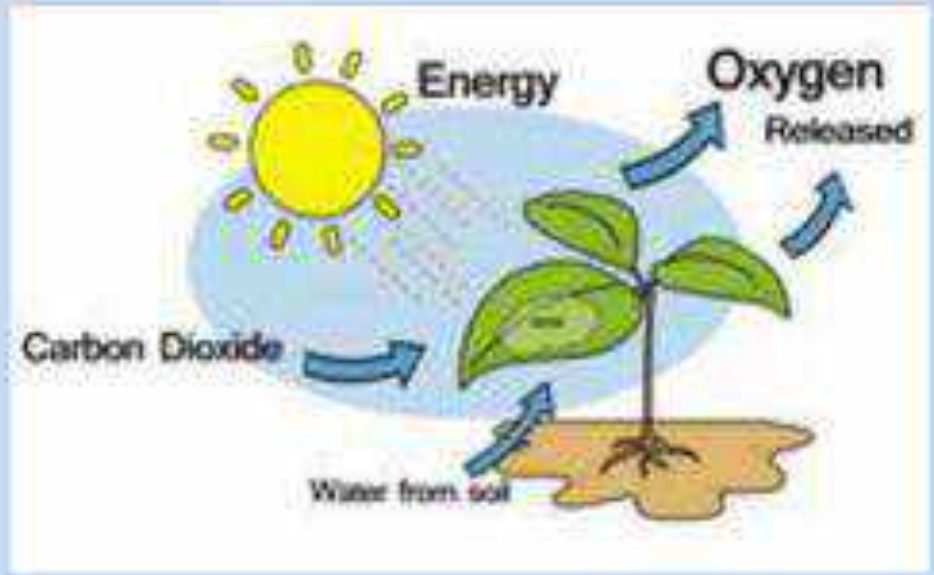
Carbohydrates are broadly defined as polyhydroxy aldehydes or ketones and their derivatives or as substances that yields one of these compounds

- Composed of carbon, hydrogen, and oxygen
- Functional groups present include hydroxyl groups
- -ose indicates sugar

Carbohydrates contained in foods such as pasta and bread provide energy for the body.

Carbohydrates

Most carbohydrates come from plant products.



Glucose is a carbohydrate made by plants. It ends up vegetable leaves and roots , fruits, nuts and grains we eat.

Carbohydrates are the most abundant of all the organic compounds in nature.

- In plants, energy from the Sun is used to convert carbon dioxide and water into the carbohydrate glucose.
- Many of the glucose molecules are made into long-chain polymers of starch that store energy.
- About 65% of the foods in our diet consist of carbohydrates.
- Each day we utilize carbohydrates in foods such as bread, pasta, potatoes, and rice.
- Other carbohydrates called disaccharides include sucrose (table sugar) and lactose in milk.
- During digestion and cellular metabolism, carbohydrates are converted into glucose,
- which is oxidized further in our cells to provide our bodies with energy and to provide the cells with carbon atoms for building molecules of protein, lipids, and nucleic acids.
- In plants, a polymer of glucose called cellulose builds the structural framework. Cellulose has other important uses, too.
- The wood in our furniture, the pages in your notebook, and the cotton in our clothing are made of cellulose.

Function of Carbohydrates in Cells

- ✓ Major source of energy for the cell
- ✓ Major structural component of plant cell
- ✓ Immediate energy in the form of GLUCOSE
- ✓ Reserve or stored energy in the form of GLYCOGEN

Classification of Carbohydrates

- Carbohydrates are classified according to the number of subunits that make them up

3 Types of Carbohydrates

- Monosaccharides
- Oligosaccharides
  - Disaccharides
  - Trisaccharides
  - Tetrasaccharides
- Polysaccharides

Monosaccharides are simple sugars, or the compounds which possess a free aldehyde (CHO) or ketone (C=O) group and two or more hydroxyl (OH) groups. They are the simplest sugars and cannot be hydrolysed further into smaller units.

Monosaccharides contain a single carbon chain and are classified on the basis of number of carbon atoms they possess, and as aldoses or ketoses depending upon their groups.

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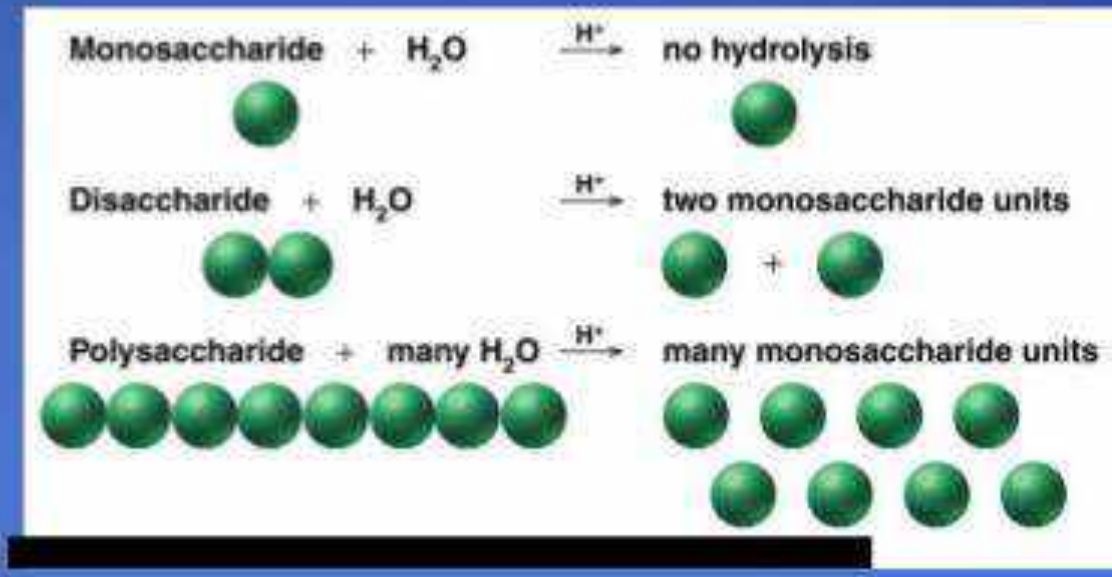
Classification

According to the number of sugar molecules:

- **Simple Carbohydrates**
  - Monosaccharides (1 unit)
  - Disaccharides (2 Units)
- **Complex Carbohydrates**
  - Oligosaccharides (3-10 Units)
  - Polysaccharides (More than 10 Units)
  - Glycogen
  - Starches
  - Fibers



Monosaccharides

Classification by Carbon Atoms

	Sugar	Structure formula	Aldoses	Ketoses
1.	Triose	$C_3H_6O_3$	Glyceraldehydes	Dehydroxy acetone
2.	Tetroses	$C_4H_8O_4$	Erythrose, Threose	Erthrulose
3.	Pentoses	$C_5H_{10}O_5$	Xylose Ribose Arabinose	Ribulose
4.	Hexoses	$C_6H_{12}O_6$	Glucose Galactose Mannose	Fructose

Monosaccharides

Hexoses

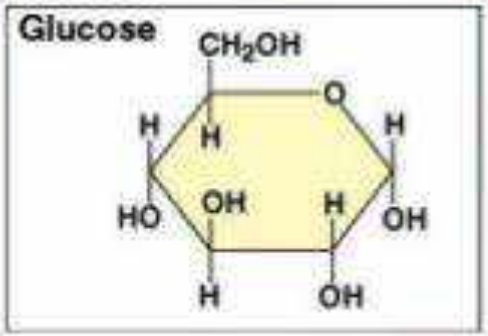
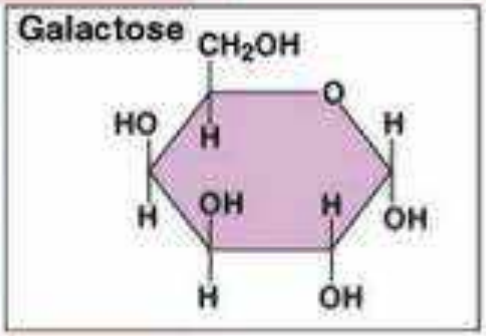
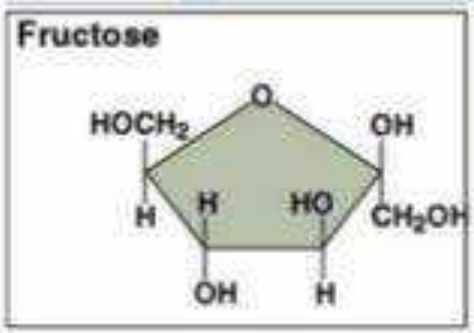
Glucose

- The essential energy source for all body functions.
- Other names: Dextrose and Blood Sugar.
- A component of each disaccharide.

- Galactose
  - Seldom occurs freely in nature
  - Binds with glucose to form sugar in milk: lactose.
  - Once absorbed by the body, galactose is converted to glucose to provide energy.

Fructose:

- The sweetest of all sugars
  - (1.5 X sweeter than sucrose)
- Occurs naturally in fruits and honey "the fruit sugar"

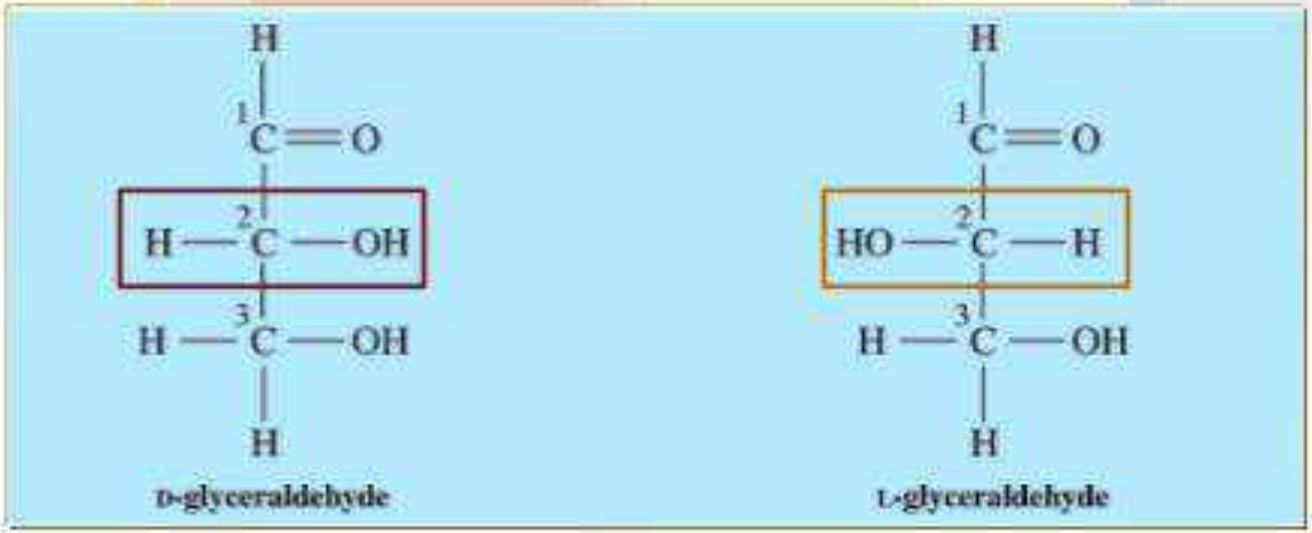
D-glucose "dextrose" Blood sugar	D-galactose	D-fructose "Levulose" Fruit sugar
		

Steriochemistry

Optical isomers (= enantiomers) differ from each other in the disposition of the various atoms or groups of atoms in space around the asymmetric carbon atom. These are, in fact, the mirror image of each other. These may also be likened to left- and right-handed gloves.

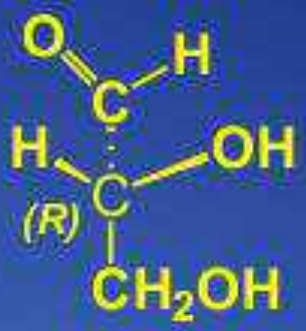
One form in which H atom at carbon 2 is projected to the left side and OH group to the right is designated as D-form and the other form where H atom is projected to the right side and OH group to the left is called as L-form (note the use of small capital letters D and L)

For example, the glyceraldehyde has only one asymmetric carbon atom (numbered as 2) and it can, therefore, exist in 2 isomeric forms :

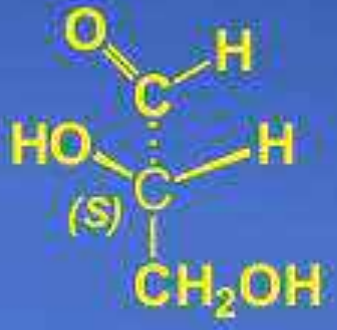


D and L Designations of Monosaccharides

- The simplest monosaccharide is glyceraldehyde, which contains a stereocenter. Therefore, it exist in two enantiomeric forms.
- In 1906, (+)-glyceraldehyde is designated D-(+)- glyceraldehyde and (–)- glyceraldehyde is designated L-(–)- glyceraldehyde.
- These two compounds serve as configurational standards for all monosaccharides.



(+)-Glyceraldehyde



(-)-Glyceraldehyde

- A monosaccharide whose highest numbered stereocenter (the penultimate carbon) has the same configuration as D-(+)- glyceraldehyde is designated as a D sugar; one whose highest numbered stereocenter has the same configuration as L-(–)- glyceraldehyde is designated as an L sugar.

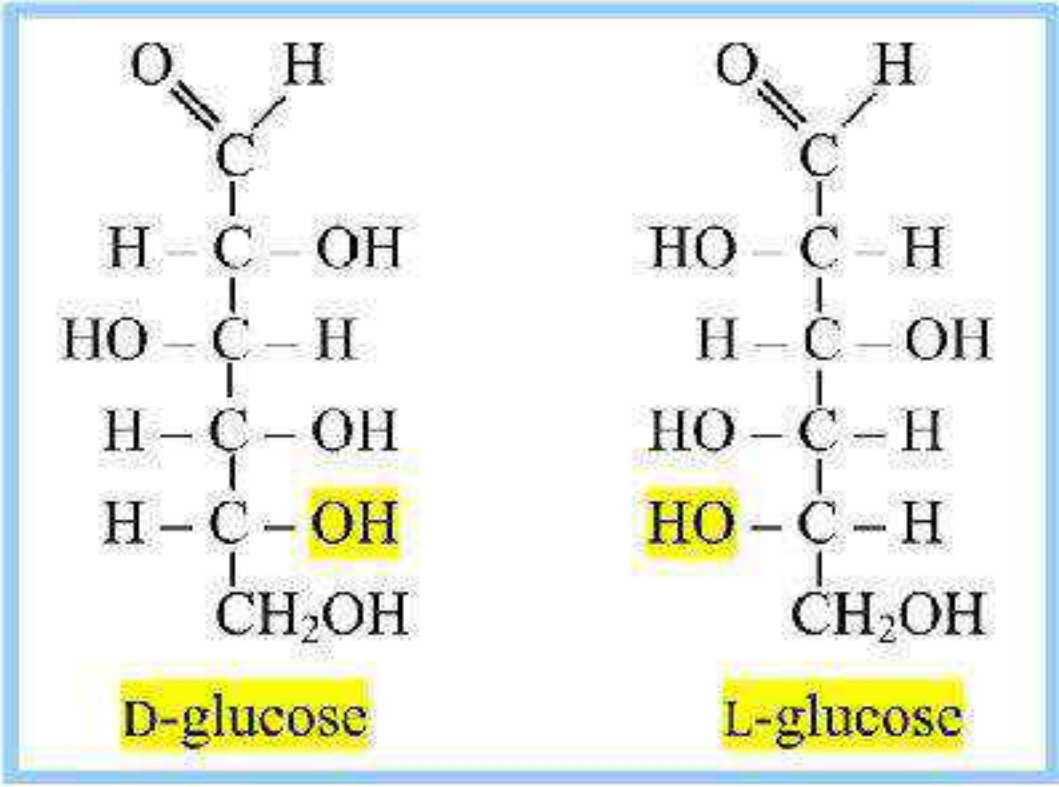
- D and L designations are not related to the optical rotations of the sugars to which they are applied.

- One may encounter other sugars that are D-(+)- or D-(–)- and ones that are L-(+)- or L-(–)-.

Sugar Nomenclature

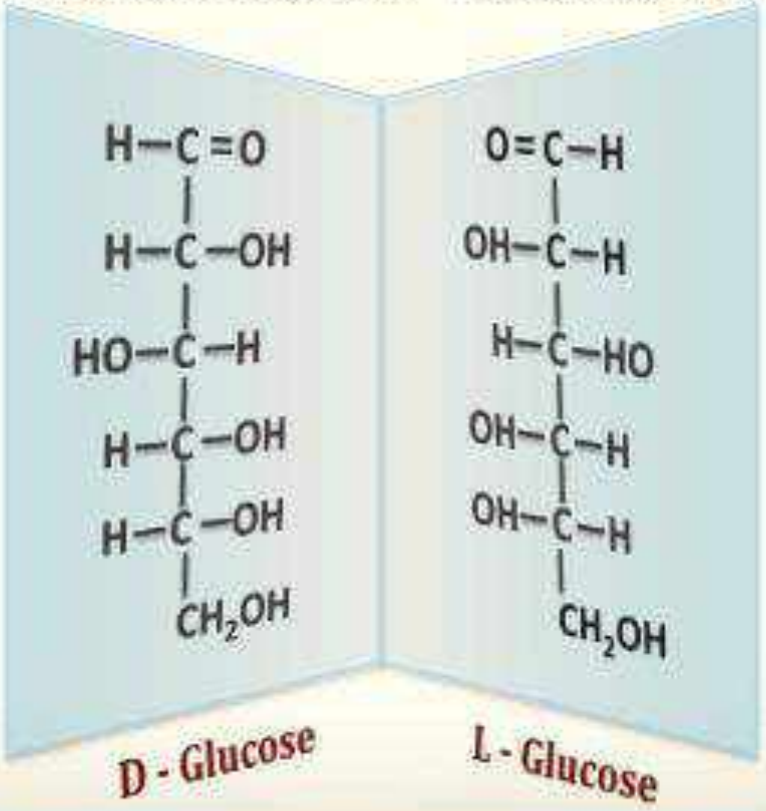
For sugars with more than one chiral center, **D** or **L** refers to the asymmetric **C** farthest from the aldehyde or keto group.

Most naturally occurring sugars are D isomers.



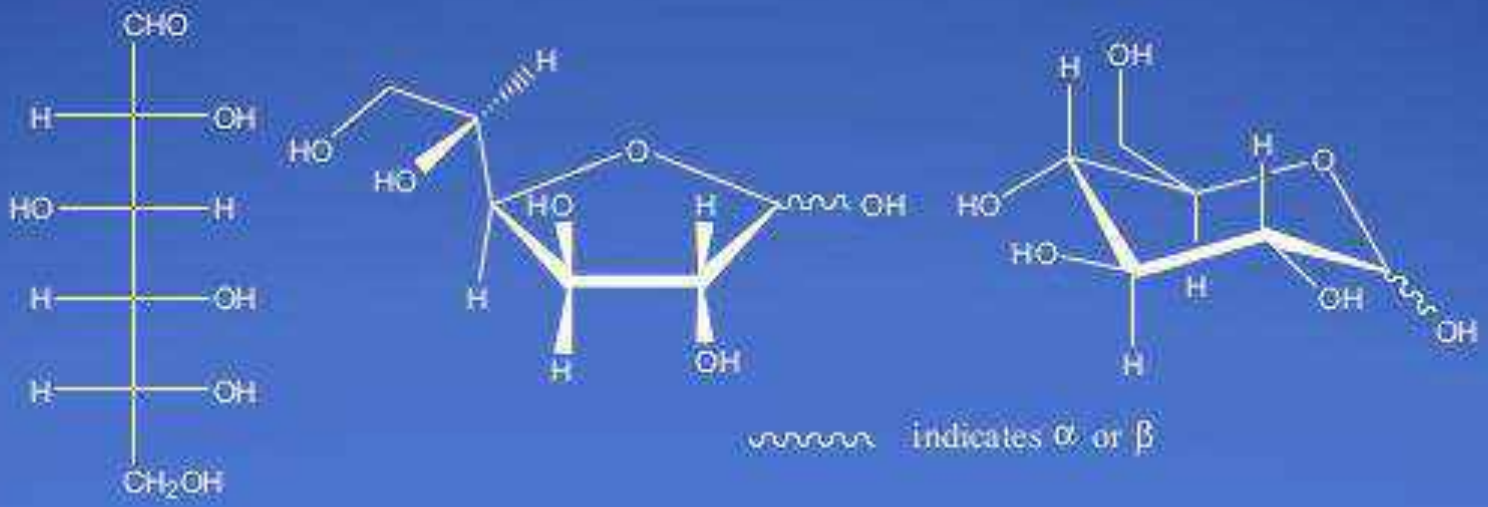
D and L isomerism (enantiomer)

- D and L isomers are mirror images of each other.
- These two forms are called Enantiomers.



Structural formulas for Monosaccharides

- **Fisher projection**
- **Haworth formulas**
- **α anomer or β anomer**



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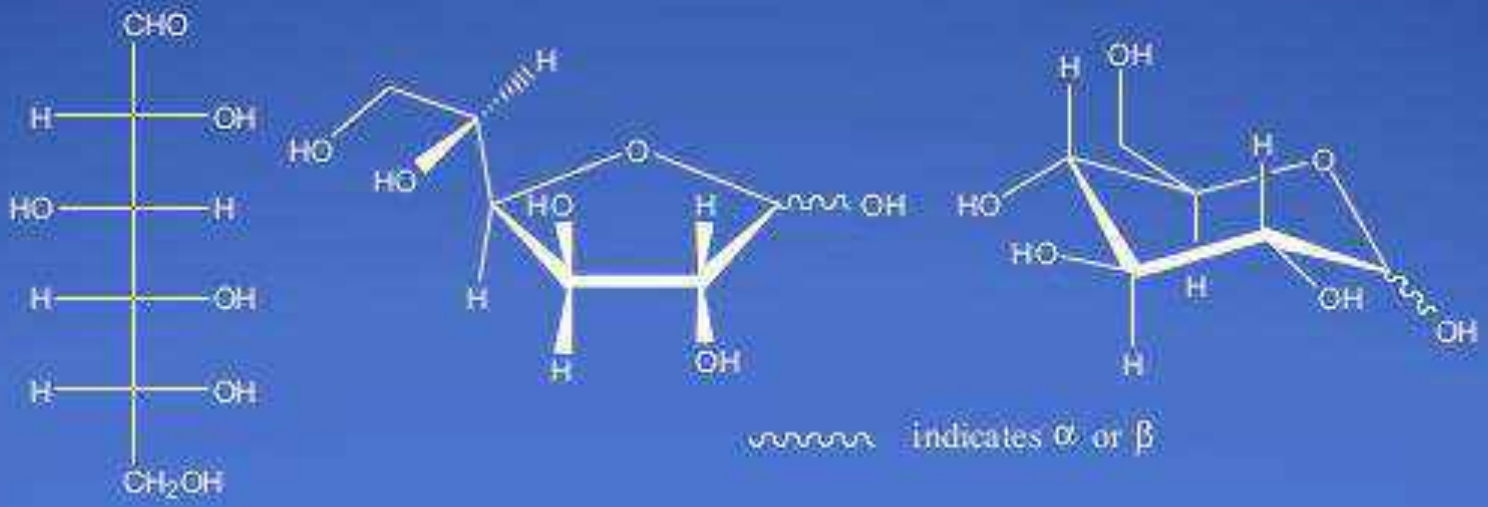
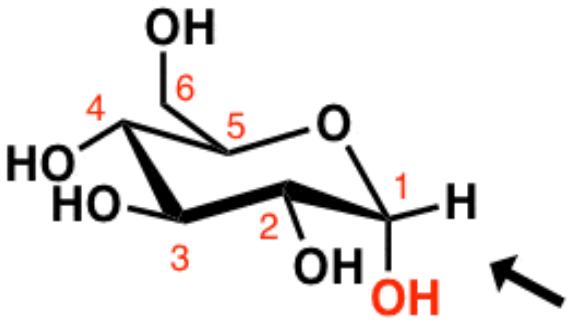
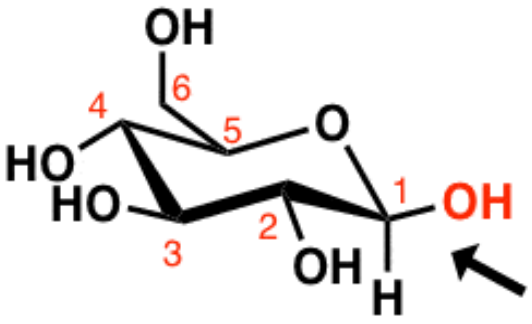


Anomer

• two sugars that differ in configuration only at the anomeric carbon (i.e. the carbon bearing a hemiacetal)
e.g. in glucose, the anomers differ only in configuration at C-1 :

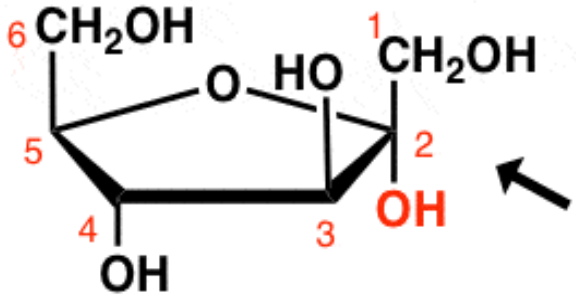


α-D-glucopyranose

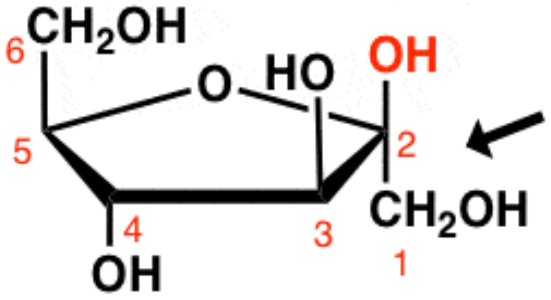


β-D-glucopyranose

In fructose (a ketose) the sugars differ in configuration at C-2 :

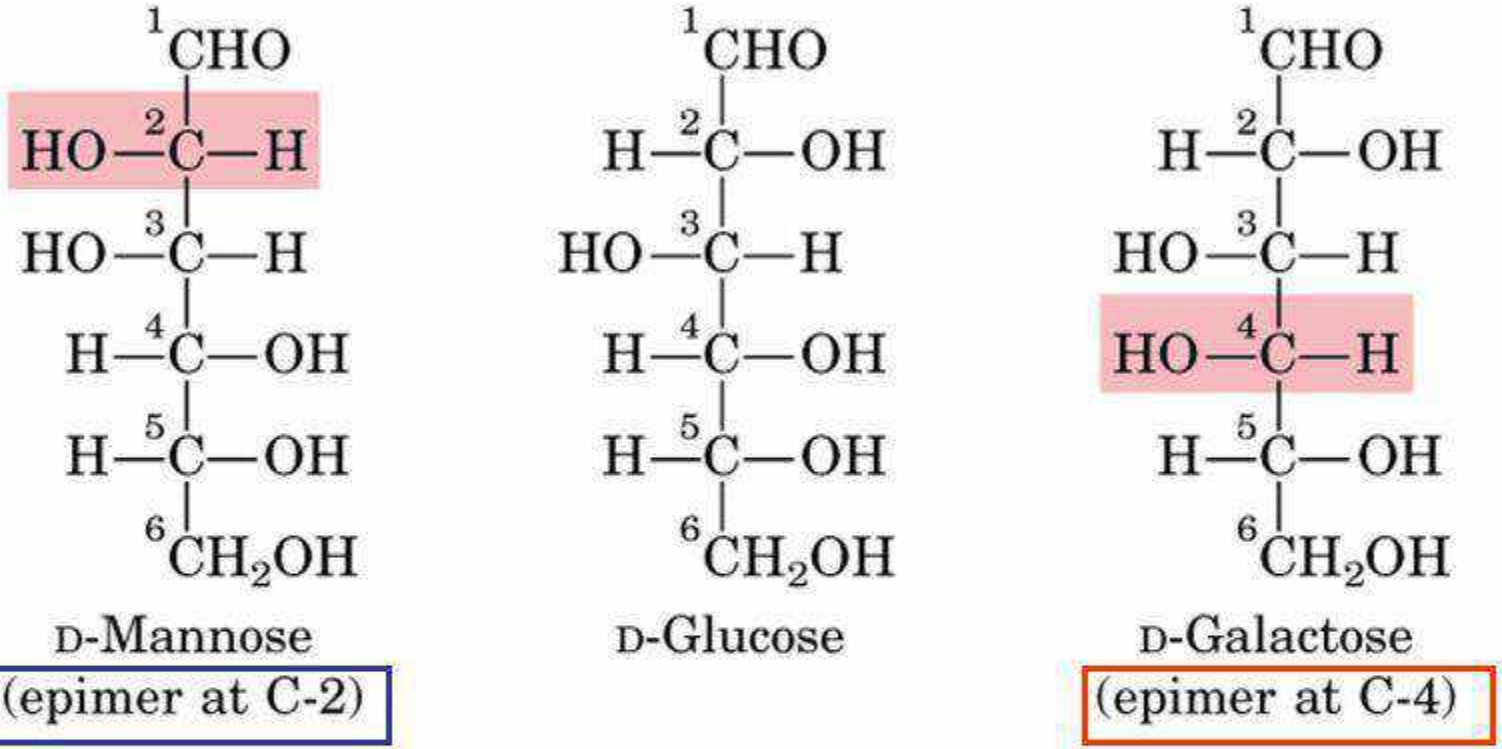


α-D-fructose

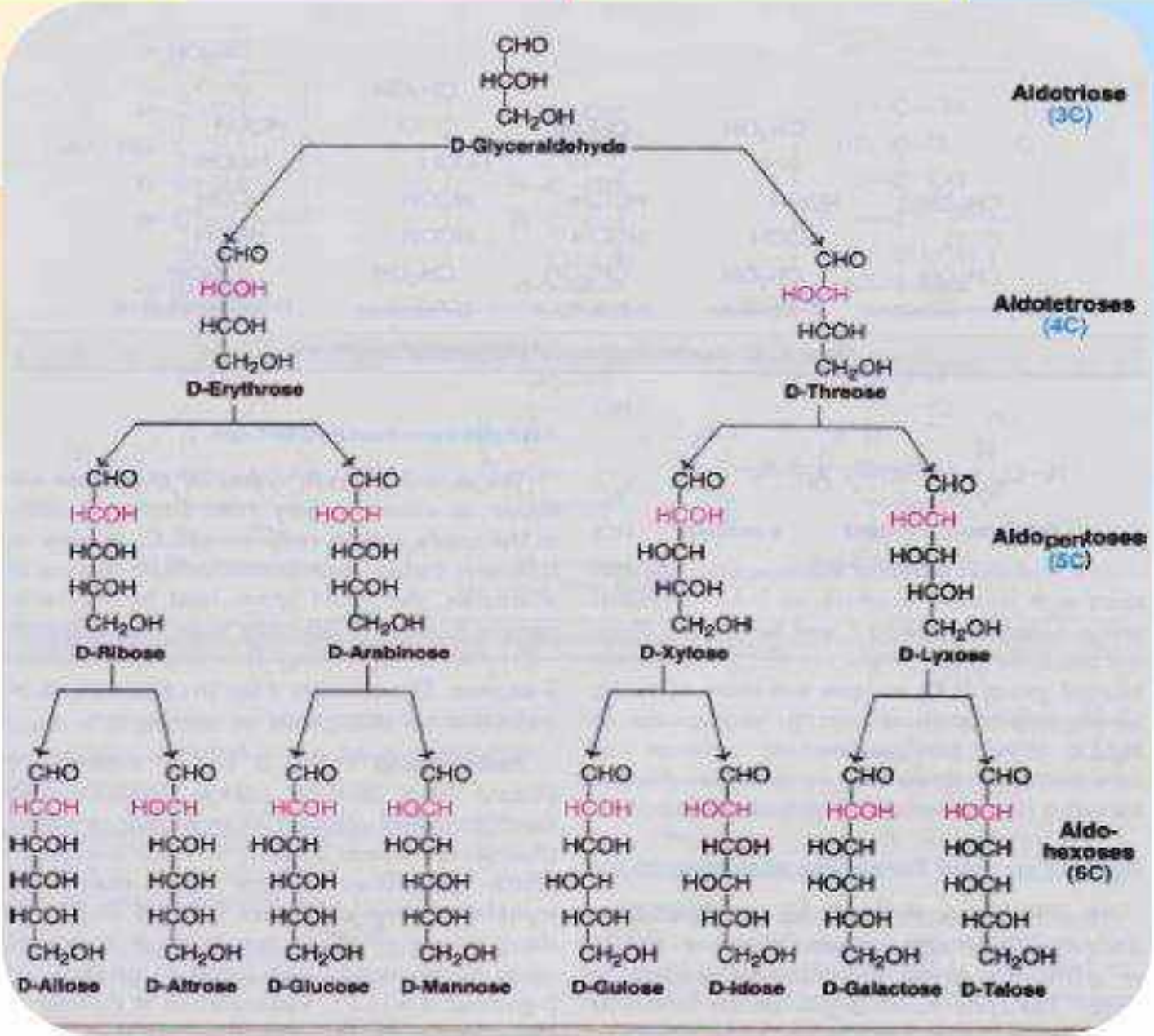


β-D-fructose

Epimers



- Epimers: Two sugars that different only in the configuration around one carbon atom.
- D-glucose and D-mannose, which differ only in the stereochemistry at C-2, are epimers, as are D-glucose and D-galactose (which differ at C-4)



D-Aldoses containing three , four , five and six atoms

Properties of monosaccharides

1. *Mutarotation* : when a monosaccharide is dissolved in water, the optical rotatory power of the solution gradually changes until it reaches a constant value. For ex : when D-glucose is dissolved in water, a specific rotation of +112.2° is obtained, but this slowly changes , so that at 24h the value has become +52.7°. This gradual change in specific rotation is known as mutarotation. This phenomenon is shown by number of pentoses, hexoses and reducing disaccharides.

2. *Glucoside formation* : when D-glucose solution is treated with methanol and HCl, two compounds are formed, these are α – and β-D- glucosides. Thus, formed glucosides are not reducing sugar and also doesnot show phenomenon of mutarotation

3. *Reducing power* : Sugars having free or potentially free aldehyde or ketone group have an ability to reduce the cupric copper to cuprous

Reducing sugar + 2 Cu^{++} → oxidized + 2Cu^{+}

(cupric) sugar (cuprous)

4. *Oxidation / Reduction*: The alcoholic OH, aldehyde (COH) or keto (C=O) group are oxidized to carboxyl group with certain oxidizing agents. The oxidation may be brought under mild or with vigorous oxidizing condition

i. <u>With mild oxidant like BrH_2O</u> : In this group only aldehyde is oxidized to produce gluconic acid (monocarbonic). Ketoses do not respond to this reaction.

Properties of monosaccharides

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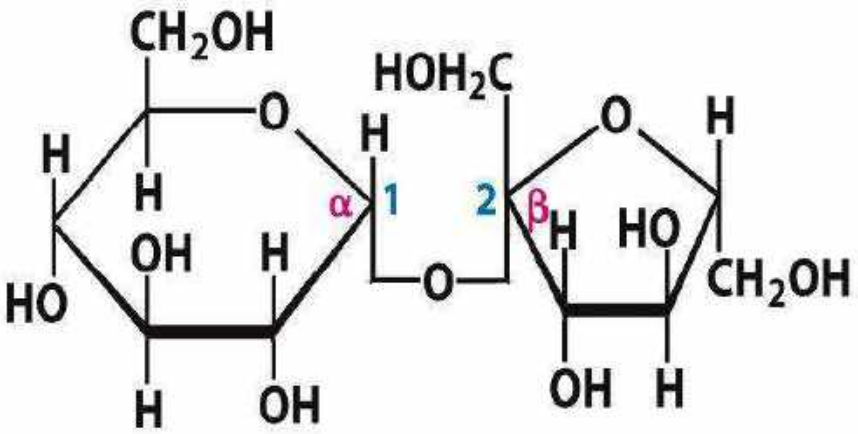
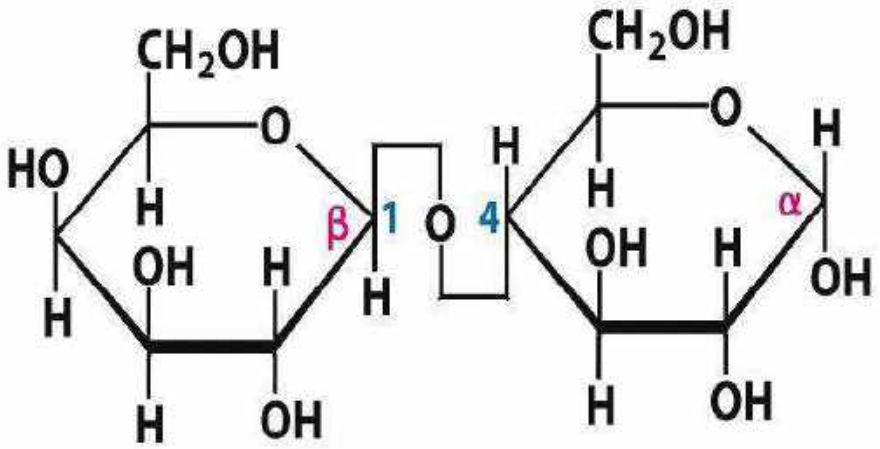
Carbohydrates with free carbonyl groups or in hemiacetal form give positive tests to Benedict's and Fehling's reagents without having been hydrolyzed are referred as **'reducing'** sugars; others (*i.e.*, the acetal types) are then **'non-reducing'** sugars

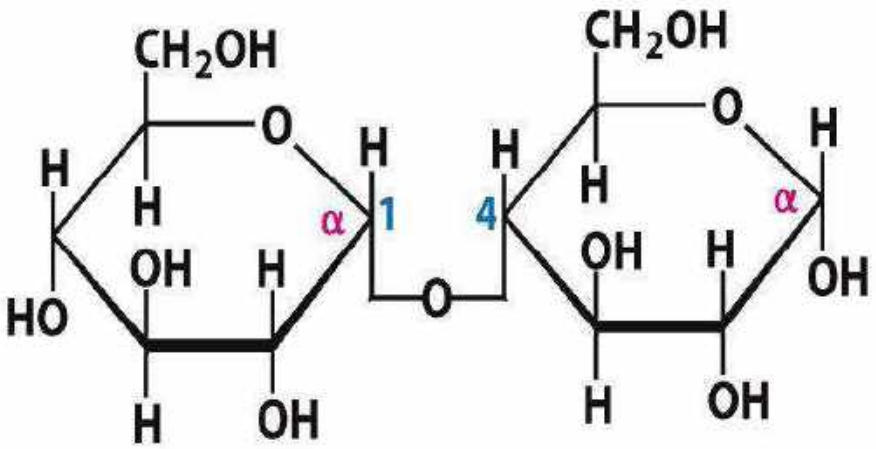
Reducing sugar	*Nonreducing sugar*
1. Carbohydrates with a free aldehyde (at C-1) or a free ketone (at C-2) group. 2. They are in hemiacetal or hemiketal form. 3. Do exhibit mutarotation. 4. Do form osazones with phenyl hydrazine. 5. Do form oximes with hydroxylamine. *Examples* – Glucose, Fructose, Lactose, Maltose, Cellobiose	1. Aldehyde or ketone group is not free but instead utilized in bond formation. 2. They are in acetal or ketal form. 3. Do not exhiblit mutarotation. 4. Do not form osazones. 5. Do not form oximes. Examples – Sucrose, Glycogen, Inulin



Sucrose
(α-D-Glucopyranosyl-(1→ 2)-β-D-fructofuranose



Lactose
(β-D-Galactopyranosyl-(1→ 4)- α-D-glucopyranose



Maltose
(α-D-Glucopyranosyl-(1→ 4)-α-D-glucopyranose

Figure 11.11
***Biochemistry*, Seventh Edition**

Oligosaccharides

These are *compound sugars* that yield 2 to 10 molecules of the same or different monosaccharides on hydrolysis. Accordingly, an oligosaccharide yielding 2 molecules of monosaccharide on hydrolysis is designated as a disaccharide, and the one yielding 3 molecules of monosaccharide as a trisaccharide and so on.

Disaccharides – Sucrose, Lactose, Maltose, Cellobiose, Trehalose, Gentiobiose, Melibiose
Trisaccharides – Rhamninose, Gentianose, Raffinose (= Melitose), Rabinose, Melezitose
Tetrasaccharides – Stachyose, Scorodose
Pentasaccharide – Verbascose
The molecular composition of the 3 legume oligosaccharides (viz., raffinose, stachyose and verbascose) is shown below :
α-Galactose (1–6) α-Glucose (1–2) β-Fructose Raffinose
α-Galactose (1–6) α-Galactose (1–6) α-Glucose (1–2) β-Fructose Stachyose
α-Galactose (1–6) α-Galactose (1–6) α-Galactose (1–6) α-Glucose (1–2) β-Fructose Verbascose

$$\text{Maltose} + H_2O \xrightarrow{H^+ \text{ or maltase}} \text{glucose} + \text{glucose}$$

$$\text{Lactose} + H_2O \xrightarrow{H^+ \text{ or lactase}} \text{glucose} + \text{galactose}$$

$$\text{Sucrose} + H_2O \xrightarrow{H^+ \text{ or sucrase}} \text{glucose} + \text{fructose}$$

Disaccharides

- Composed of 2 monosaccharides
- cells can make disaccharides by joining two monosaccharides by biosynthesis.

Glucose + fructose = sucrose

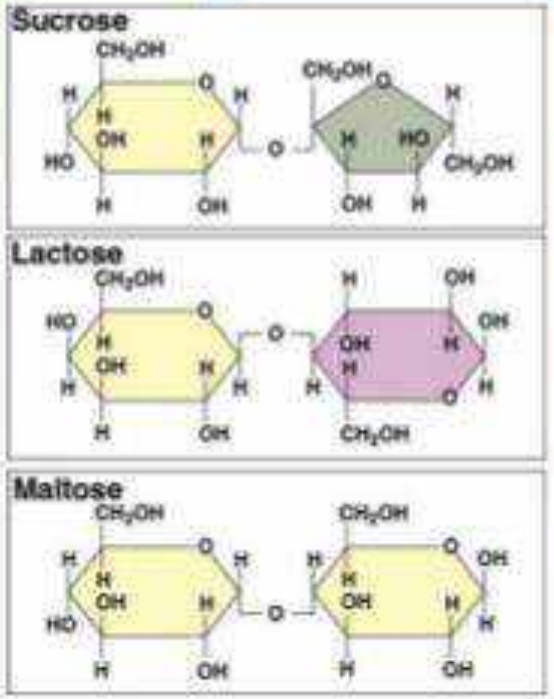
- Table sugar
- Found naturally in plants: sugar cane, sugar beets, honey, maple syrup
- Sucrose may be purified from plant sources into Brown, White and Powdered Sugars.

Glucose + galactose = lactose

- The primary sugar in milk and milk products.
- Many people have problems digesting large amounts of lactose (lactose intolerance)

Glucose + glucose = Maltose

- Produced when starch breaks down.
- Used naturally in fermentation reactions of alcohol and beer manufacturing.



Trisaccharides: Composed of three monosaccharide ex: Raffinose (Formed by one mole of each i.e. glu, fruc, galac)

Tetrasaccharides :

ex: Stachyose (composed of two moles of galactose one mole of glu & one mole of fruct)

TABLE 13.2 Relative Sweetness of Sugars and Artificial Sweeteners

	Sweetness Relative to Sucrose
Monosaccharides	
Galactose	30
Glucose	75
Fructose	175
Disaccharides	
Lactose	16
Maltose	33
Sucrose	100 ← reference standard
Sugar Alcohols	
Sorbitol	60
Maltitol	80
Xylitol	100
Artificial Sweeteners (Noncarbohydrate)	
Aspartame	18 000
Saccharin	45 000
Sucralose	60 000
Neotame	1 000 000

Artificial sweeteners are used as sugar substituents.

Polysaccharides

Containing 10 or more monosaccharide units attached together

- Examples

1. **Starch- digestible**
2. **Glycogen- digestible**
3. **Fiber- indigestible**

Long chains of glucose units form these polysaccharides

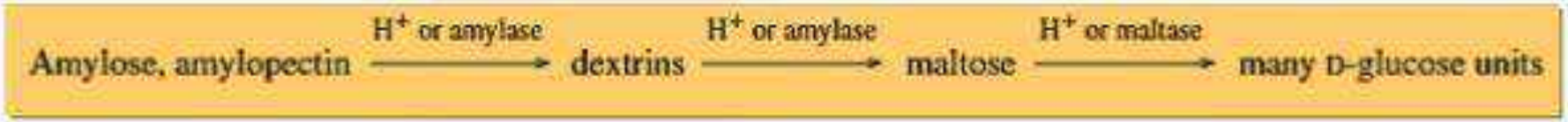
- Cellulose gives structure to plants, fiber to our diet
- Glycogen is an energy storage sugar produced by animals
- Liver cells synthesize glycogen after a meal to maintain blood glucose levels

A great majority of carbohydrates of nature occur as polysaccharides

Chemically, the polysaccharides may be distinguished into **homopolysaccharides**, which yield, on hydrolysis, a single monosaccharide and **heteropolysaccharides** ,which produce a mixture of monosaccharides on hydrolysis. Based on their functional aspect, the polysaccharides may be grouped under two heads :

(*a*) **Nutrient** (or **digestible**) **polysaccharides.** These act as metabolic reserve of monosaccharides in plants and animals, *e.g.*, starch, glycogen and inulin.

(*b*) **Structural** (or **indigestible**) **polysaccharides.** These serve as rigid mechanical structures in plants and animals, *e.g.*,cellulose, pectin and chitin and also hyaluronic acid and chondroitin.



Types of Polysaccharides

1. Starch

- The major digestible polysaccharide in our diet.
- The storage form of carbohydrate in plants.
- Sources: Wheat, rice, corn, rye, barley, potatoes, tubers, yams, etc.
- Two types of plant starch: 1. Amylose
  2. Amylopectin

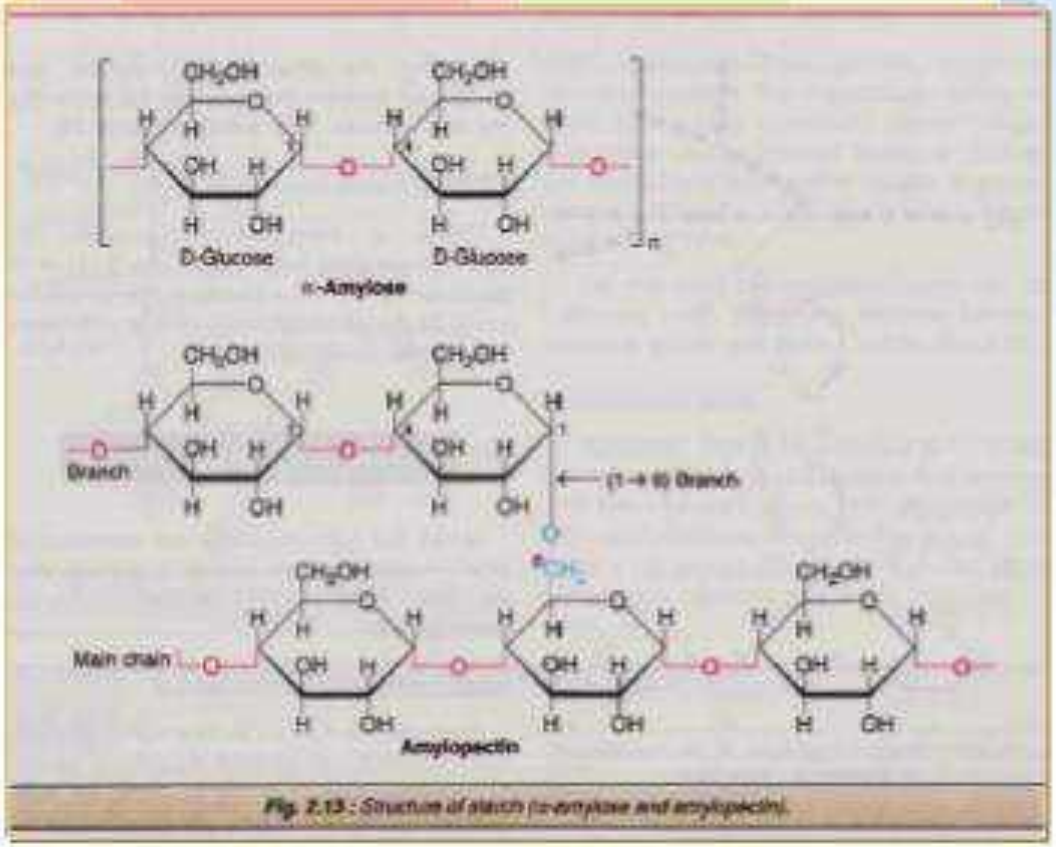


Fig. 2.13 : Structure of starch (α-amylose and amylopectin)

Amylose: is in the form of straight chain linked together with α- 1-4, linkages indicating 300 – 5,500 glucose units per molecules, molecular wt range from 10^5 to 10^6. Generally it is water soluble and gives blue colour with iodine.

Amylopectins: It contain beside straight chain several branched chains, which are arranged in α—1-4 and β-1-6 linkage units, one molecule of amylopectin contains 50,000 to 5,00,000 glucose molecules, molecular wt. range from 10^7 to 10^8, it is insoluble in water and gives purple colour with iodine .

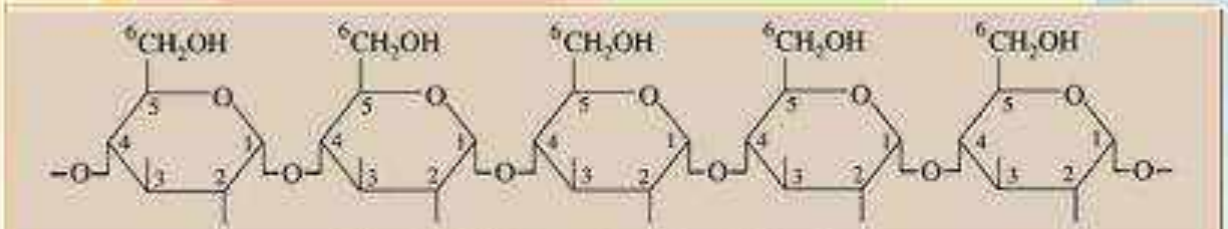


Fig. 8–1. Structure of amylose (= A-fraction)

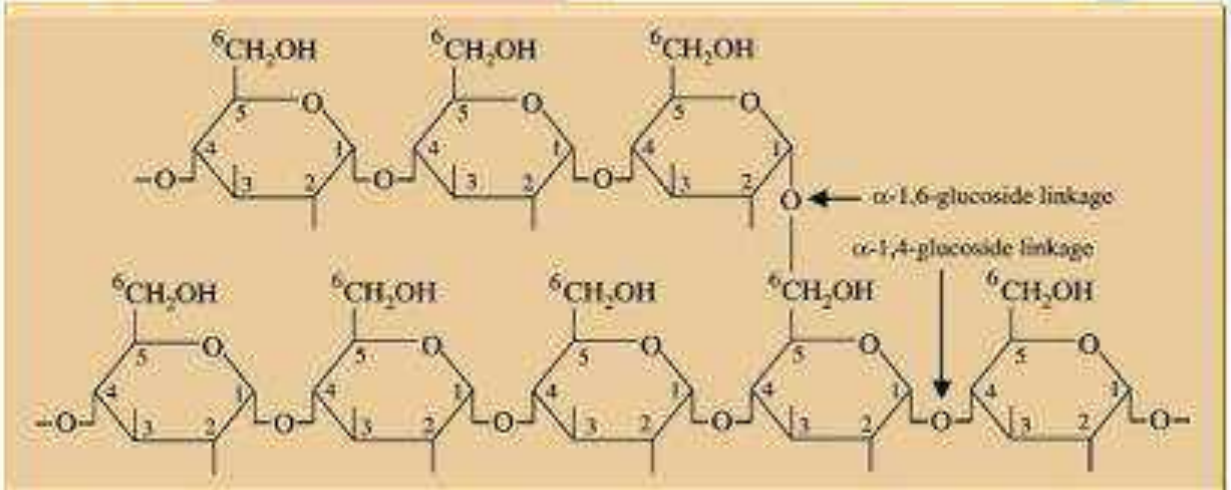
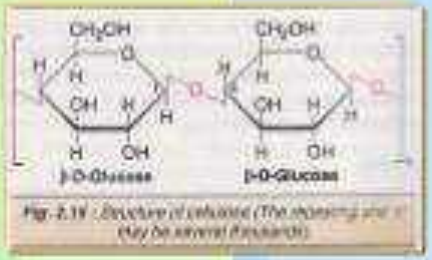


Fig. 8–2. Structure of amylopectin (= B-fraction)

Types of Polysaccharides

2. **Cellulose** - form cell walls in plant cells
   - also called fiber or ruffage
   - indigestible by humans



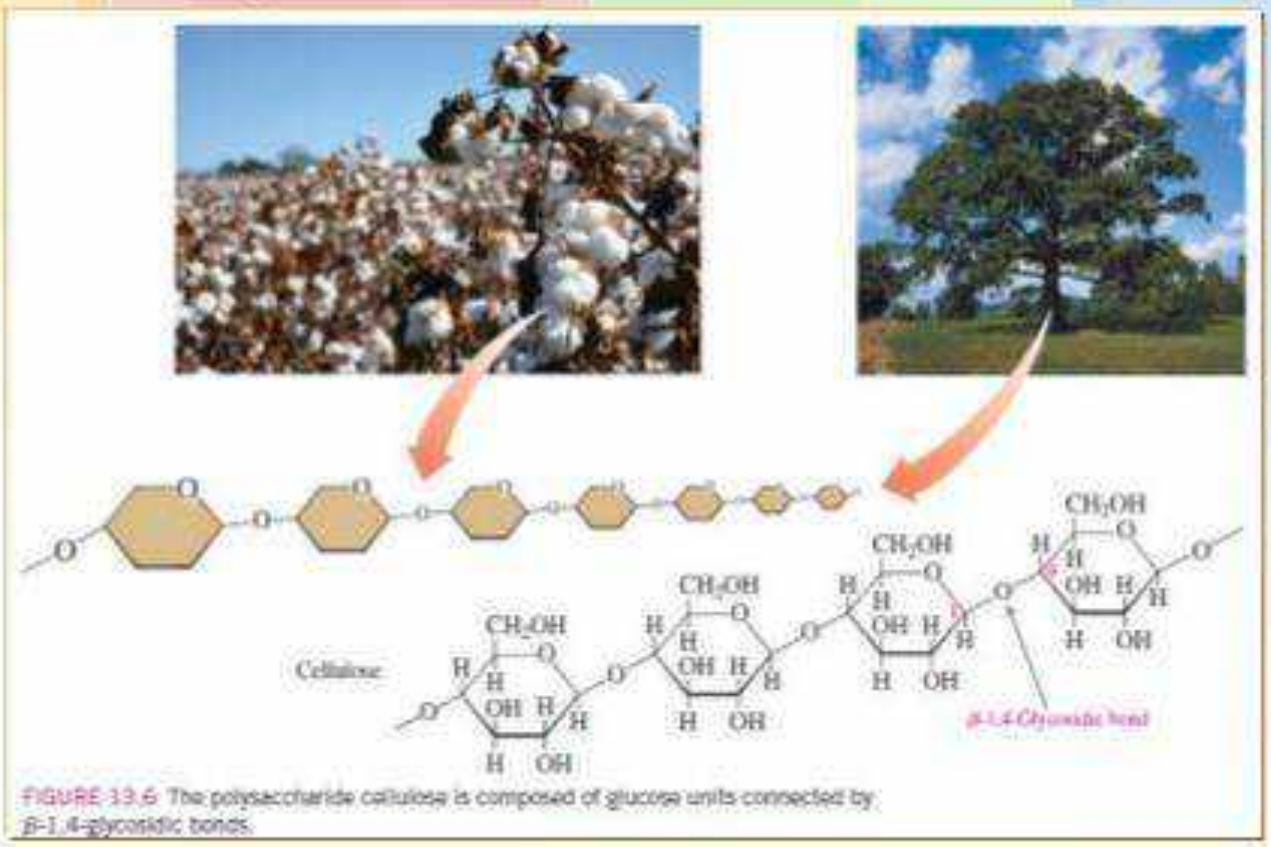


FIGURE 13.6 The polysaccharide cellulose is composed of glucose units connected by β-1,4-glycosidic bonds.

Types of Polysaccharides

3. Glycogen

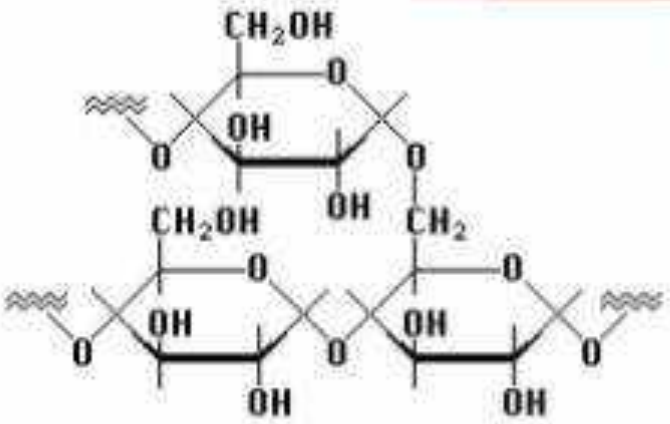
The storage form of glucose in the body.

Stored in the liver and muscles.

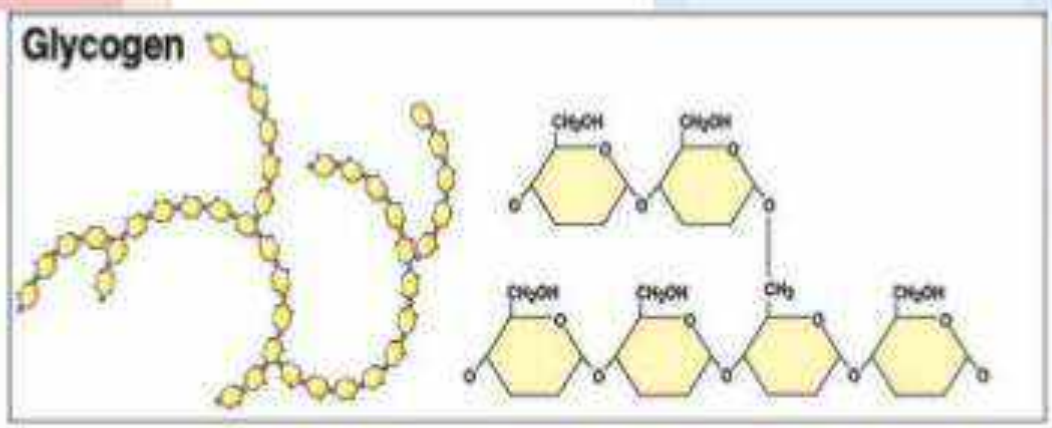
Found in tiny amounts in meat sources.

Not found in plants.

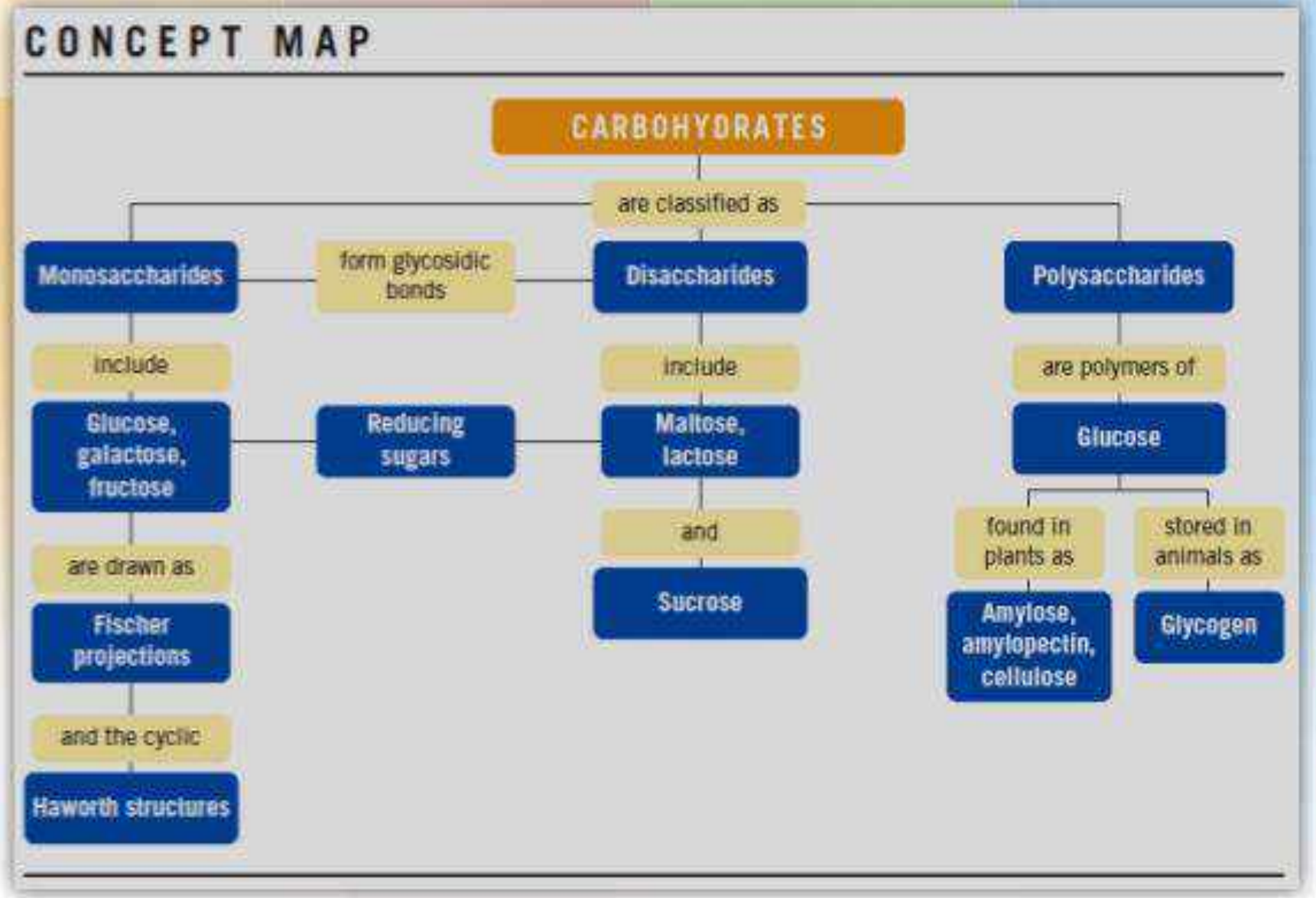
Not a significant food source of carbohydrate.

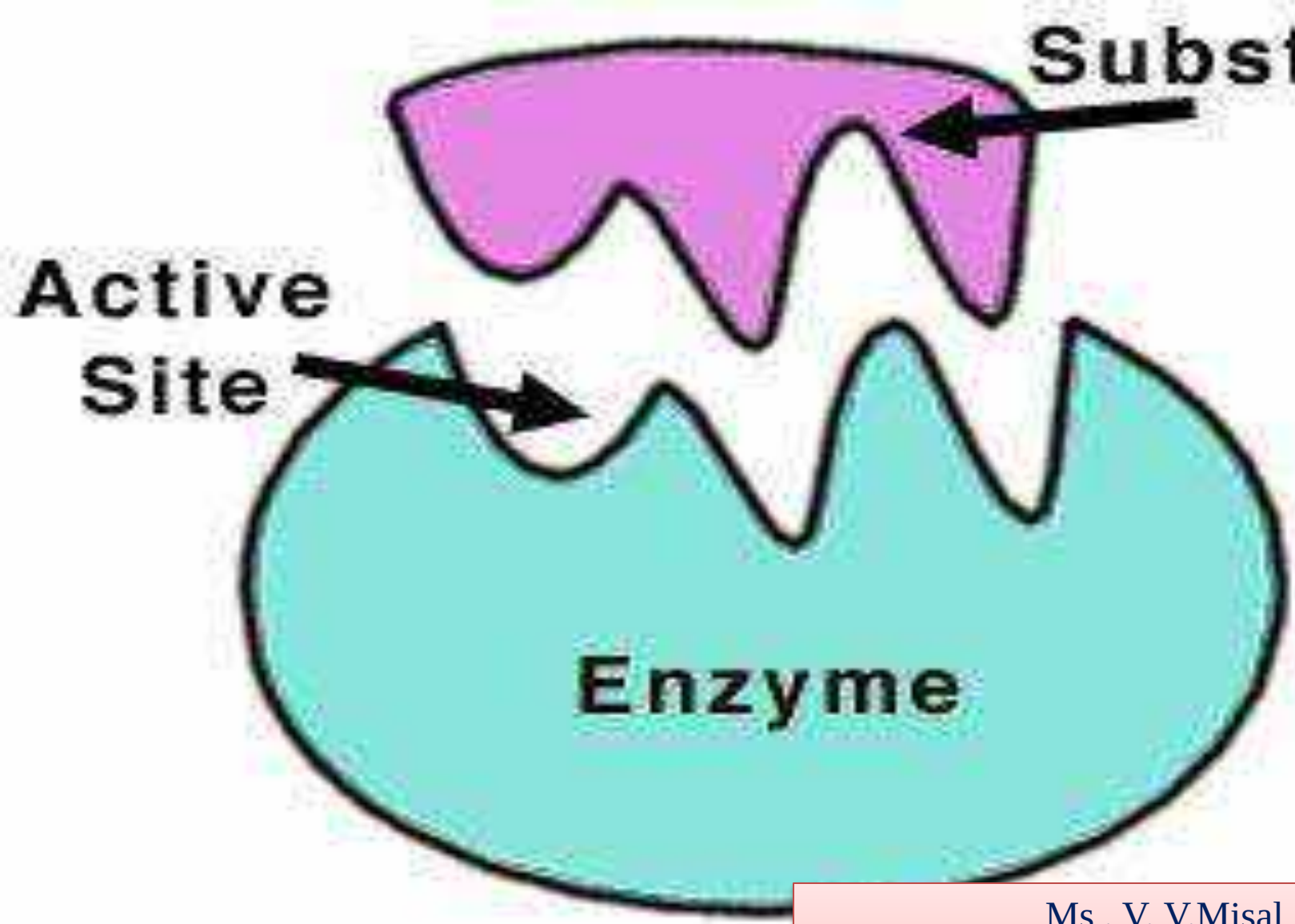


glycogen



CONCEPT MAP


CARBOHYDRATES
are classified as
Monosaccharides
form glycosidic bonds
Disaccharides
Polysaccharides
include
include
are polymers of
Glucose, galactose, fructose
Reducing sugars
Maltose, lactose
Glucose
are drawn as
and
found in plants as
stored in animals as
Fischer projections
Sucrose
Amylose, amylopectin, cellulose
Glycogen
and the cyclic
Haworth structures



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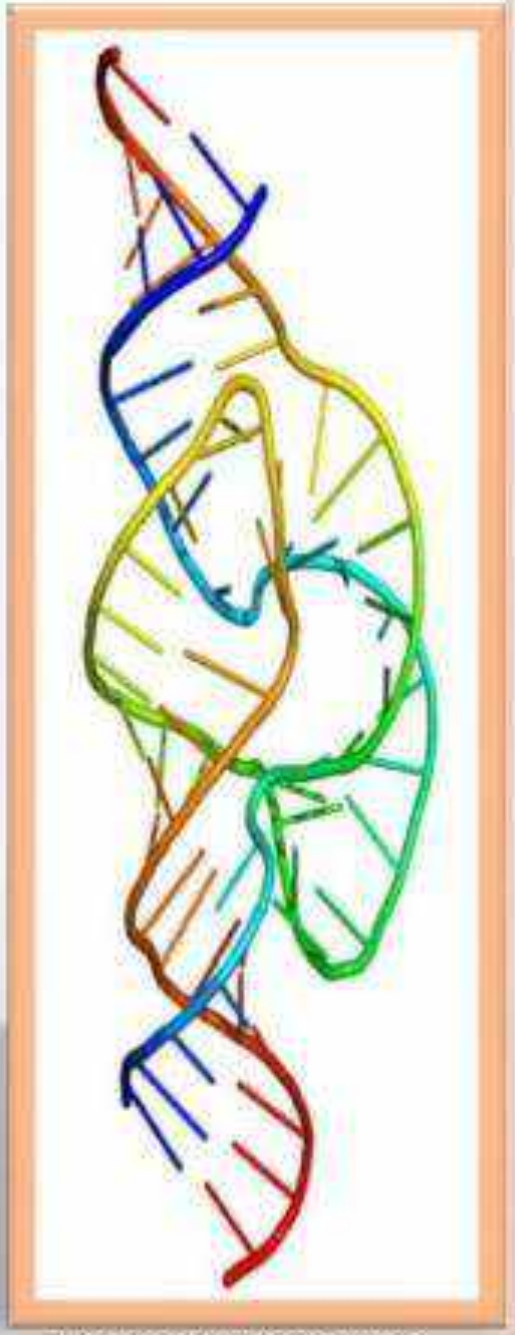
History of Enzyme

- The term 'enzyme' was coined in 1878 by Friedrich Wilhelm Kuhne
- 'biological catalysts' that had previously been called 'ferments'
- "manifestations of nature's impatience".
- The name 'enzyme' (en (G) = in ; zyme (G) = yeast) literally means 'in yeast'
- Because of most recognizable reaction popularly known as alcohol fermentation by zymase enzyme in yeast

Friedrich Wilhelm Kuhne

Introduction

- Enzymes are *biological catalysts* that speed up the rate of the biochemical reaction.
- Most enzymes are three dimensional *globular proteins* (tertiary and quaternary structure).
- Some special RNA species also act as enzymes and are called *Ribozymes* e.g. hammerhead ribozyme.



Hammerhead enzyme

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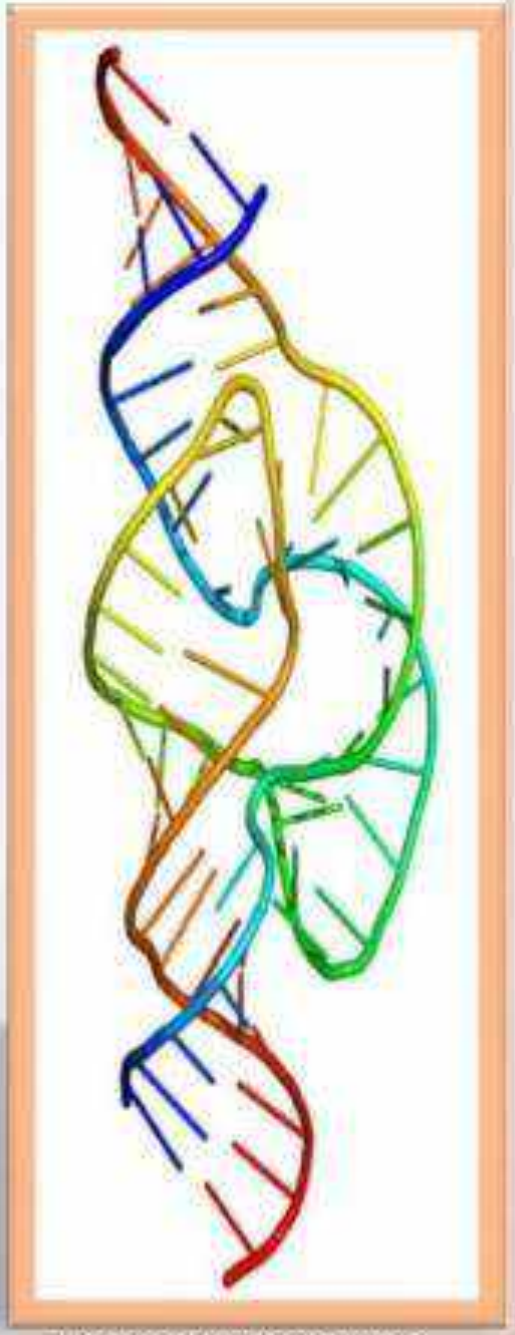


Hammerhead enzyme

Difference from catalysts

- Like catalysts, the enzymes do not alter the chemical equilibrium point of a reversible reaction but only the speed of the reaction is changed
- Differ from catalysts in being the biological products, i.e., produced from the living cells.
- the enzymes are all protein and, unlike catalysts, cannot last indefinitely in a reaction system since they, being colloidal in nature, often become damaged or inactivated by the reactions they catalyze. they must be replaced constantly by further synthesis in the body.
- unlike catalysts, most individual enzymes are very specific in that they act either on a single or at the most on some structurally related substrates.

Enzyme

- Enzymes are characterized by three distinctive features:
- **Catalytic Power**
  - Ability to catalyses biochemical reaction
  - Accelerating reaction rates as much as 10^{16} over uncatalyzed levels - far greater than any synthetic catalysts
- **Specificity**
  - A given enzyme is very selective
  - Both in the substances with which it interacts and in the reaction that it catalyzes
- **Regulation**
  - Metabolic inhibitors and activators

CHARACTERISTICS

- Enzymes *speed up* the reaction by lowering the activation energy of the reaction.
- Their presence *does not effect* the nature and properties of *end product*.
- They are *highly specific* in their action that is each enzyme can catalyze one kind of substrate.
- Small amount of enzymes can accelerate chemical reactions.
- Enzymes are *sensitive* to change in pH, temperature and substrate concentration.
- *Turnover number* is defined as the number of substrate molecules transformed per minute by one enzyme molecule.

Catalase turnover number = 6 x106/min

Structure of Enzymes

- The *active site* of an enzyme is the region that binds substrates, co-factors and prosthetic groups and contains residue that helps to hold the substrate.
- Active sites generally occupy less than 5% of the total surface area of enzyme.
- Active site has a *specific shape* due to tertiary structure of protein.
- A change in the shape of protein affects the shape of active site and function of the enzyme.

ACTIVE SITE

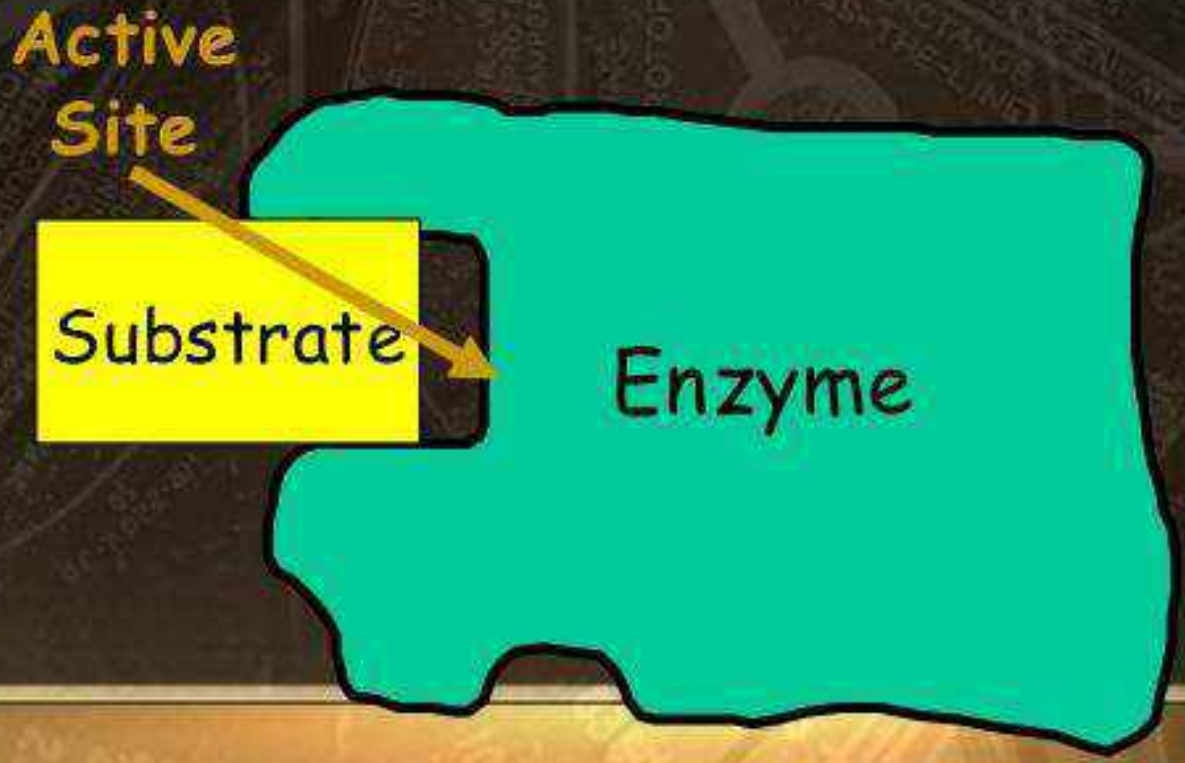
- Active site can be further divided into:

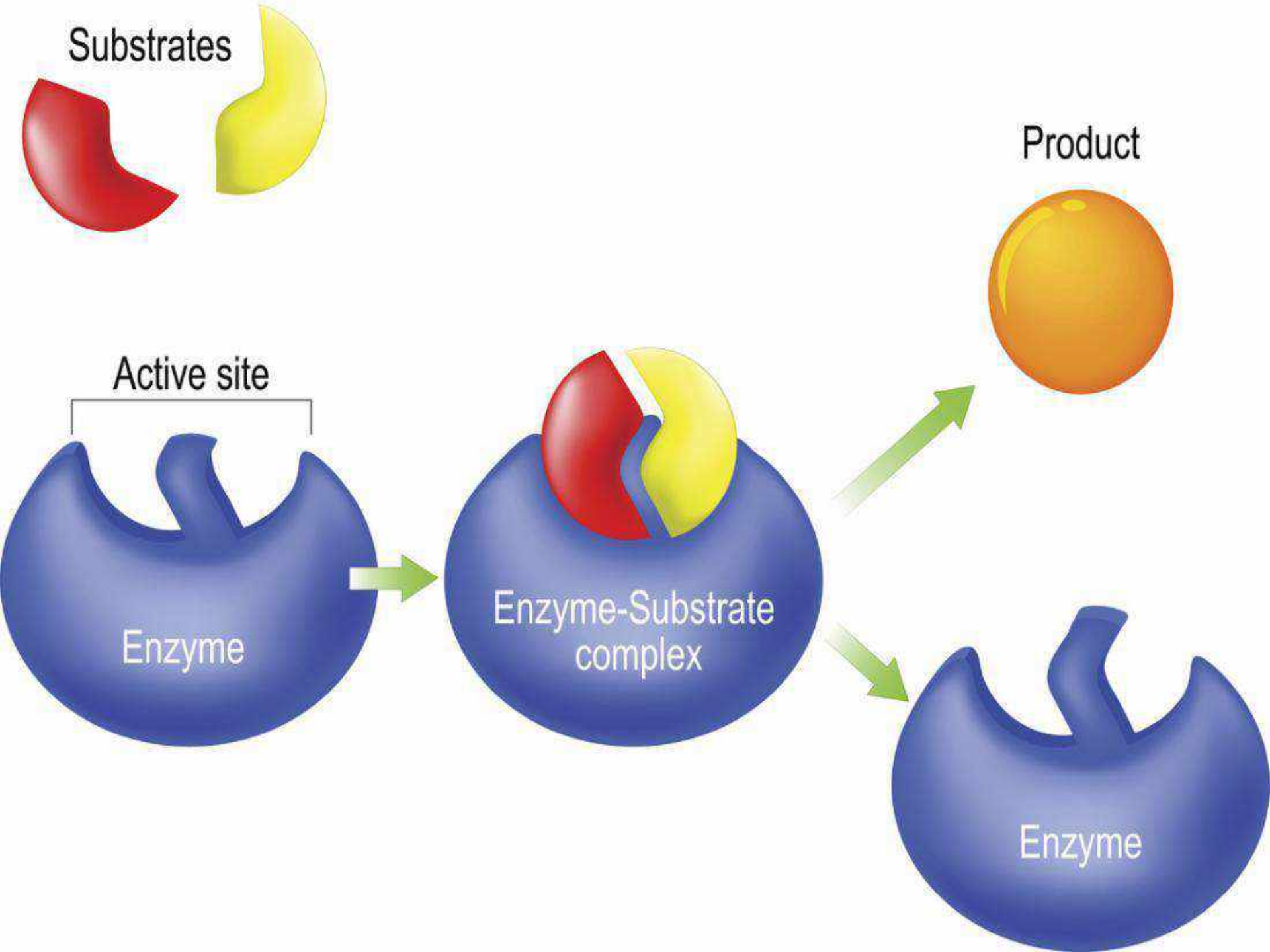
It chooses the substrate and binds it to active site.

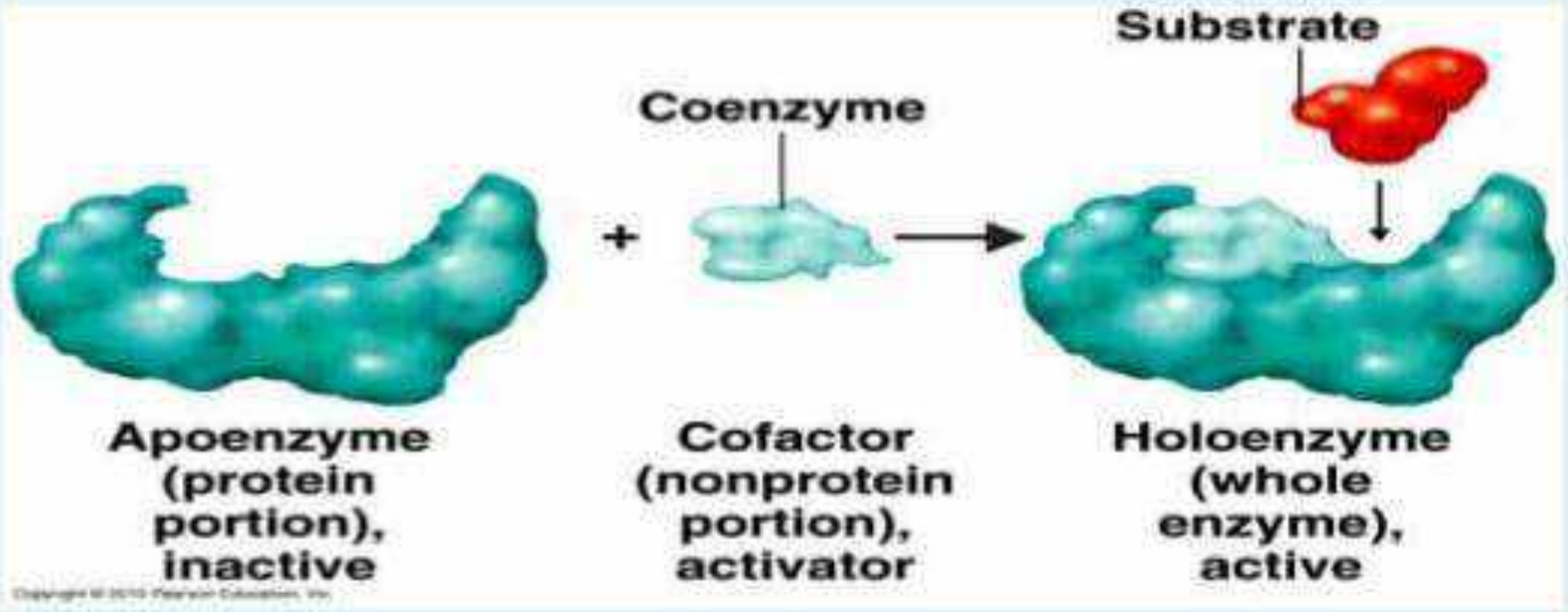
It performs the catalytic action of enzyme.

Active Site

- A **restricted region** of an **enzyme** molecule which **binds** to the **substrate**.




Substrates
Product
Active site
Enzyme
Enzyme-Substrate complex
Enzyme

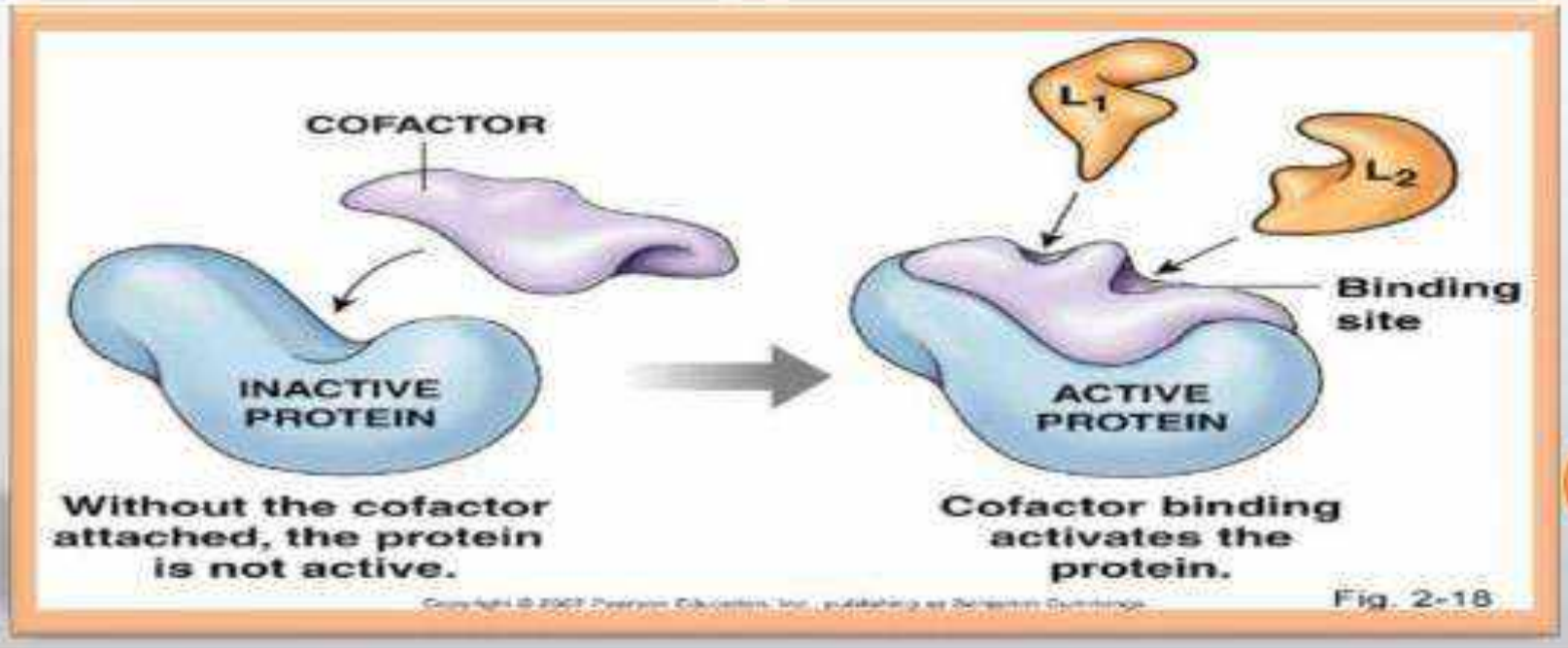

Substrate
Coenzyme
+
Apoenzyme (protein portion), inactive
Cofactor (nonprotein portion), activator
Holoenzyme (whole enzyme), active


Apo enzyme
coenzyme
Holo enzyme

CO-FACTORS

- Co-factor is the non protein molecule which carries out chemical reactions that can not be performed by standard 20 amino acids.

- Co-factors are of two types:
  - Organic co-factors
  - Inorganic cofactors



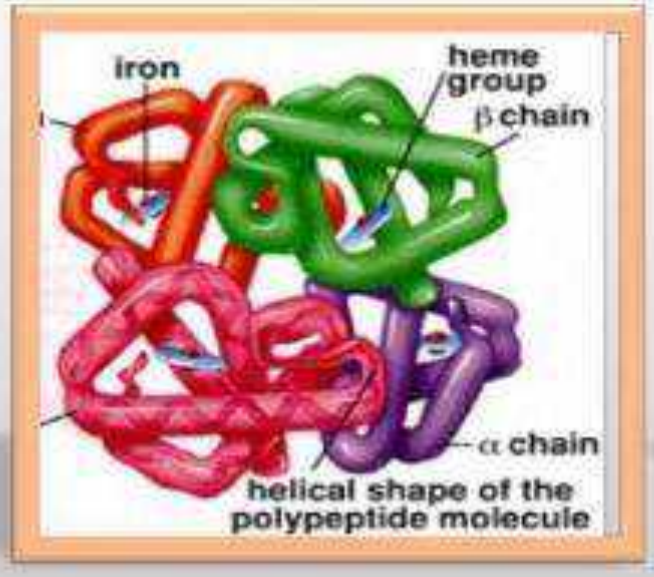
Types of Organic co-factors

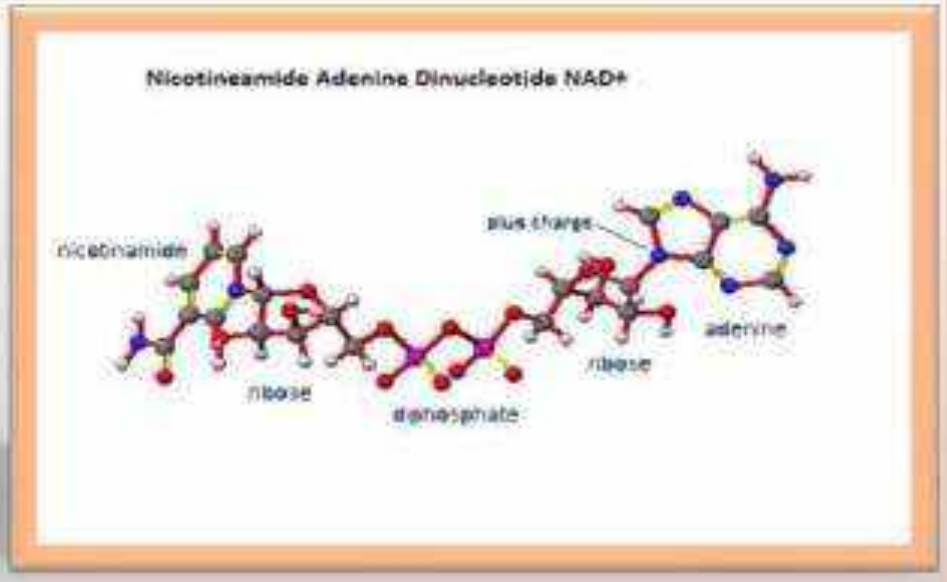
Prosthetic Group

- A prosthetic group is a tightly bound organic co-factor e.g. Flavins, heme groups and biotin.

Coenzyme

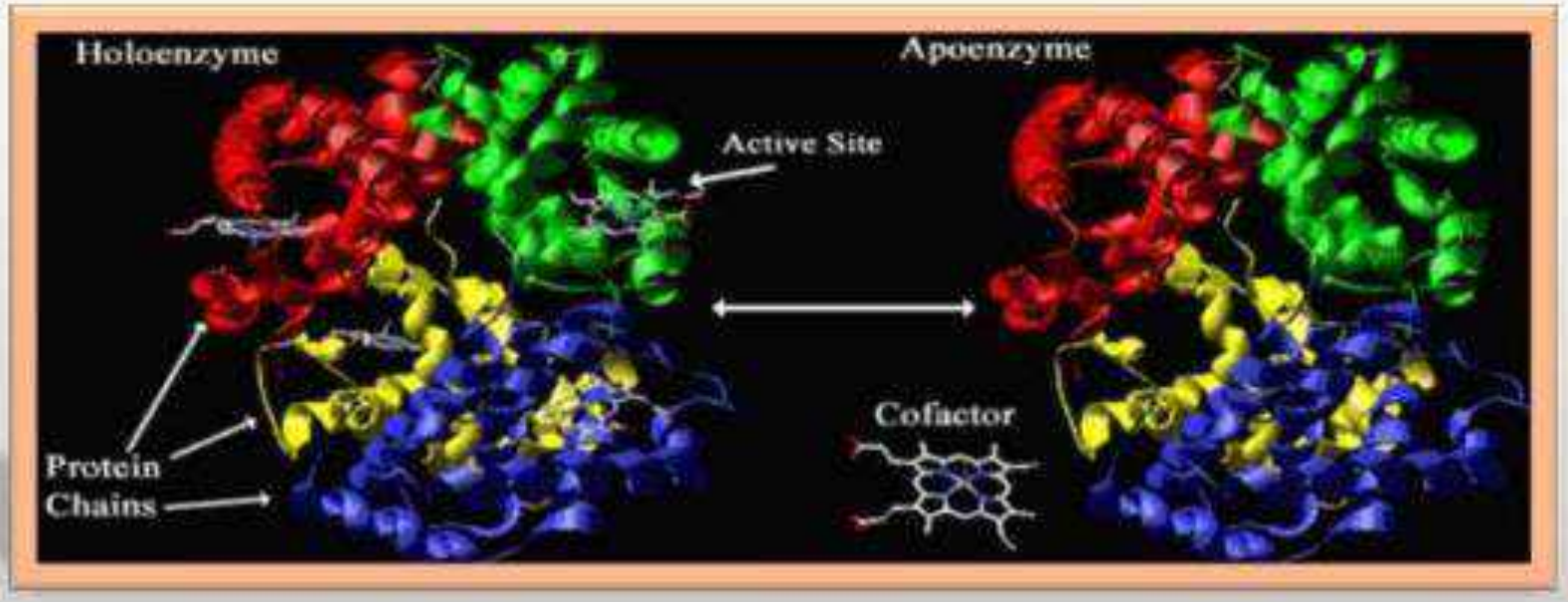
- A coenzyme is loosely bound organic co-factor. E.g. NAD^+





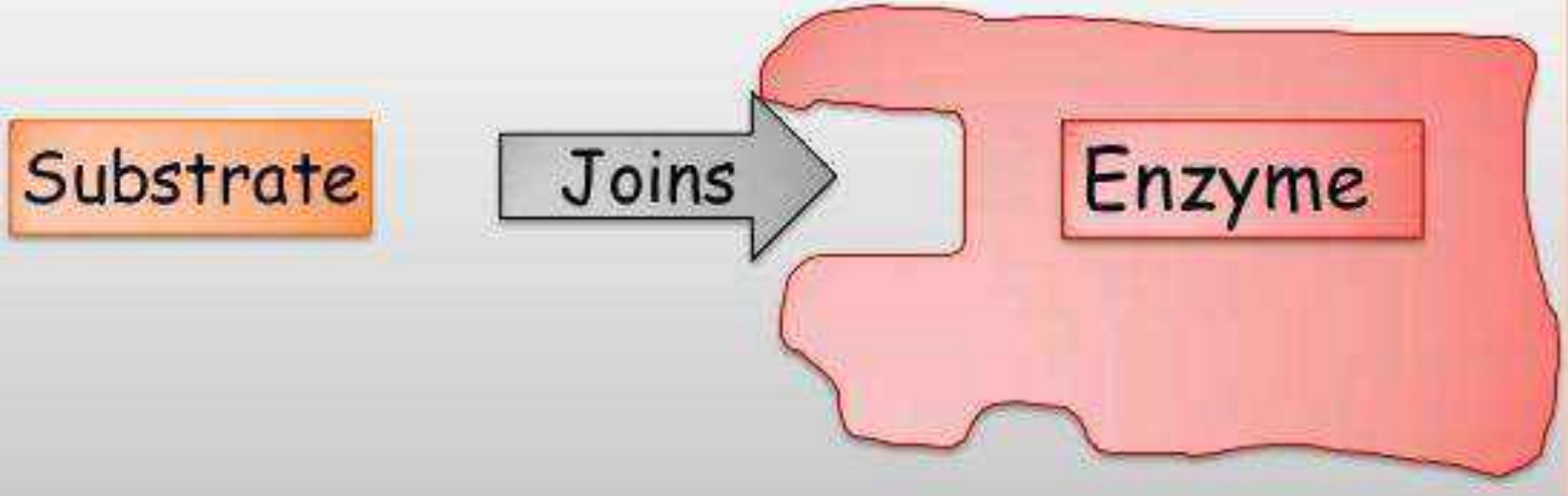
Types of co-factors Continued…

- An enzyme with it's co-factor removed is designated as *apoenzyme*.
- The complete complex of a protein with all necessary small organic molecules, metal ions and other components is termed as *holoenzyme* of *holoprotein*.



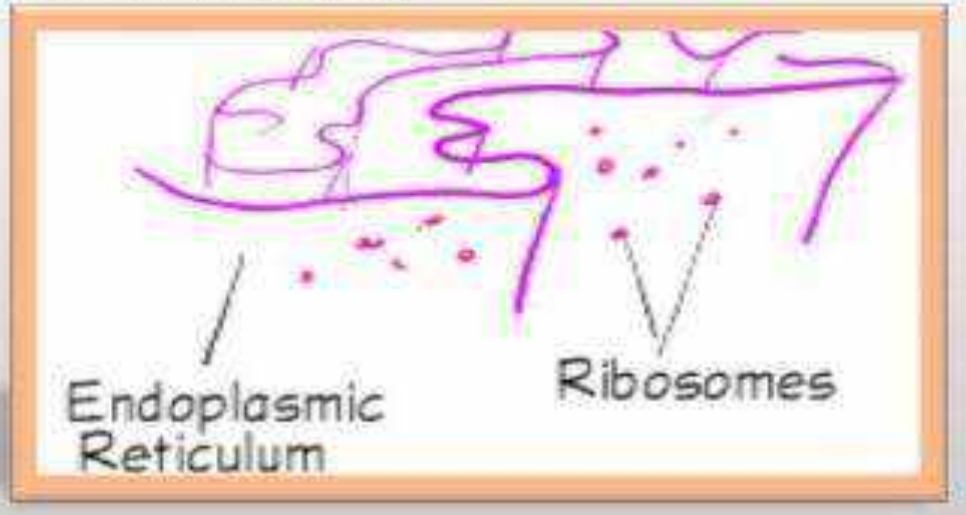
SUBSTRATE

- The reactant in biochemical reaction is termed as substrate.
- When a substrate binds to an enzyme it forms an enzyme-substrate complex.



SITES OF ENZYME SYNTHESIS

- Enzymes are synthesized by *ribosomes* which are attached to the rough endoplasmic reticulum.
- Information for the synthesis of enzyme is *carried by DNA*.
- Amino acids are bonded together to form specific enzyme according to the DNA's codes.



INTRACELLULAR AND EXTRACELLULAR ENZYMES

- **Intracellular** enzymes are *synthesized and retained in the cell for the use of cell itself.*
- They are found in the cytoplasm, nucleus, mitochondria and chloroplast.

Example :

- Oxydoreductase catalyses biological oxidation.
- Enzymes involved in reduction in the mitochondria.

- **Extracellular** enzymes are *synthesized in the cell but secreted from the cell to work externally.*

Example :

- Digestive enzyme produced by the pancreas, are not used by the cells in the pancreas but are transported to the duodenum.

INTRACELLULAR AND EXTRACELLULAR ENZYMES

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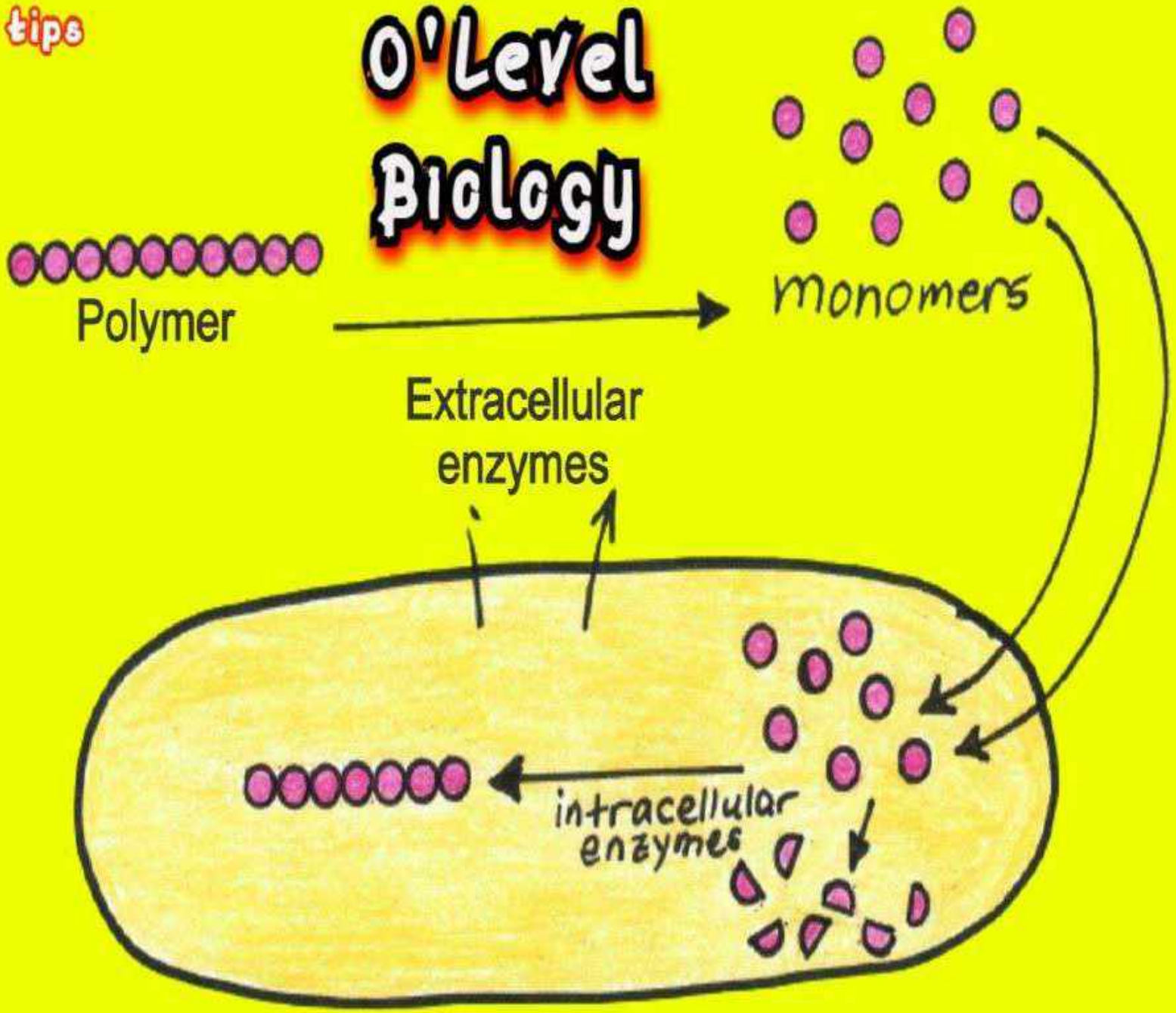
- **Extracellular** enzymes are *synthesized in the cell but secreted from the cell to work externally.*

Example :

- Digestive enzyme produced by the pancreas, are not used by the cells in the pancreas but are transported to the duodenum.

Ladder tips

O'Level Biology



TYPES OF ENZYMES

- **CONSTITUTIVE ENZYME** : produced independently of the composition of the medium.
- **INDUCIBLE ENZYME** : produced when they are needed, only in the presence of substrate.
- **REPRESSIBLE ENZYME** : this is downregulated (turned off) when there is the formation of the end product.

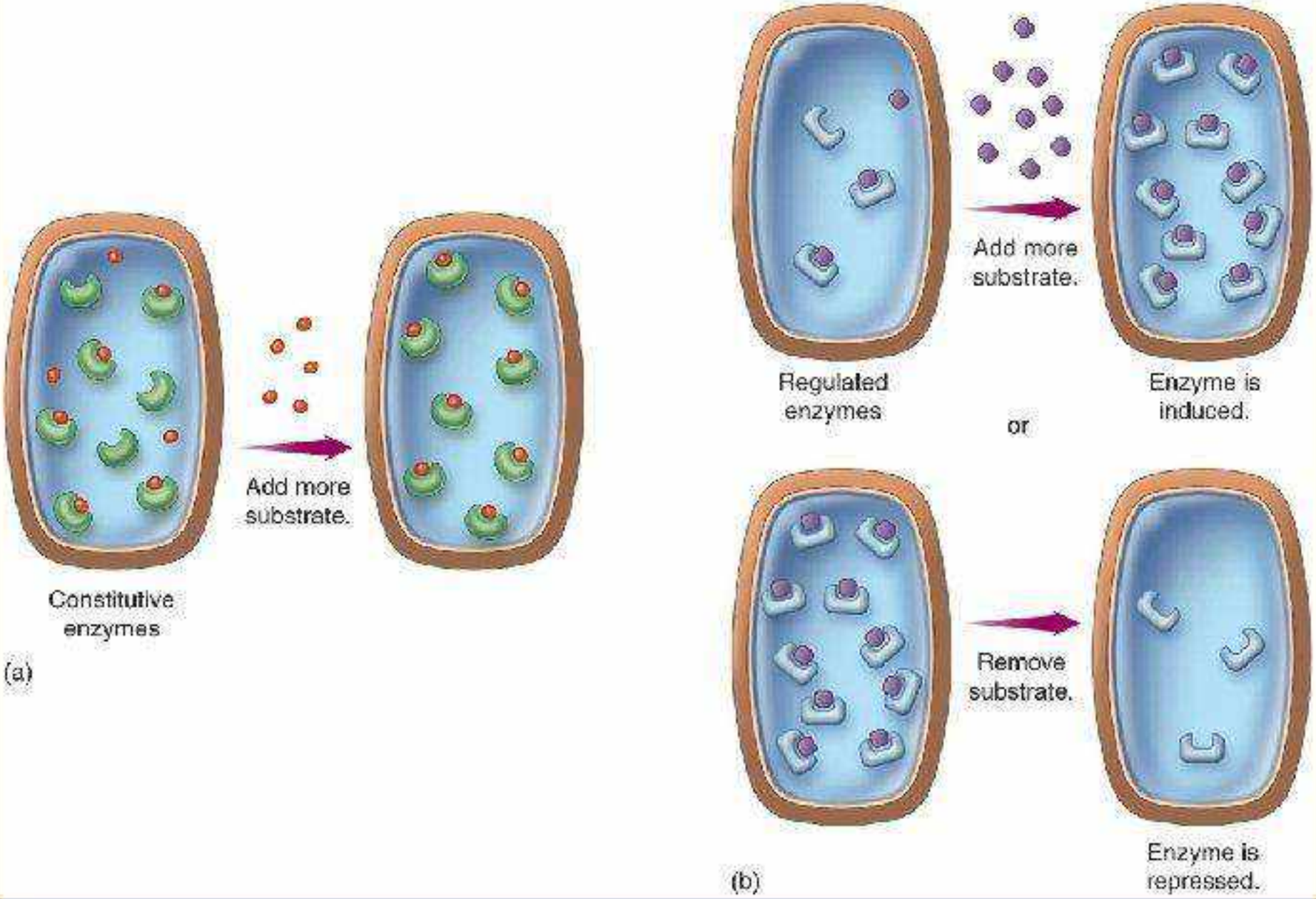


Figure 8.6

MECHANISM OF ENZYME ACTION

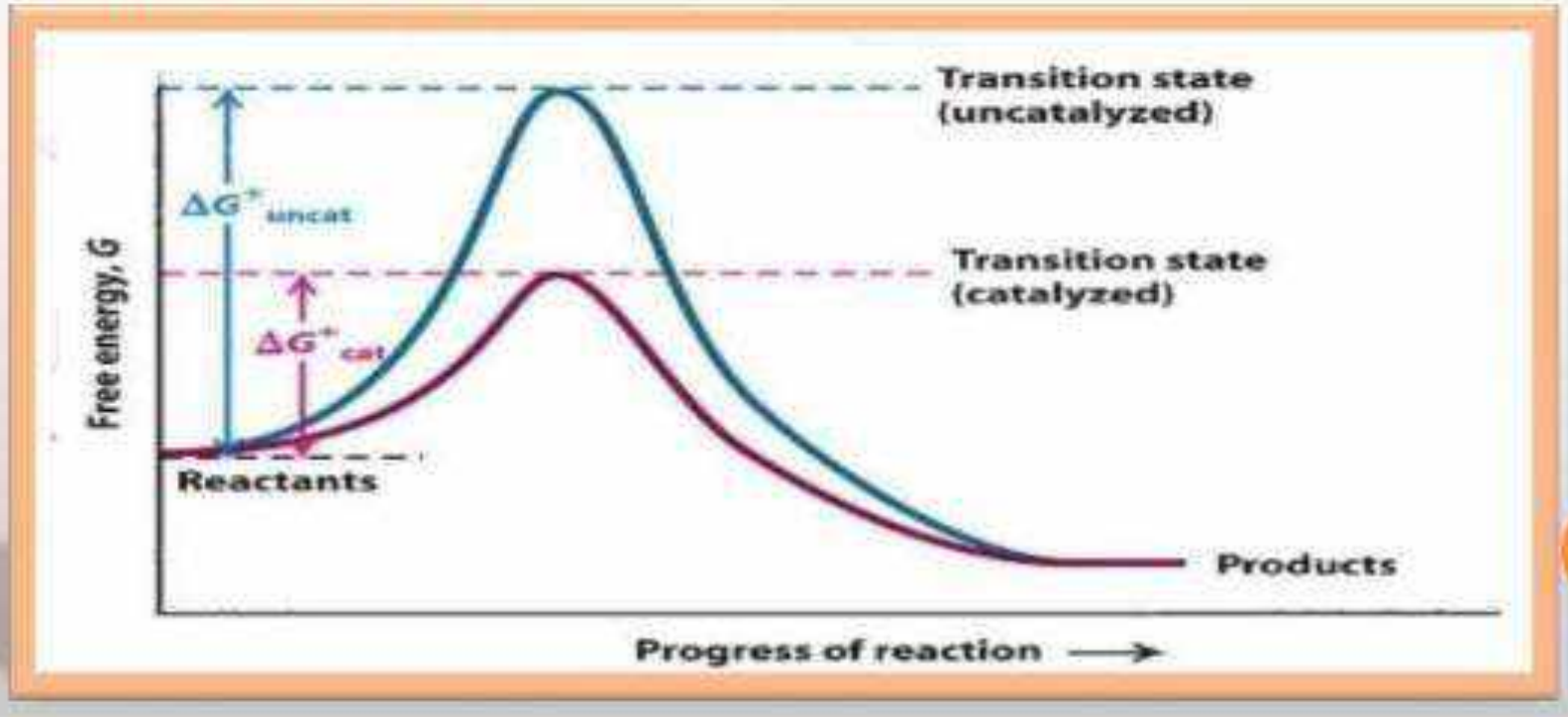
- The catalytic efficiency of enzymes is explained by two perspectives:

Thermodynamic changes

Processes at the active site

THERMODYNAMIC CHANGES

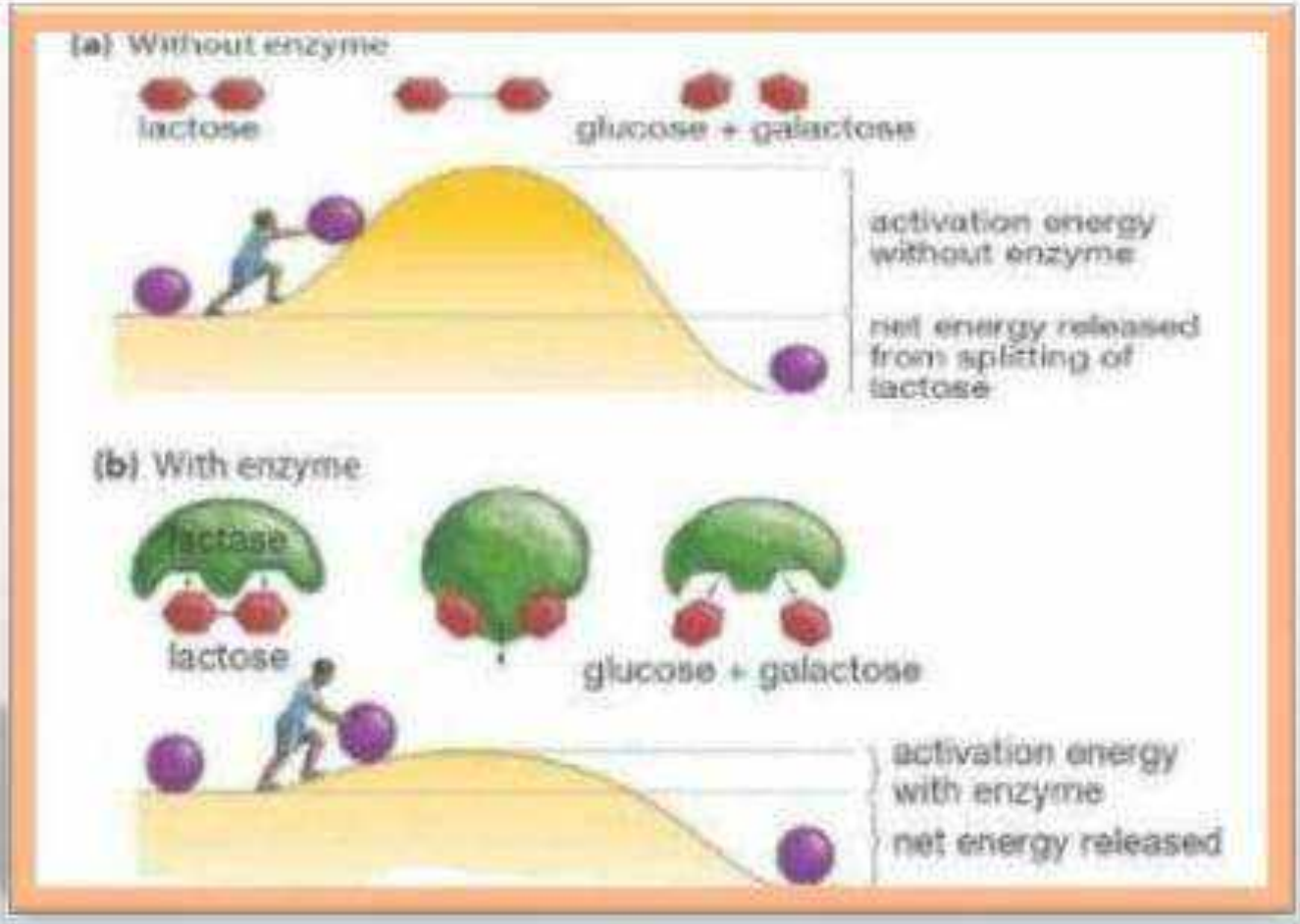
- All chemical reactions have energy barriers between reactants and products.
- The difference in transitional state and substrate is called *activational barrier*.



THERMODYNAMIC CHANGES

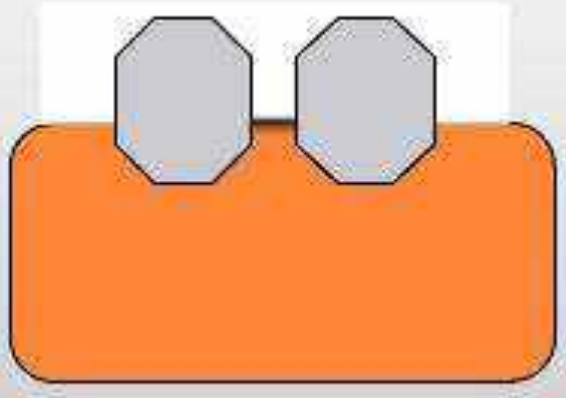
- Only a few substances cross the activation barrier and change into products.
- That is why rate of uncatalyzed reactions is much slow.
- Enzymes provide an alternate pathway for conversion of substrate into products.
- Enzymes accelerate reaction rates by forming transitional state having low activational energy.
- Hence, the reaction rate is increased many folds in the presence of enzymes.
- The total energy of the system remains the same and equilibrium state is not disturbed.

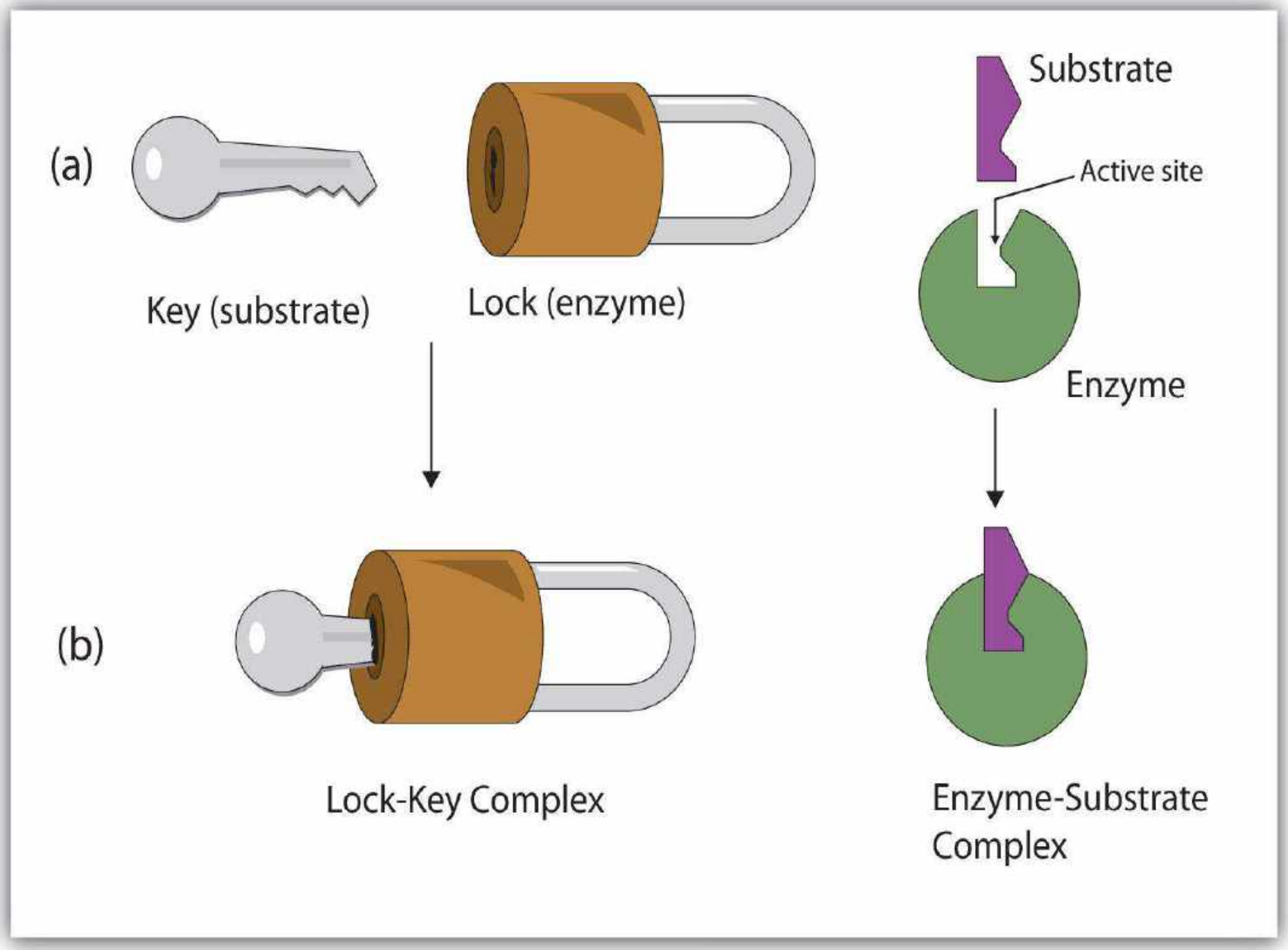
THERMO-DYNAMIC CHANGES OVERVIEW



LOCK AND KEY MODEL

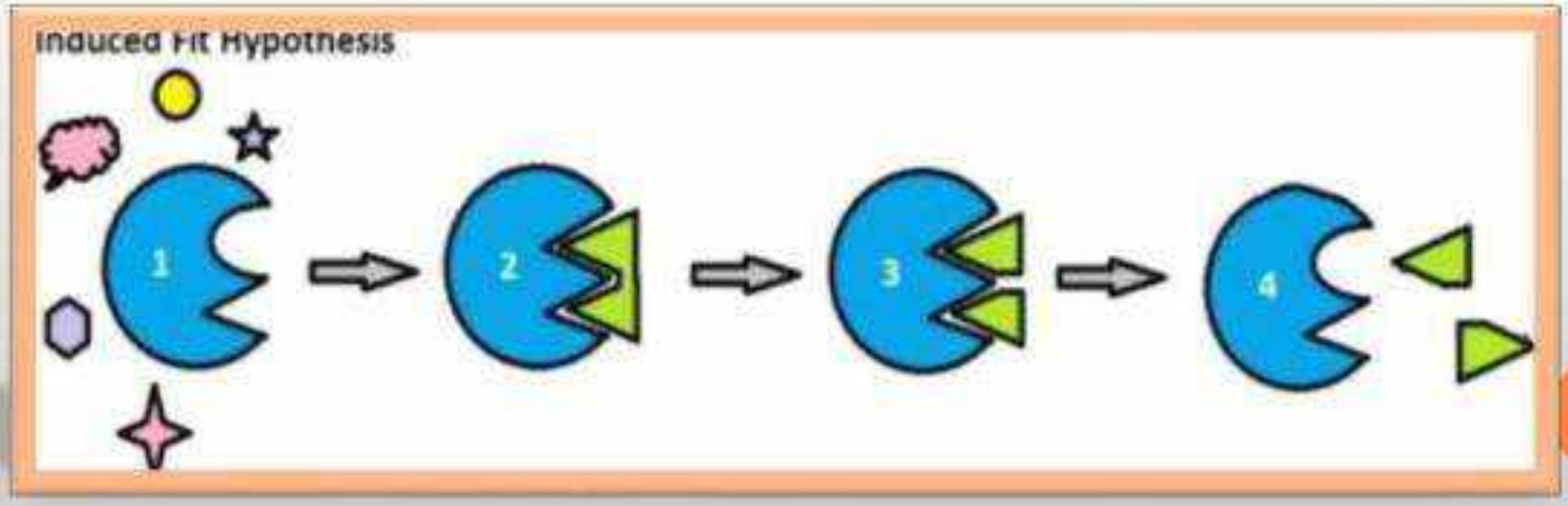
- Proposed by EMIL FISCHER in 1894.
- Lock and key hypothesis assumes the active site of an enzymes are rigid in its shape.
- There is no change in the active site before and after a chemical reaction.




(a)
Key (substrate)
Lock (enzyme)
Substrate
Active site
Enzyme
(b)
Lock-Key Complex
Enzyme-Substrate Complex

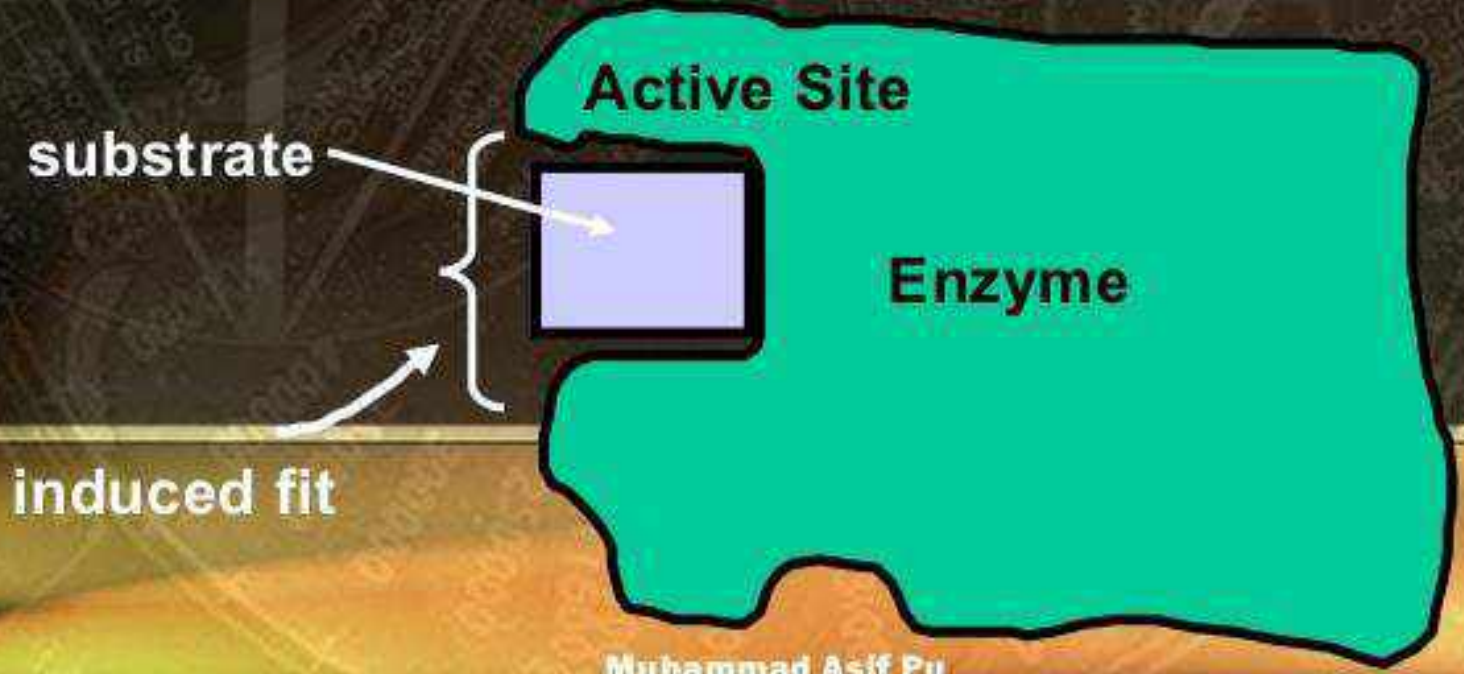
INDUCED FIT MODEL

- More recent studies have revealed that the process is much more likely to involve an induced fit model(proposed by DANIAL KOSH LAND in 1958).
- According to this exposure of an enzyme to substrate cause a change in enzyme, which causes the active site to change it's shape to allow enzyme and substrate to bind.



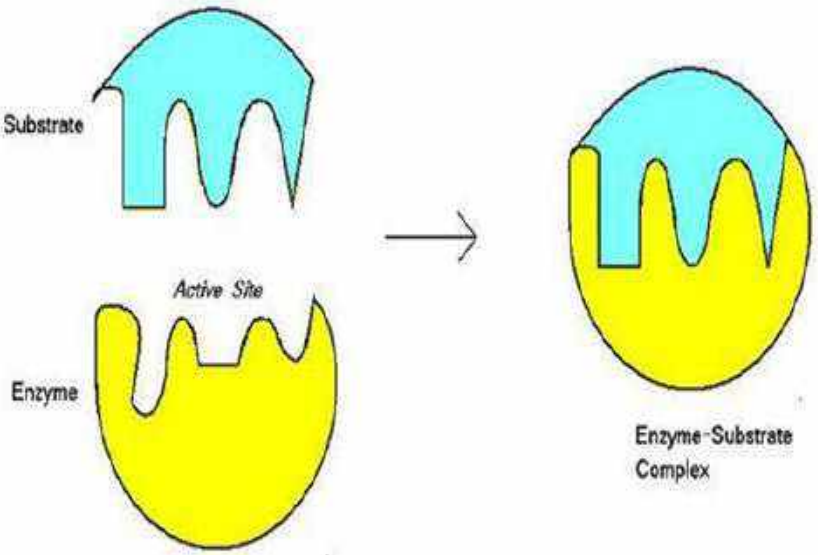
Induced Fit

- A change in the configuration of an enzyme's active site (H+ and ionic bonds are involved).
- Induced by the substrate.

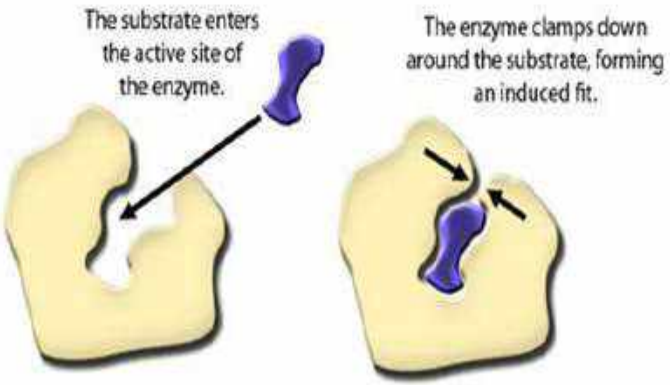


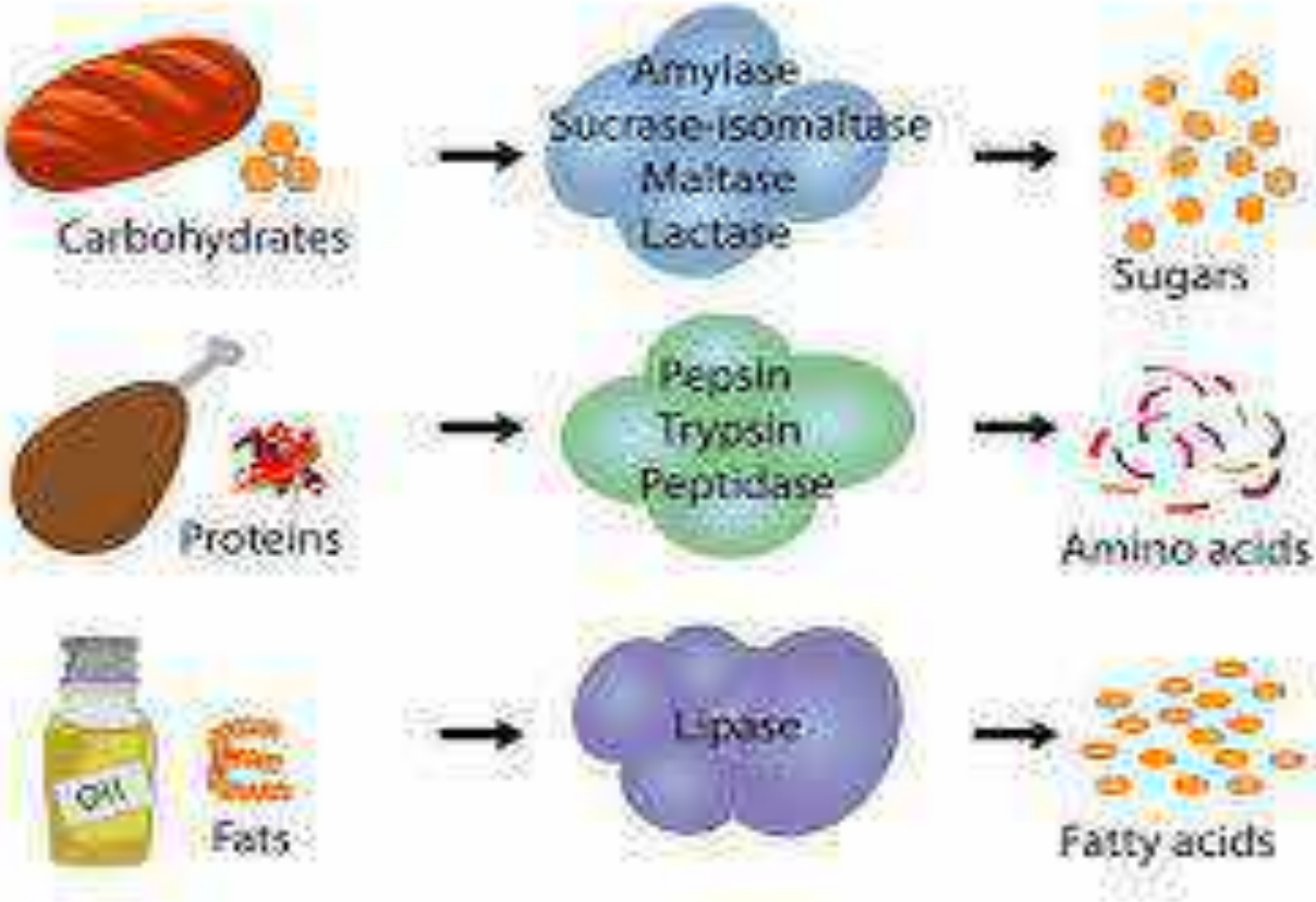
5) What is the **Induced Fit Hypothesis**?

- The enzyme changes shape to enhance the ability of the enzyme to catalyze the chemical reaction
- The enzyme returns back to its original shape after the reaction occurs

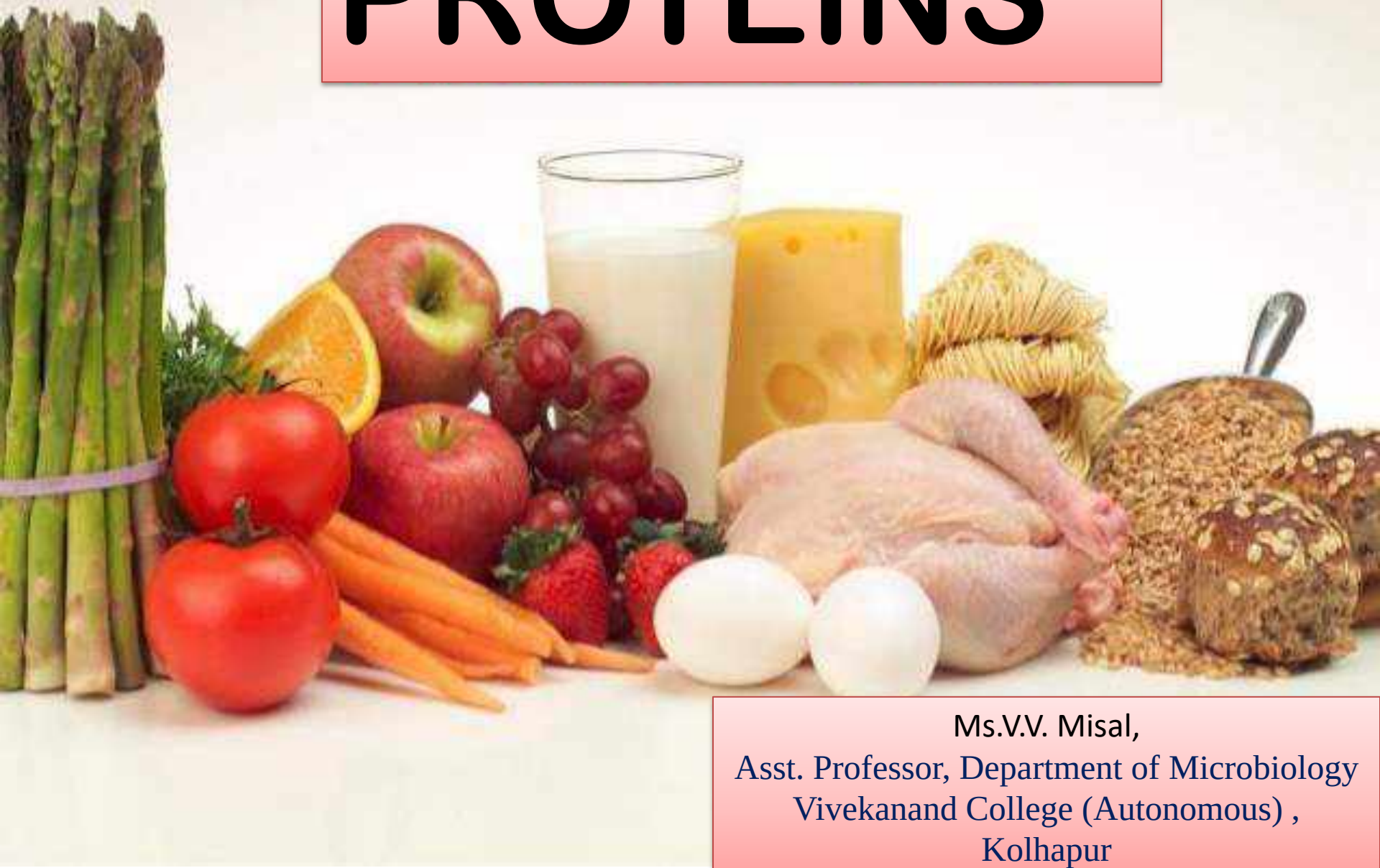


Induced-fit Model. - The enzyme active site forms a complementary shape to the substrate after binding.




Carbohydrates
Amylase
Sucrase-isomaltase
Maltase
Lactase
Sugars
Proteins
Pepsin
Trypsin
Peptidase
Amino acids
OIL
Fats
Lipase
Fatty acids

PROTEINS



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Kolhapur

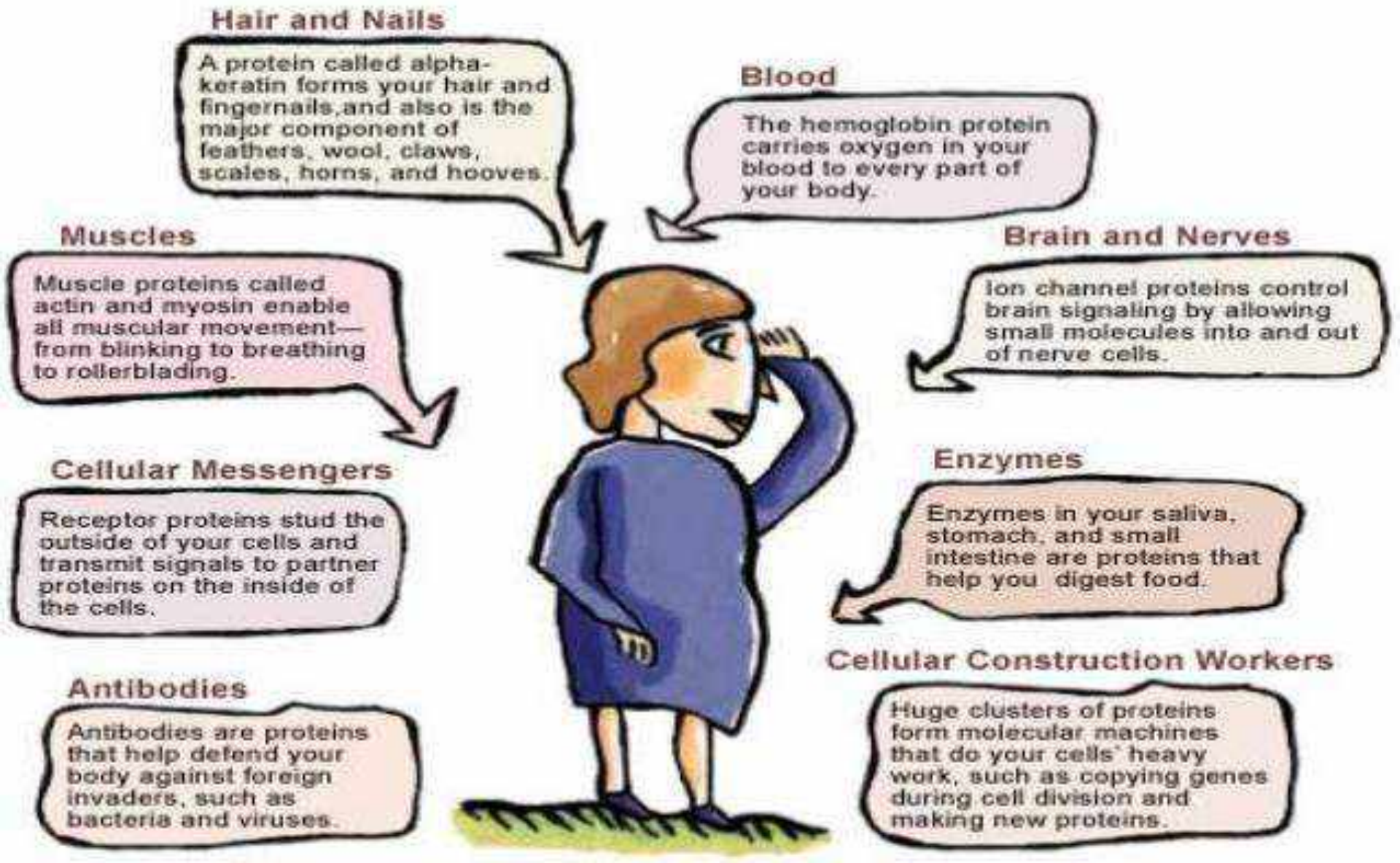
What is Protein?

The word protein came from a Greek word “Proteios”

Proteins are like long necklaces with differently shaped beads. Each "bead" is a small molecule called an amino acid.

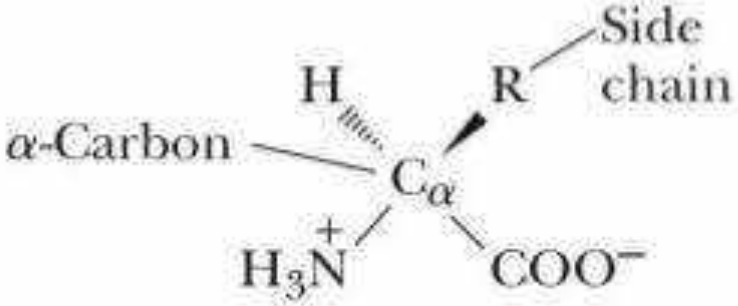
Compounds composed of carbon, hydrogen, oxygen, and nitrogen and arranged as strands of amino acids

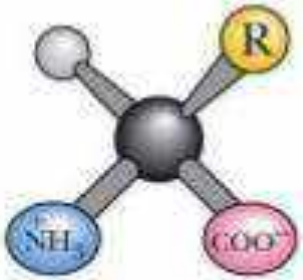
Protein for Our Body



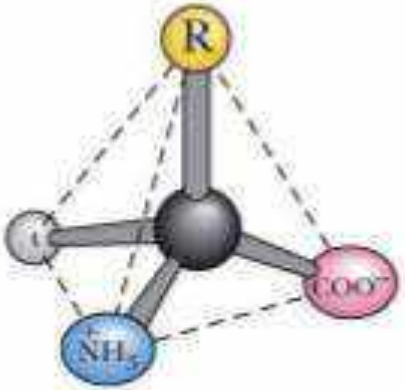
Amino Acids

- **Amino acid:** a compound that contains an amino group, a carboxyl group and a side-chain that is specific to each amino acid.
- **□-Amino acid:** an amino acid in which the amino group is on the carbon adjacent to the carboxyl group
- The are 20 common α-amino acids used by the ribosomes to make proteins. These 20 have L chirality at the α-carbon.





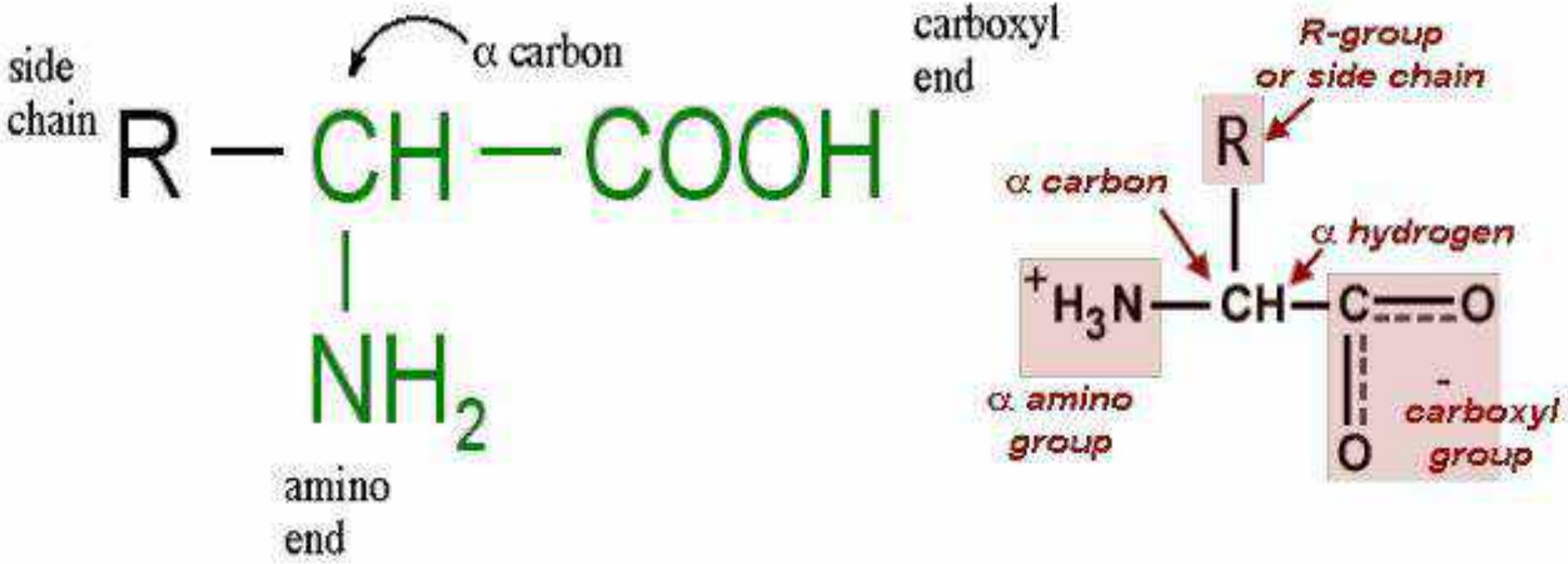
Ball-and-stick model



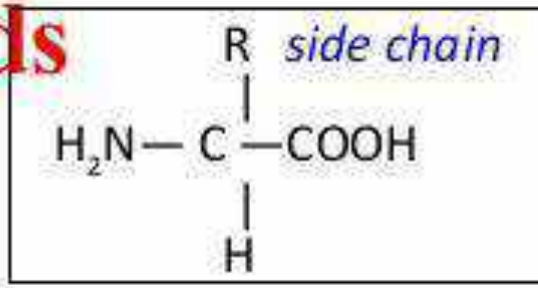
Amino acids are tetrahedral structures

What is Amino Acid?

- Amino acids are derivatives of carboxylic acids formed by substitution of α-hydrogen for amino functional group



Chemistry of Amino Acids



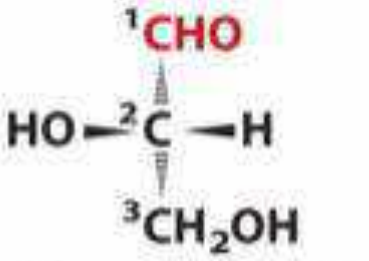
- The building blocks of proteins
- 20 amino acids are naturally incorporated into polypeptides and are called proteinogenic or standard amino acids. These 20 are encoded by universal genetic code.
- 10 standard amino acids (Lys, Met, His, Leu, Ile, Thr, , Try, Phe, Val & Arg) are called "essential" for humans because they cannot be created from other compounds by the human body, and so must be taken in as food.
- From these building blocks different organisms can make such widely diverse products as enzymes, hormones, antibodies, antibiotics, and a myriad of other substances having distinct biological activities.
- Also used as single molecules in biochemical pathways

Chemistry of Amino Acids

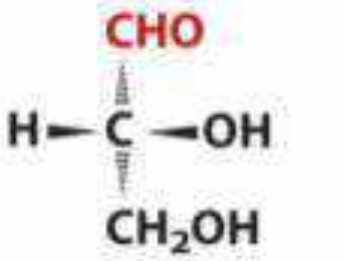
- Two functional groups:
  - carboxylic acid group
  - amino group on the alpha (α) carbon
- Have different side groups (R)
  - Properties dictate behavior of AAs

Stereochemistry of AAs

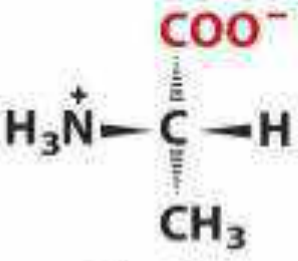
- All amino acids (except glycine) are optically active (chiral)



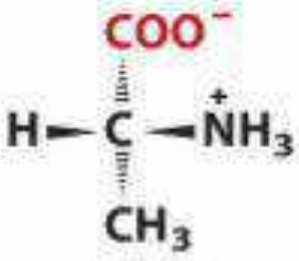
L-Glyceraldehyde



D-Glyceraldehyde



L-Alanine



D-Alanine

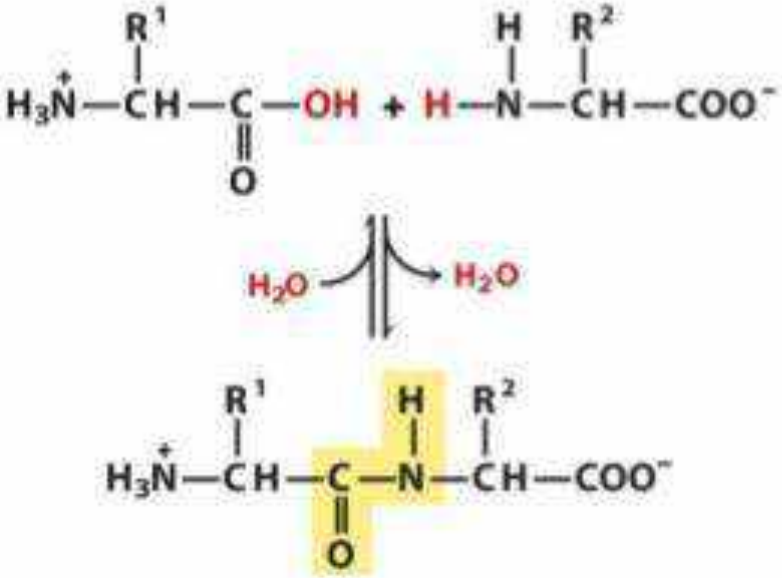
2 forms of enantiomers/stereoisomers

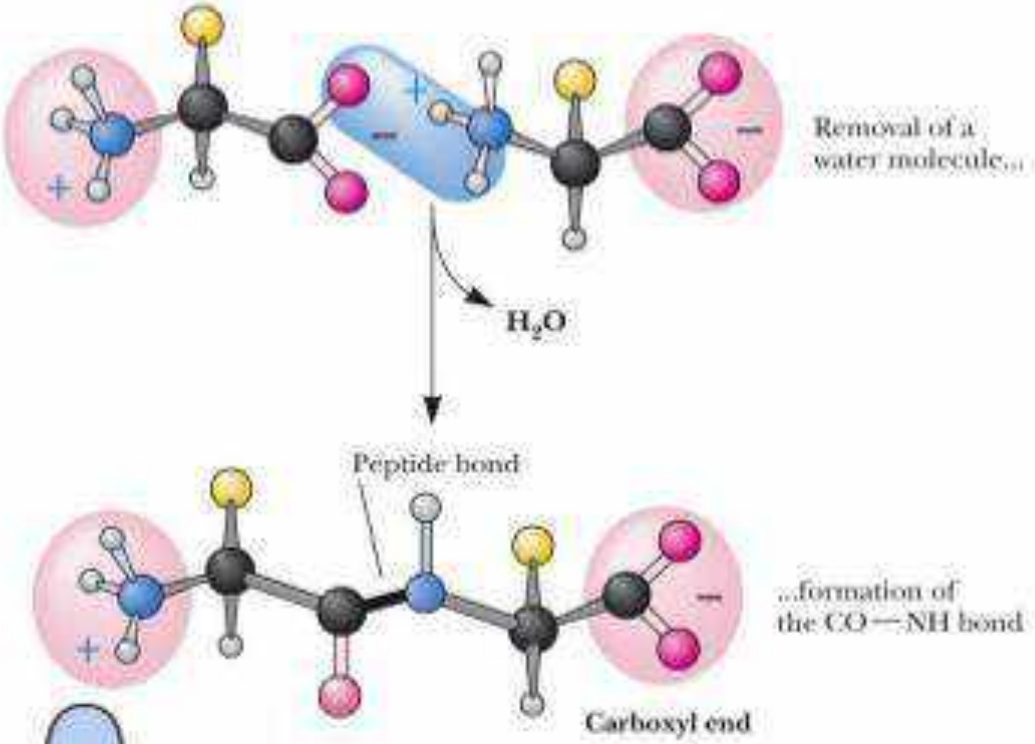
- *d* = dextrorotatory ,*dextro means right*
- *l* = levorotatory, *levo means left*
- D, L = relative to glyceraldehyde

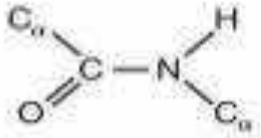
Amino acids join together via peptide bonds

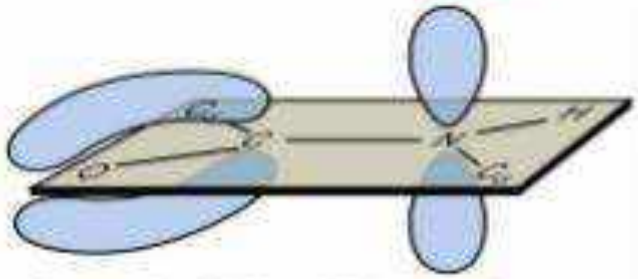
- Chain of amino acids = peptide or protein

Two amino acids can react with loss of a water molecule to form a covalent bond.







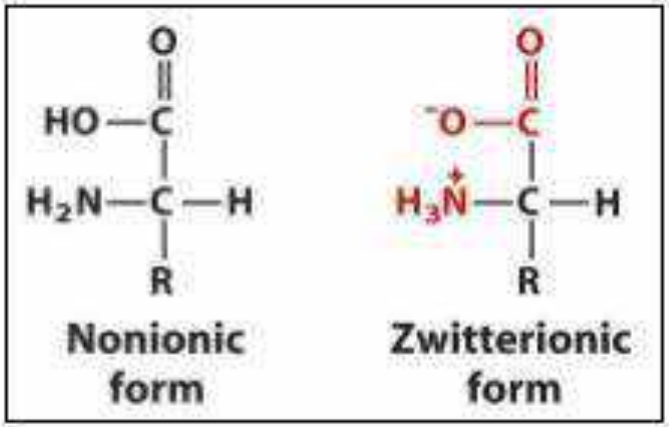


A pure double bond between C and O would permit free rotation around the C — N bond.

Amide linkage is planar
NH and CO are anti

Zwitterions

- Although α-amino acids are commonly written in the unionized form, they are more properly written in the zwitterion (internal salt) form (Germ. Zwitter means hybrid)
- Both the $-NH_2$ and the –COOH groups in an amino acid undergo ionization in water.
- At physiological pH (7.4), a zwitterion forms
  - Both + and – charges
  - Overall neutral
  - Amphoteric
    - Amino group is protonated
    - Carboxyl group is deprotonated
    - **Soluble** in polar solvents due to ionic character



Acid-Base Properties of Amino Acids

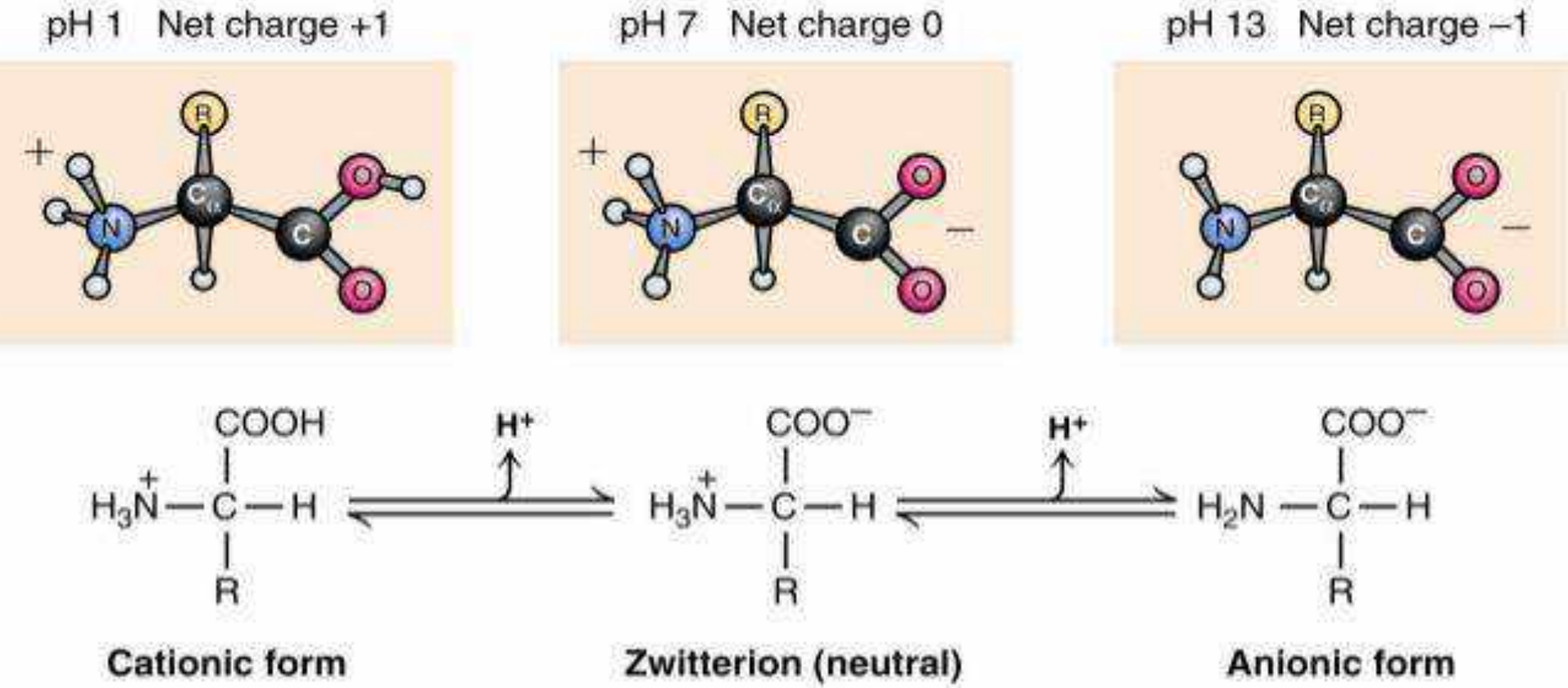


Figure : The ionic forms of the amino acids.

Classification of Amino Acids

- Classification based on side-chain structure:
  - Non-polar amino acids.
  - Polar, uncharged amino acids.
  - Acidic amino acids.
  - Basic amino acids.

- Other side chain structural classifications:
  - Aromatic, cyclic, hydroxyl, and thiol amino acids.

Classification of Amino Acids

Nonpolar side chains (predominant form at pH 7.0

R =

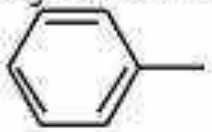
glycine (gly, G)
H-

alanine (ala, A)
CH_3-

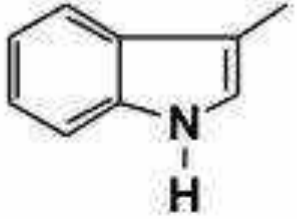
valine (val, V)
$(CH_3)_2CH$—

leucine (leu, L)
$(CH_3)_2CHCH_2$-

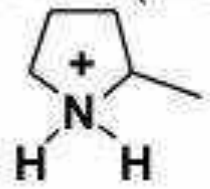
phenylalanine (phe, F



tryptophan (trp, W)



proline (Pro, P)

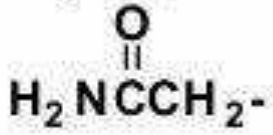


isoleucine (ile, I)
$CH_3CH_2CH(CH_3)$-

methionine (met, M)
$CH_3SCH_2CH_2$-

Polar side chains (predominant form at pH 7.0)

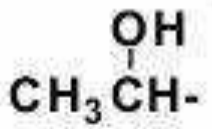
asparagine (asn, N)



glutamine (glu, G)

serine (ser, S)

threonine (thr, T)

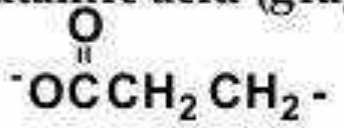


Classification of Amino Acids

Acidic side chains (predominant form at pH 7.0)

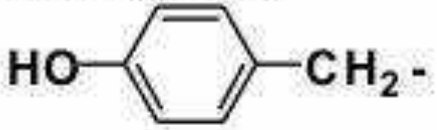
aspartic acid (asp, D)

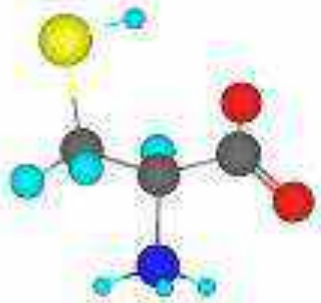
glutamic acid (glu, E)



cysteine (cys, C)

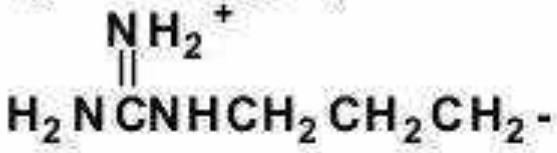
tyrosine (tyr, Y)



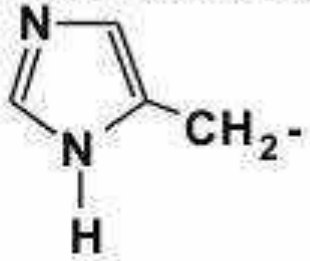


Basic side chains (predominant form at pH 7.0)

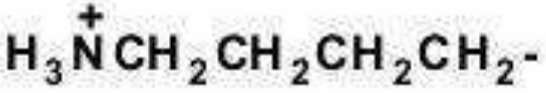
arginine (arg, R)

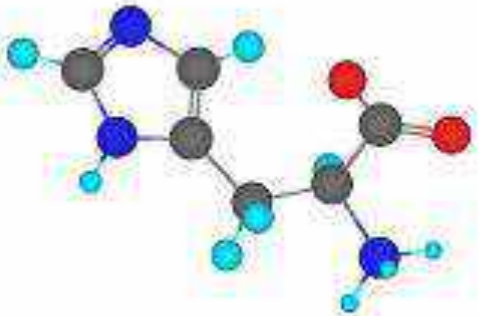


histidine (his, H)

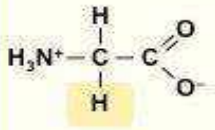


lysine (lys, K)

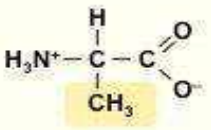




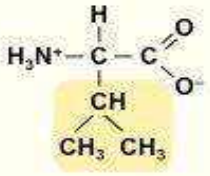
NON-POLAR



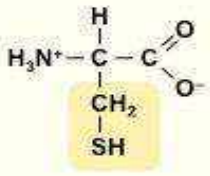
Glycine
(Gly / G)



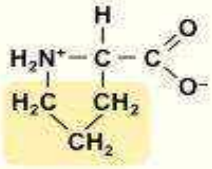
Alanine
(Ala / A)



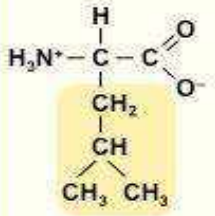
Valine
(Val / V)



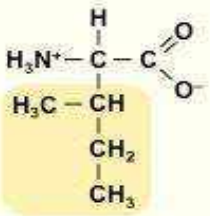
Cysteine
(Cys / C)



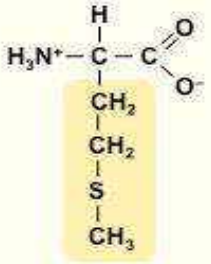
Proline
(Pro / P)



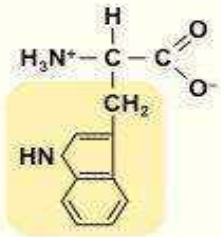
Leucine
(Leu / L)



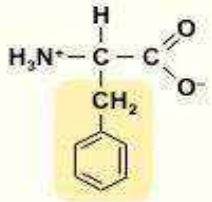
Isoleucine
(Ile / I)



Methionine
(Met / M)

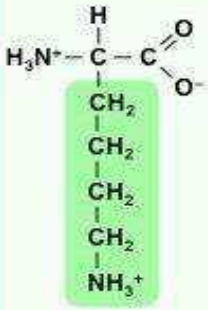


Tryptophan
(Trp / W)

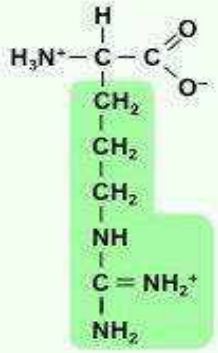


Phenylalanine
(Phe / F)

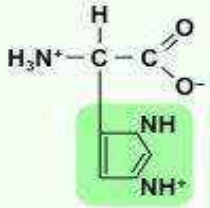
+ CHARGE



Lysine
(Lys / K)

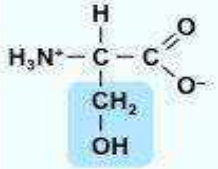


Arginine
(Arg / R)

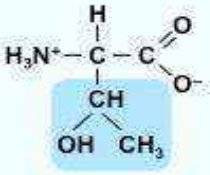


Histidine
(His / H)

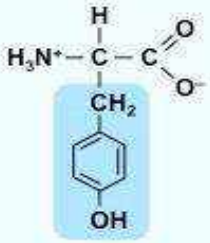
POLAR



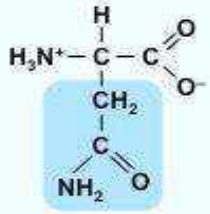
Serine
(Ser / S)



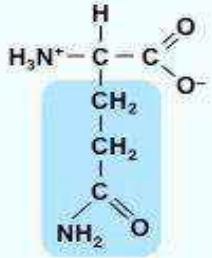
Threonine
(Thr / T)



Tyrosine
(Tyr / Y)



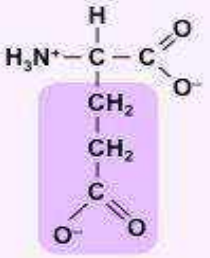
Asparagine
(Asn / N)



Glutamine
(Gln / Q)

− CHARGE

Aspartic Acid
(Asp / D)



Glutamic Acid
(Glu / E)

Essential Amino Acids

- An essential amino acid is an amino acid that cannot be synthesized itself by the organism (usually referring to humans), and therefore must be supplied in the diet.
- 10 amino acids are essential amino acid
- They are - arg, his, ile, leu, lys, met, phe, thr, trp, val
- Must obtain from the diet
- An adequate diet must contain these essential amino acids. Typically, they are supplied by meat and dairy products
- Essential amino acids help the body function and regulate neurotransmitters, chemicals in the brain that control mood and behavior.
- A lack of essential amino acids can cause emotional or physical difficulties and lead to health disorder

Peptides and Proteins

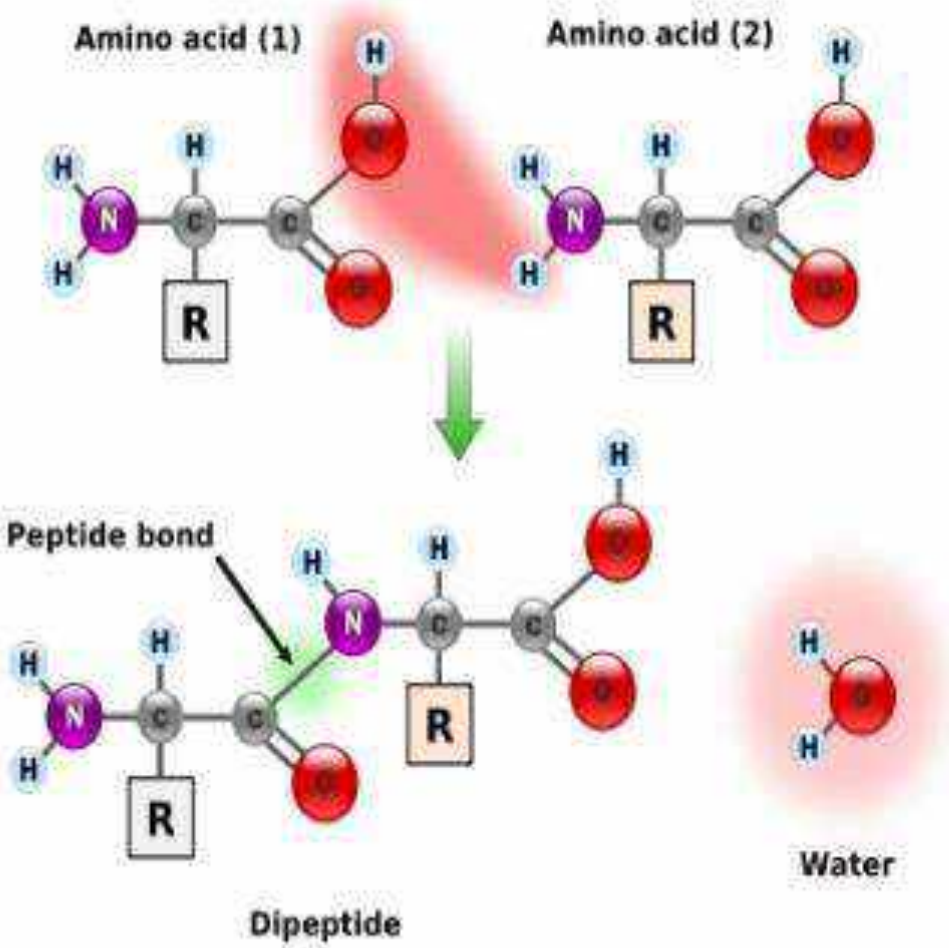
- 20 amino acids are commonly found in protein.

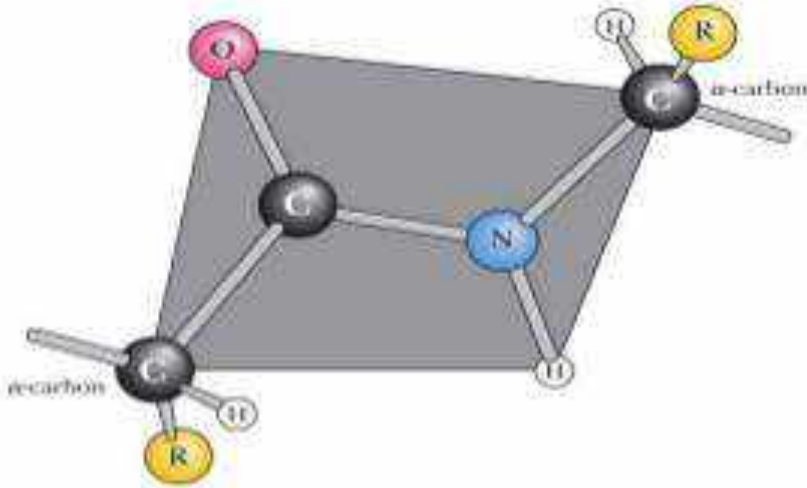
- These 20 amino acids are linked together through "peptide bond forming peptides and proteins.

- The chains containing less than 50 amino acids are called **"peptides"**, while those containing greater than 50 amino acids are called **"proteins".**

Peptide bond formation: Peptide bond is the amide bond between the □-carboxyl group of one amino acid and the □-amino group of another

Peptide bond





The *trans* conformation of the peptide bond.

Peptides and Proteins

Peptide: a short polymer of amino acids joined by peptide bonds; they are classified by the number of amino acids in the chain

- dipeptide: a molecule containing two amino acids joined by a peptide bond
- tripeptide: a molecule containing three amino acids joined by peptide bonds
- 12-20 residues (Each unit/AA) – oligopeptide.
- polypeptide: a macromolecule containing many amino acids

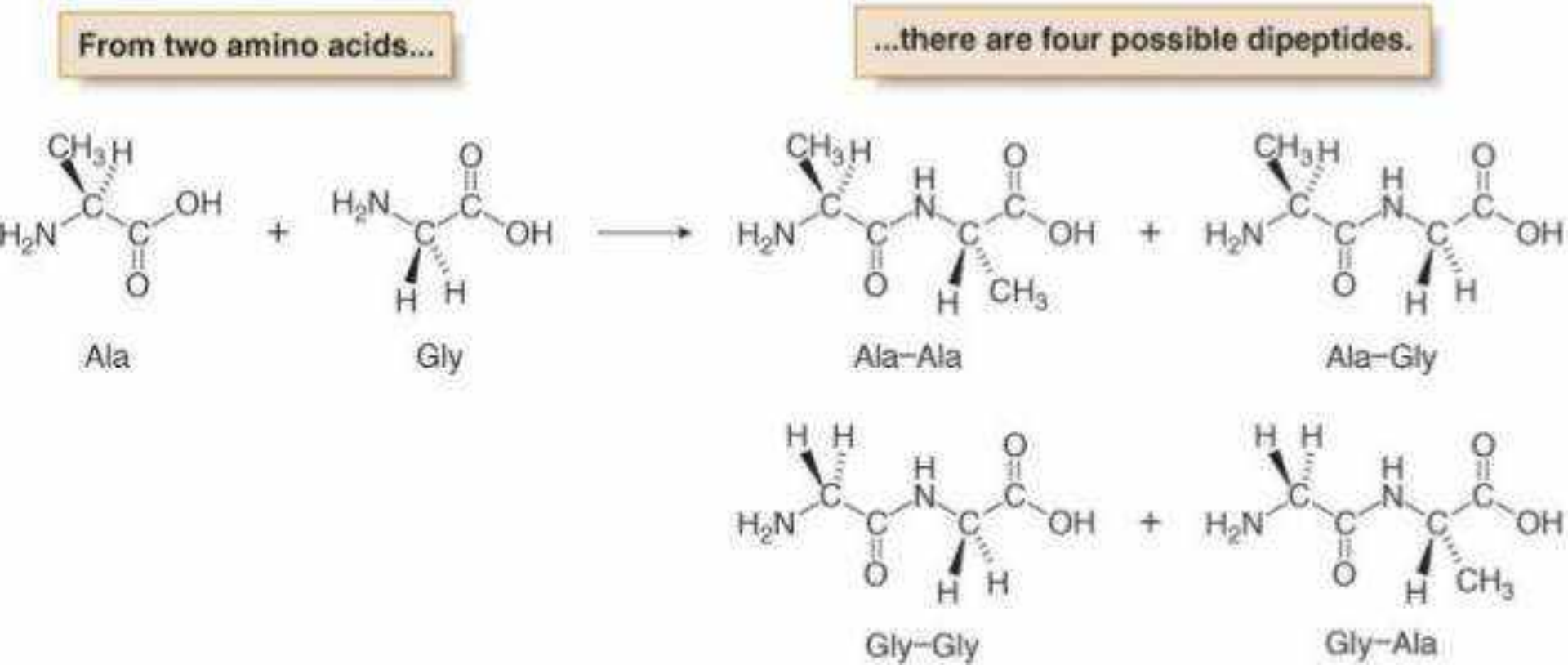
Linear polymers (no branches)

AA monomers linked head to tail through formation of peptide bonds

- protein: a biological macromolecule of molecular weight 5000 g/mol or greater, consisting of one or more polypeptide chains

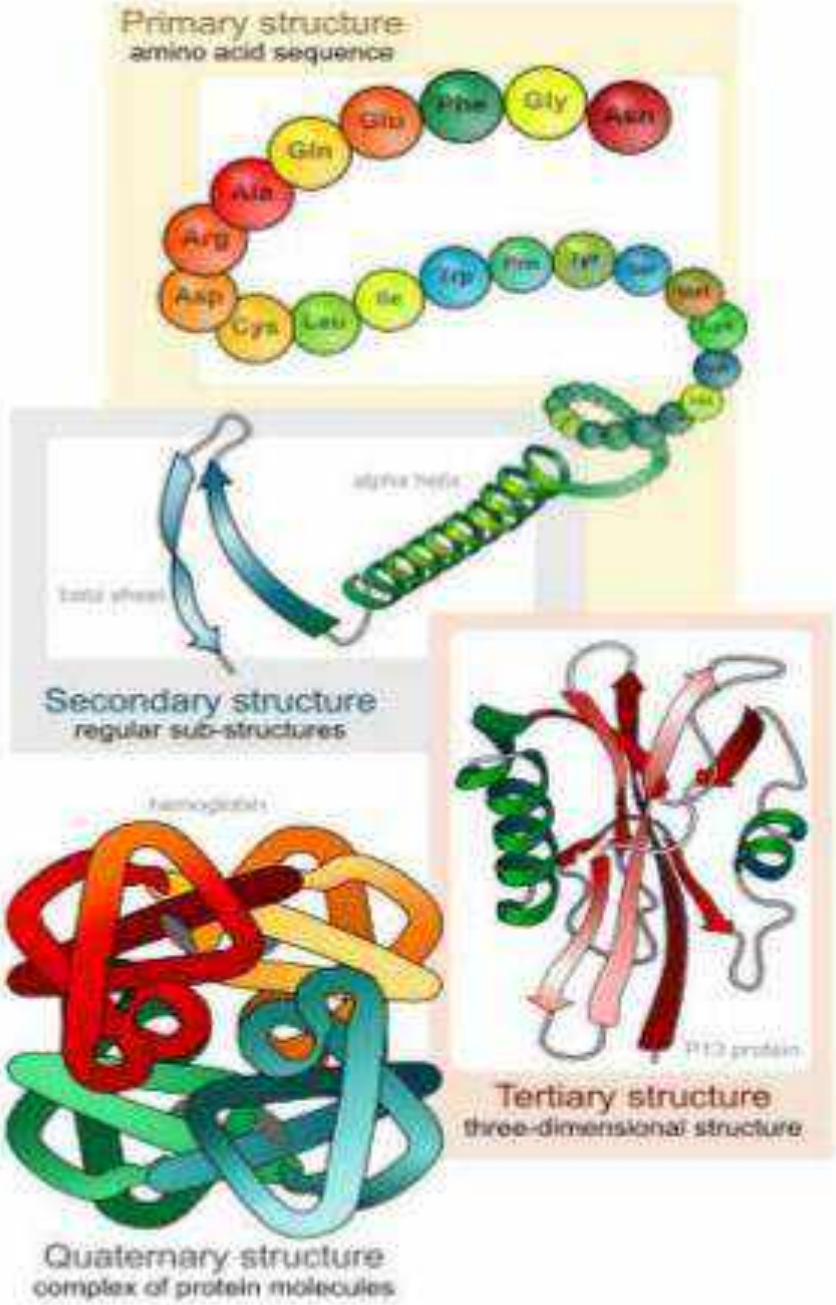
Peptide Synthesis

- The synthesis of a specific dipeptide such as Ala-Gly from alanine and glycine is complicated because both amino acids have two functional groups.
- As a result, four products—namely, Ala-Ala, Ala-Gly, Gly-Gly and Gly-Ala—are possible.



INTRODUCTION

- Proteins are an important class of biological macromolecules which are the polymers of amino acids.
- Biochemists have distinguished several levels of structural organization of proteins. They are:
  - Primary structure
  - Secondary structure
  - Tertiary structure
  - Quaternary structure



PRIMARY STRUCTURE

- The primary structure of protein refers to the sequence of amino acids present in the polypeptide chain.
- Amino acids are covalently linked by peptide bonds.
- Each component amino acid in a polypeptide is called a "residue" or "moiety"
- By convention, the 1^0 structure of a protein starts from the amino-terminal (N) end and ends in the carboxyl-terminal (C) end.

What forces determine the structure?

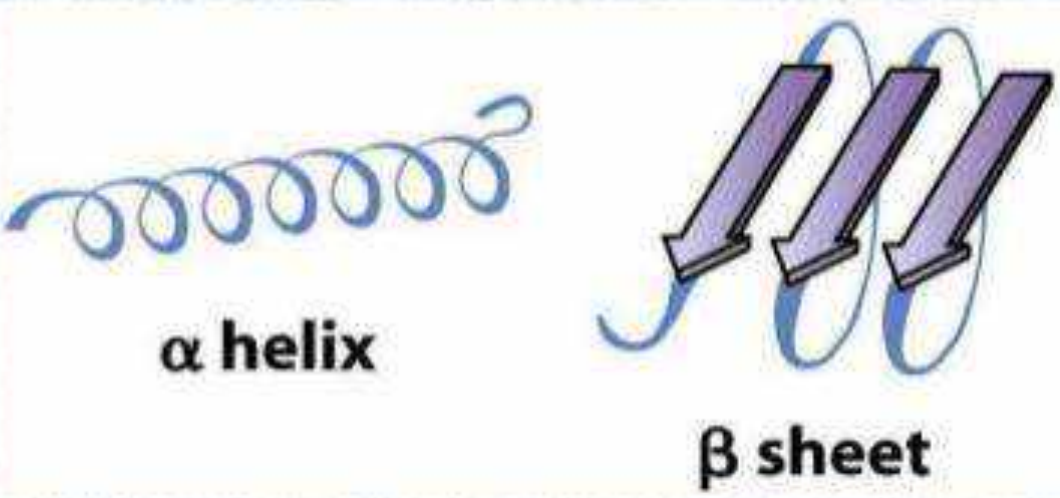
Primary structure – determined by covalent bonds

Secondary, Tertiary, Quaternary structure – determined by weak forces

Secondary Structure

- It is a local, regularly occurring structure in proteins and is mainly formed through hydrogen bonds between backbone atoms.

- Pauling & Corey studied the secondary structures and proposed 2 conformations
  - α helix
  - β sheets.



Alpha helix

- Right handed spiral structure.
- Side chain extend outwards.
- Stabilized by H bonding that are arranged such that the peptide Carbonyl oxygen (nth residue) and amide hydrogen(n+4 th residue).
- Amino acids per turn – 3.6
- Pitch is 5.4 A°
- Alpha helical segments, are found in many globular proteins like myoglobin,troponin C.
- Length ~12 residues and ~3 helical turns.
- phi = –60 degrees, psi = –45 degrees , falls within the fully allowed regions of the Ramachandran diagram.

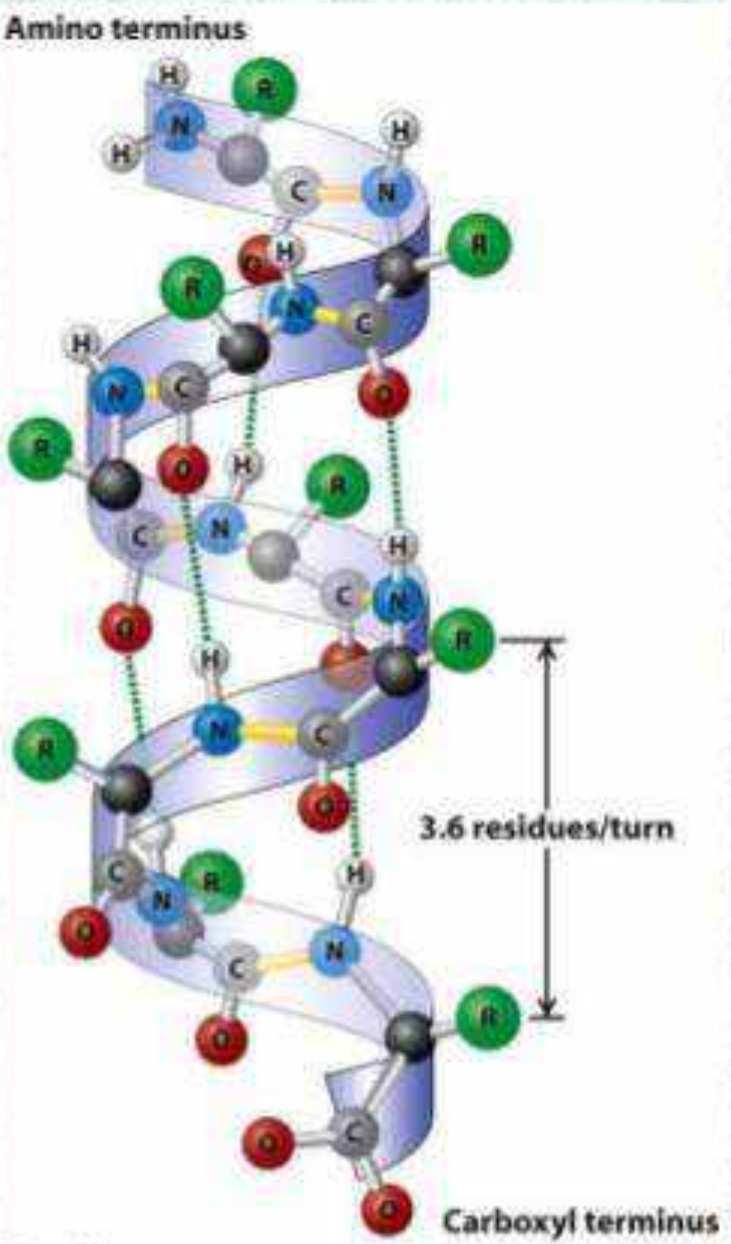
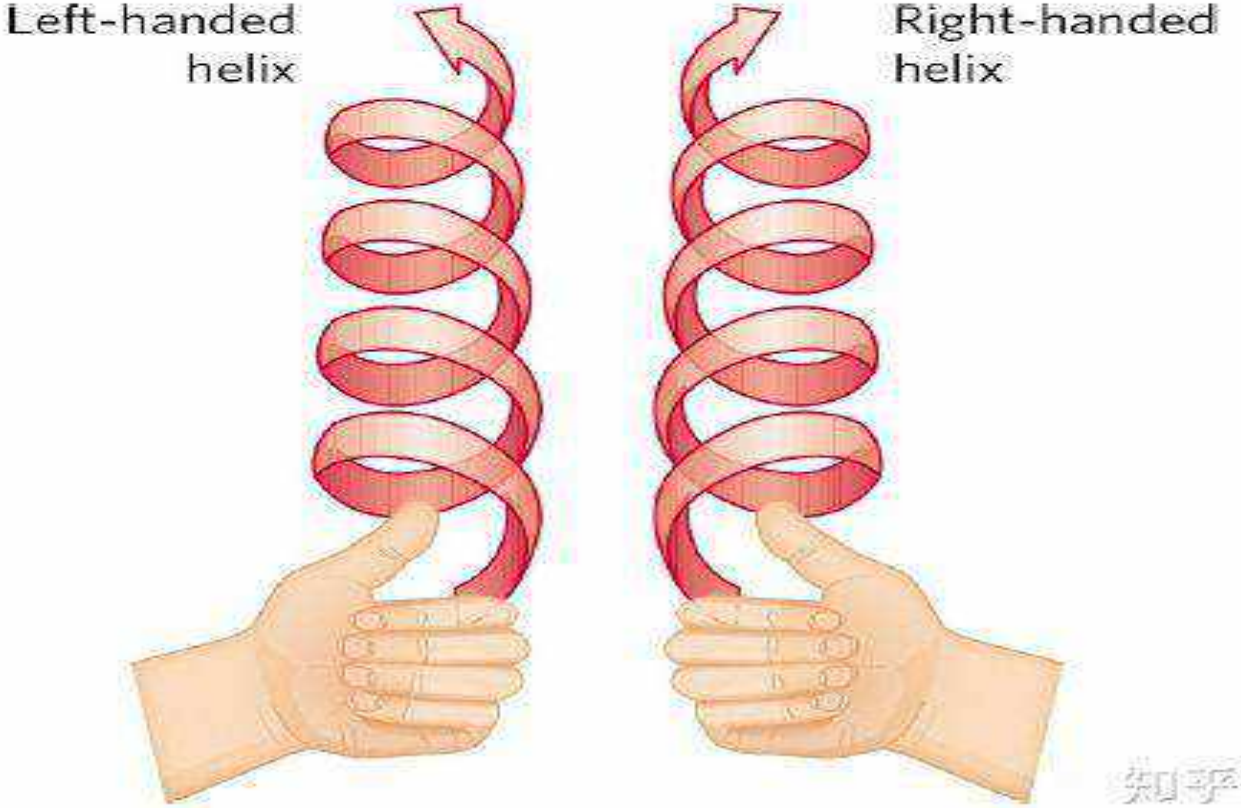
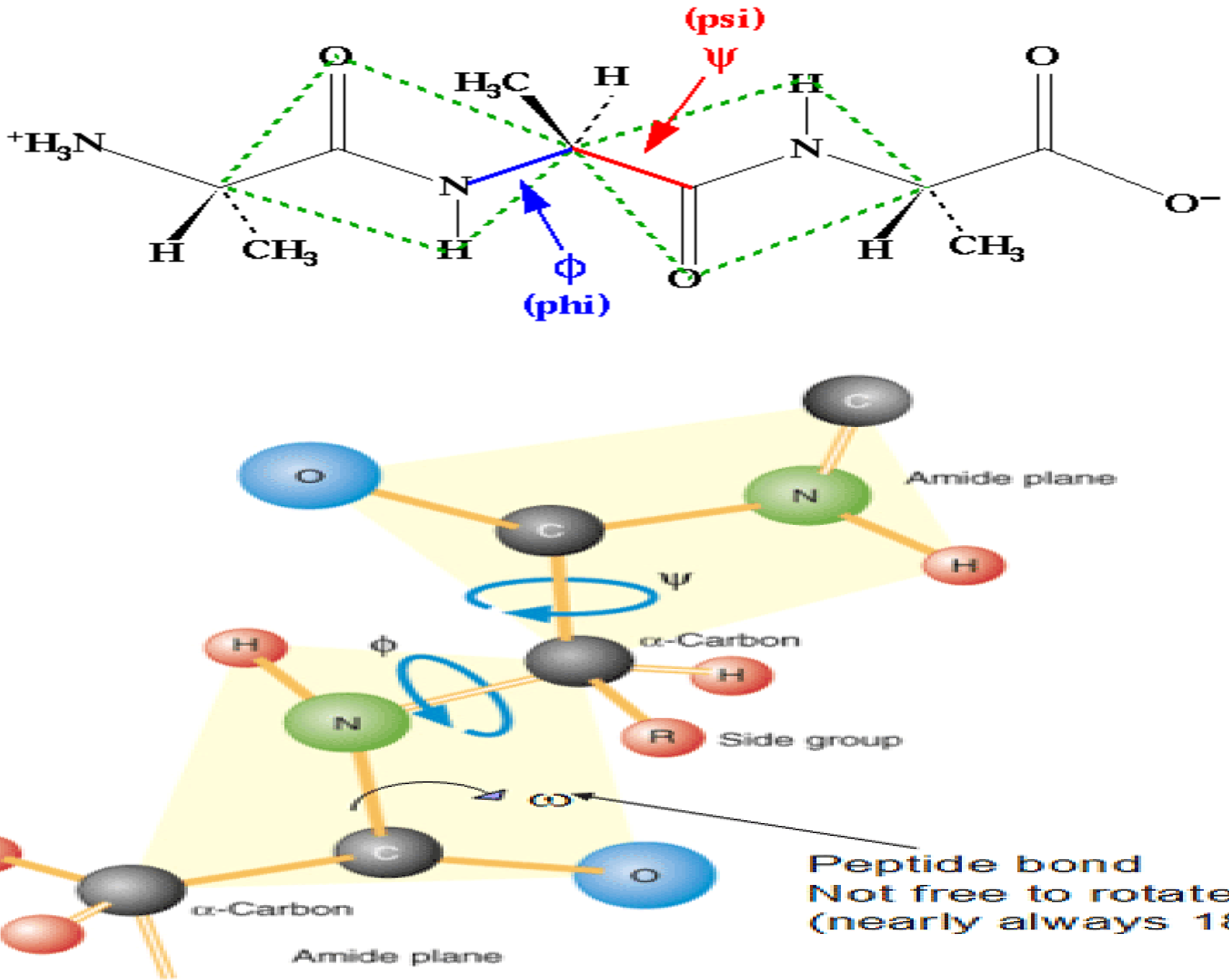


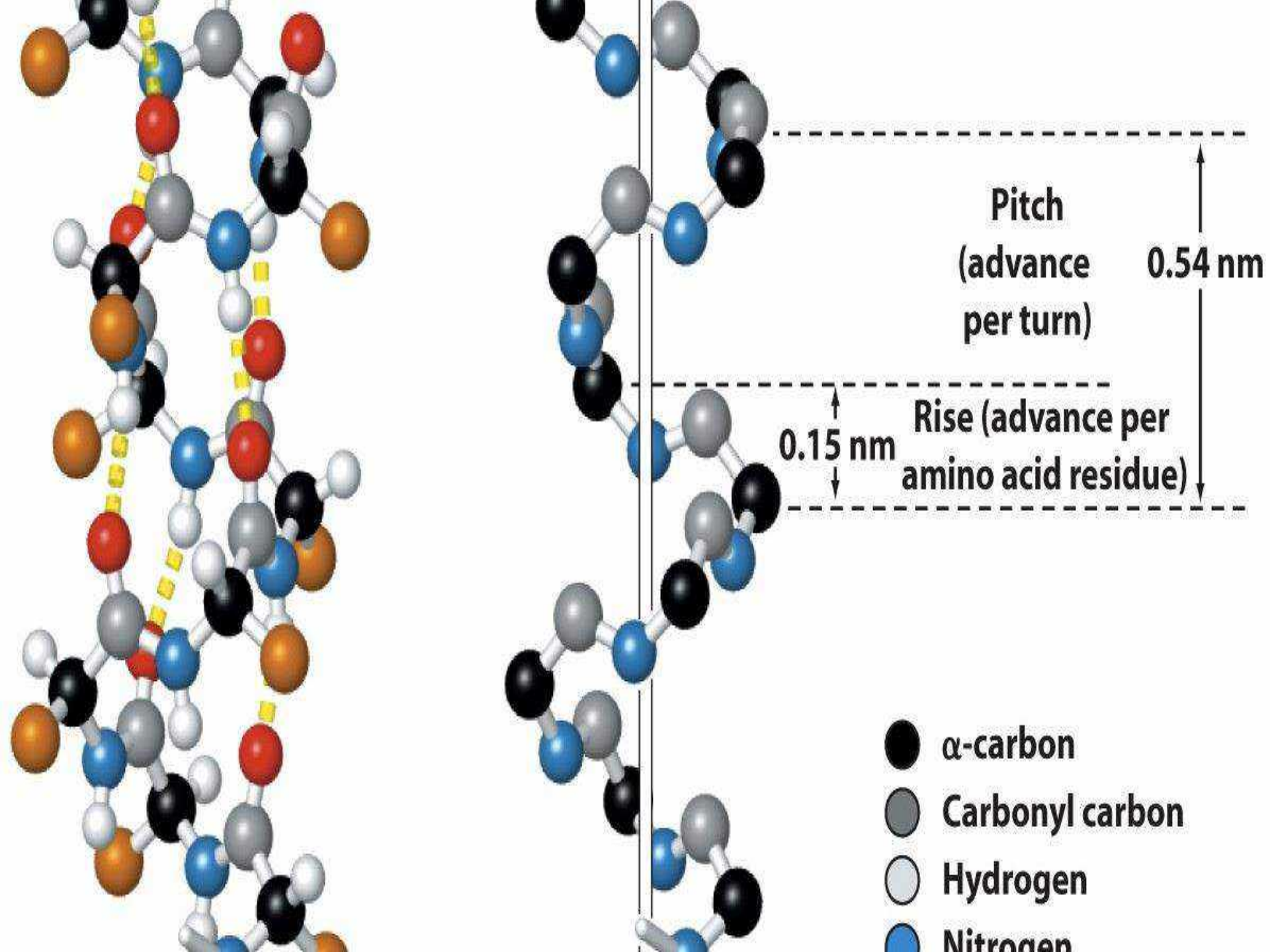
Figure 3-4
Molecular Cell Biology, Sixth Edition

BOX 4-1 METHODS Knowing the Right Hand from the Left

There is a simple method for determining whether a helical structure is right-handed or left-handed. Make fists of your two hands with thumbs outstretched and pointing away from you. Looking at your right hand, think of a helix spiraling up your right thumb in the direction in which the other four fingers are curled as shown (clockwise). The resulting helix is right-handed. Your left hand will demonstrate a left-handed helix, which rotates in the counterclockwise direction as it spirals up your thumb.

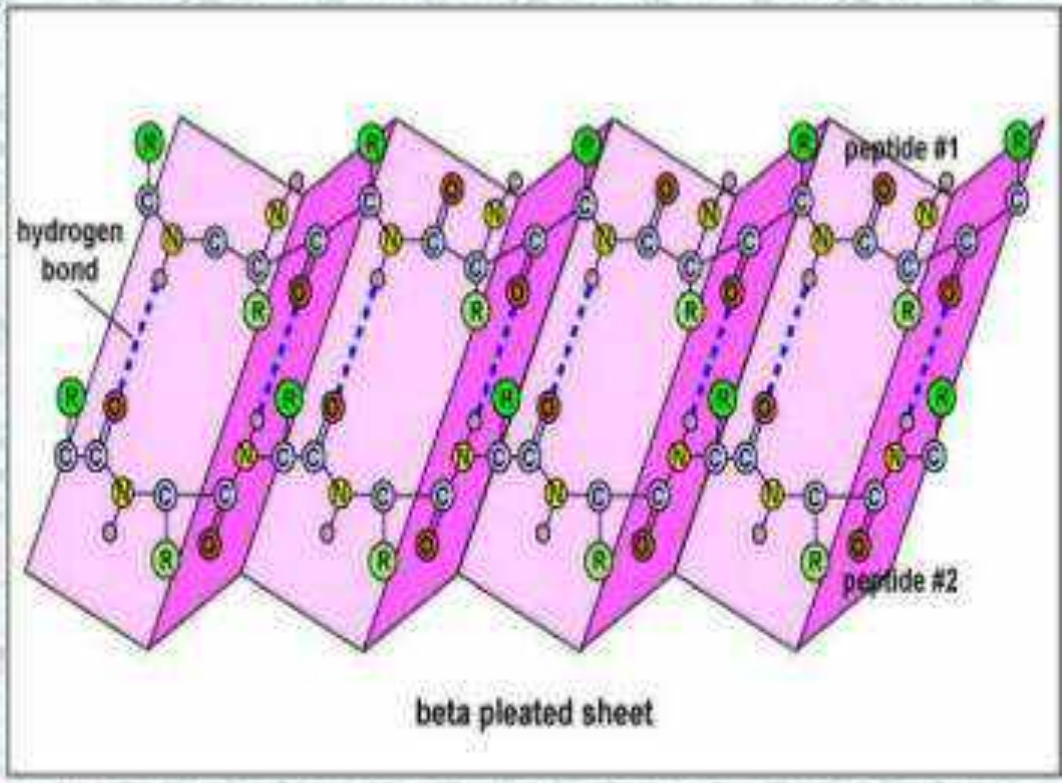



(psi)
ψ
O
H3C
H
H
O
+H3N
N
N
O−
H
CH3
H
H
CH3
ϕ
(phi)
O
C
O
Amide plane
N
H
ψ
α-Carbon
H
ϕ
H
N
R
Side group
ω
C
O
α-Carbon
Amide plane
Peptide bond
Not free to rotate
(nearly always 180)


Pitch
(advance
per turn)
0.54 nm
0.15 nm
Rise (advance per
amino acid residue)
α-carbon
Carbonyl carbon
Hydrogen
Nitrogen

BETA PLEATED SHEET

- Formed when 2 or more polypeptides line up side by side.
- Individual polypeptide – beta strand.
- Each beta strand is fully extended.
- They are stabilized by hydrogen bond between N-H and carbonyl groups of adjacent chains.

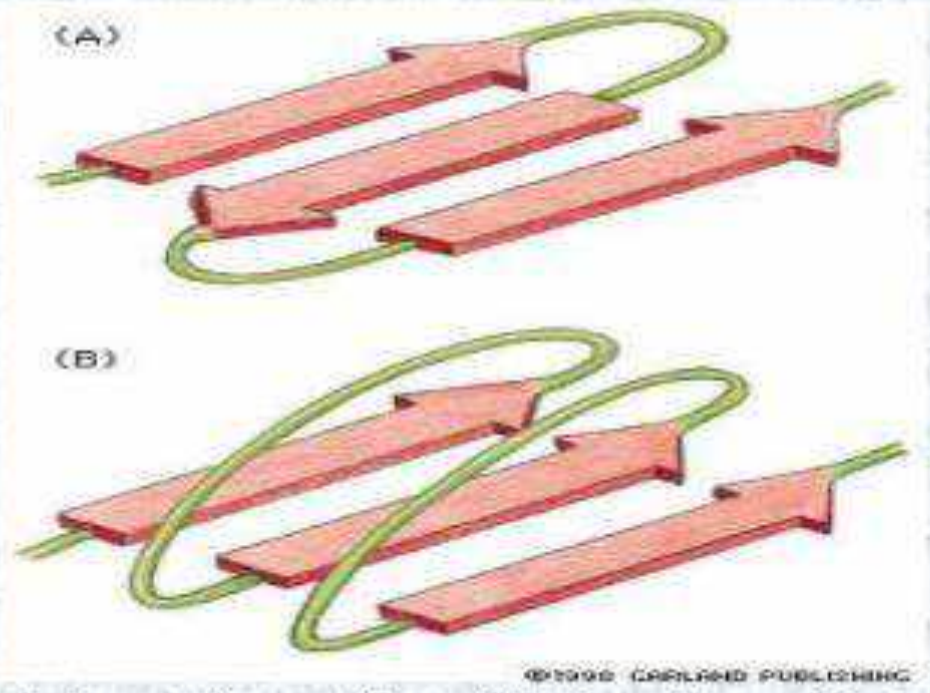


beta pleated sheet

- Beta sheets come in two varieties

Antiparallel beta sheet – neighboring hydrogen bonded polypeptide chains run in opposite direction.

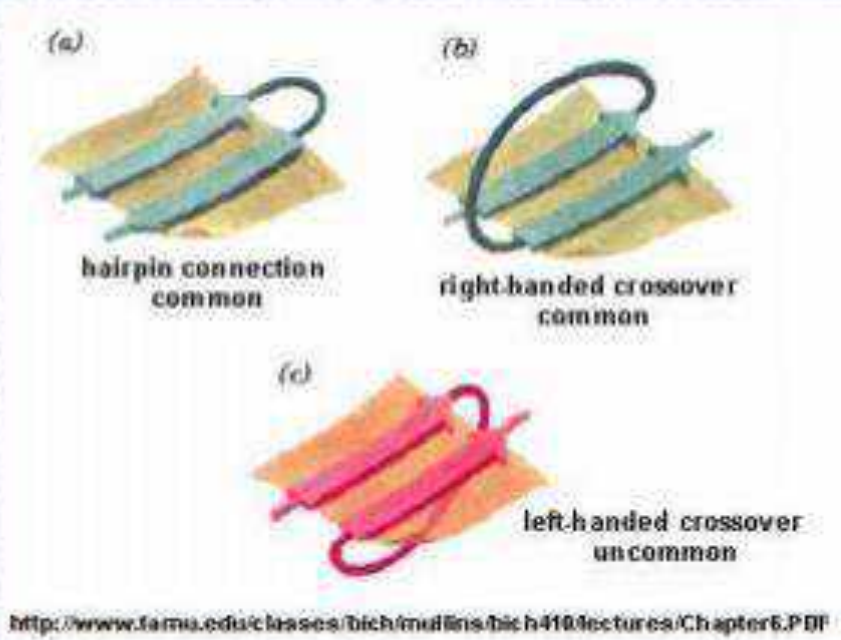
Parallel beta sheet – hydrogen bonded chains extend in the same direction.



The connection between two antiparallel strands may be just a small loop but the link between tandem parallel strands must be a crossover connection that is out of the plane of the β sheet.

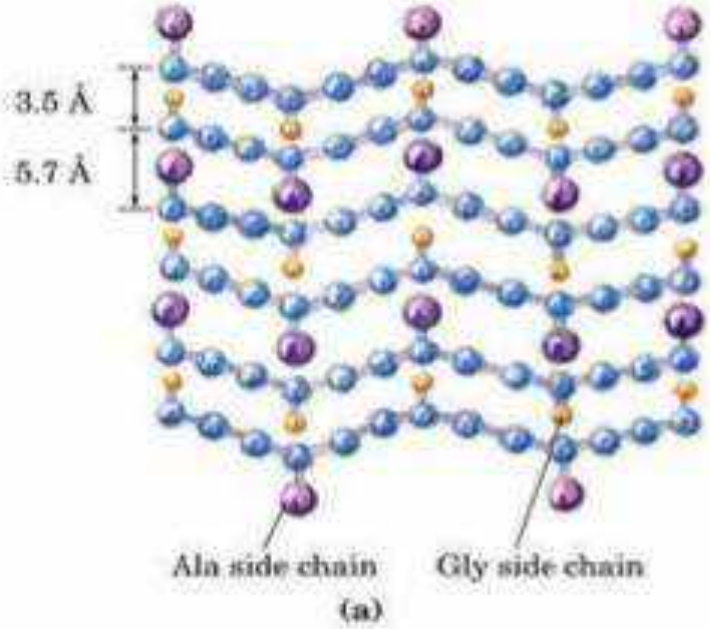
The two major sorts of connection between β strands:

- a hairpin or same end connection
- a crossover or opposite end connection.



EXAMPLES

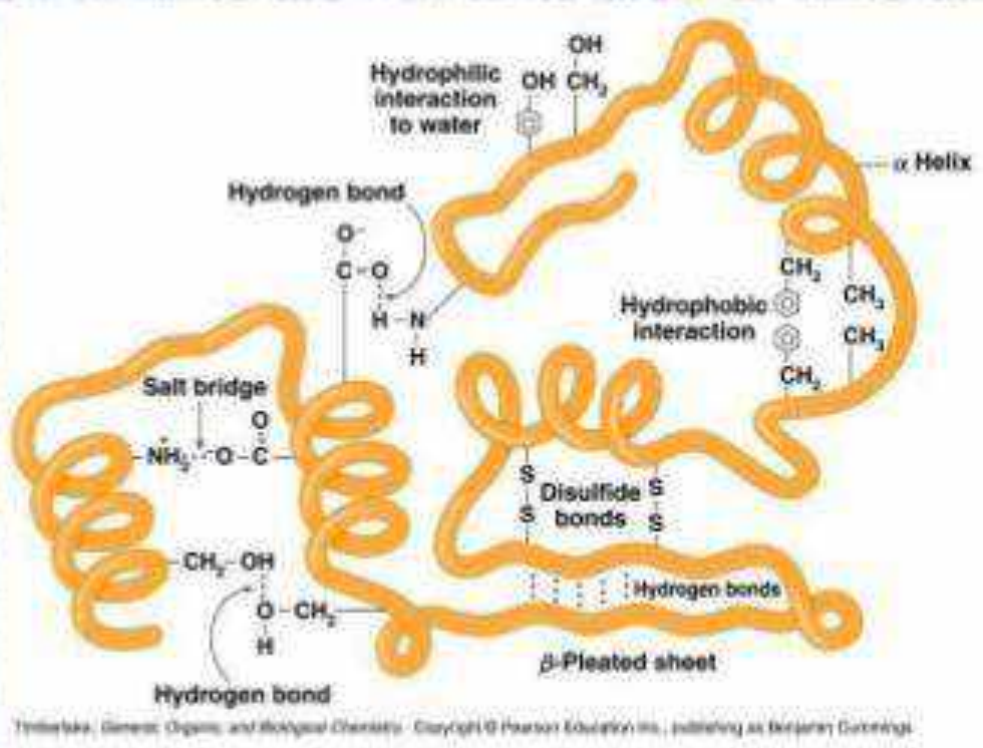
The collagen triple helix.



Silk fibroin beta sheet.

Tertiary Structure of Proteins

- The **tertiary structure** defines the specific overall **3-D shape** of the protein.
- Tertiary structure is based on various types of interactions between the side-chains of the peptide chain



Tertiary Structure Stabilization

- In globular proteins

  Tertiary interactions are frequently stabilized by sequestration of hydrophobic amino acid residues in the protein core.

  Consequent enrichment of charged or hydrophilic residues on the protein's water-exposed surface.

- In secreted proteins

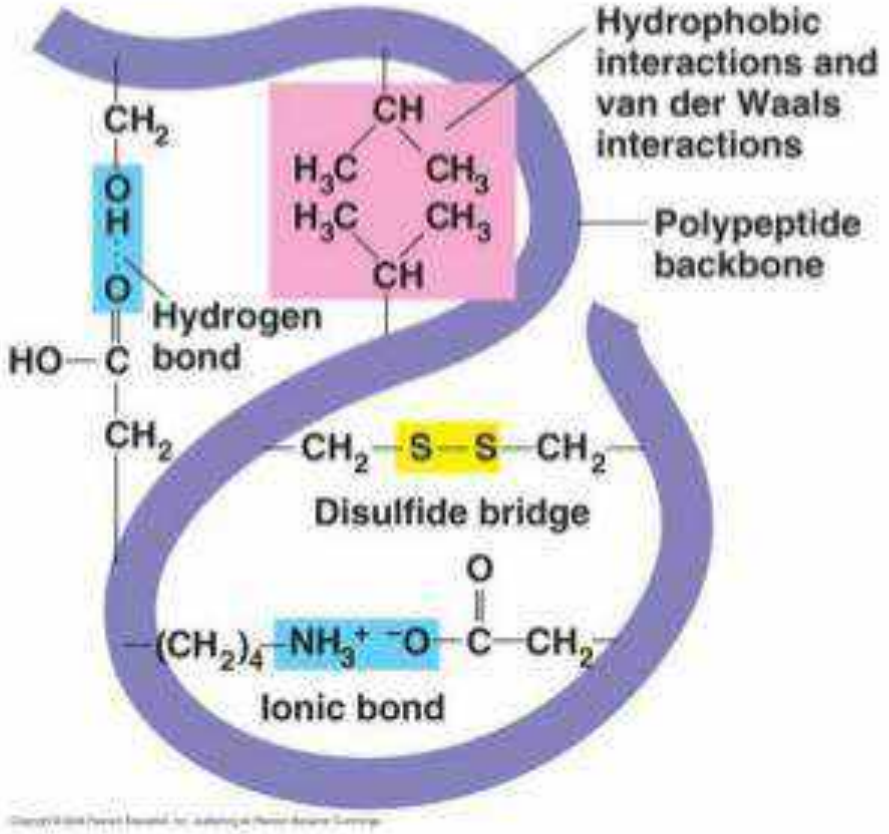
  disulfide bonds between cysteine residue helps to maintain the protein's tertiary structure

Interactions stabilizing tertiary structure :

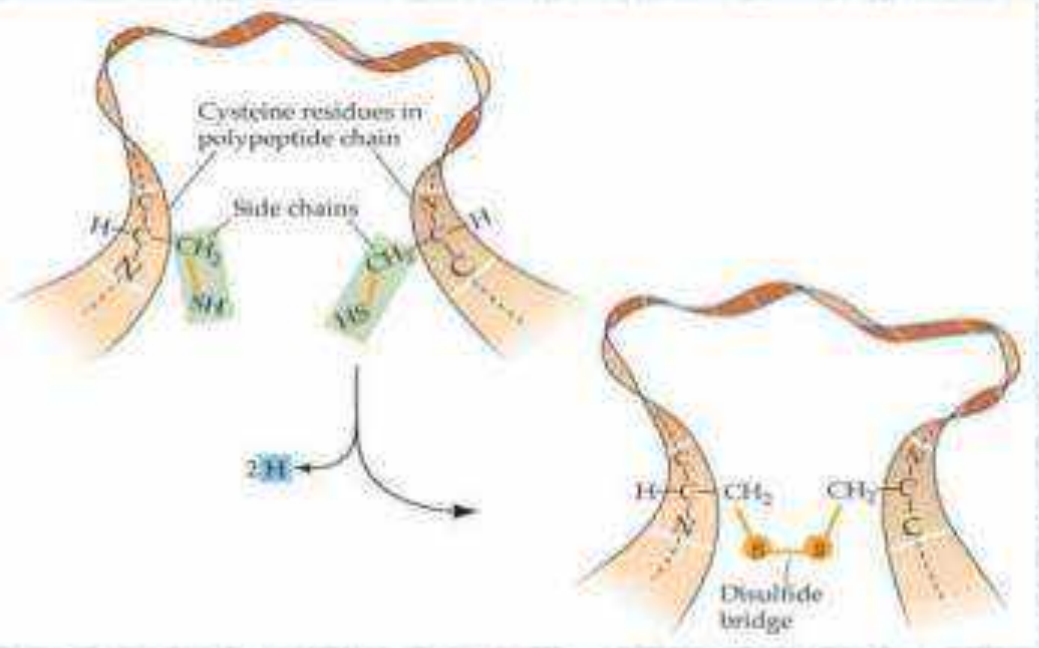
1. *Disulfide bonds*
2. *Hydrophobic interactions*
3. *Hydrogen bonds*
4. *Ionic interactions*
5. *Vander Waals force*

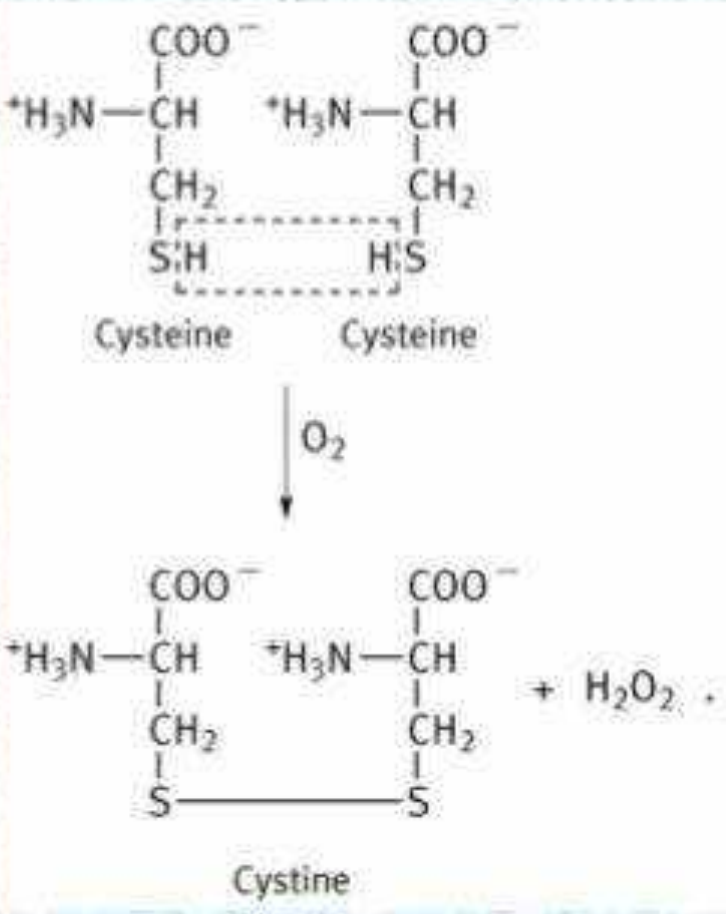
INTERACTIONS STABILIZING 3^0 STRUCTURE

- This final shape is determined by a variety of bonding interactions between the "side chains" on the amino acids.
- Hydrogen bonds
- Ionic Bonds
- Disulphide Bridges
- Hydrophobic Interactions:



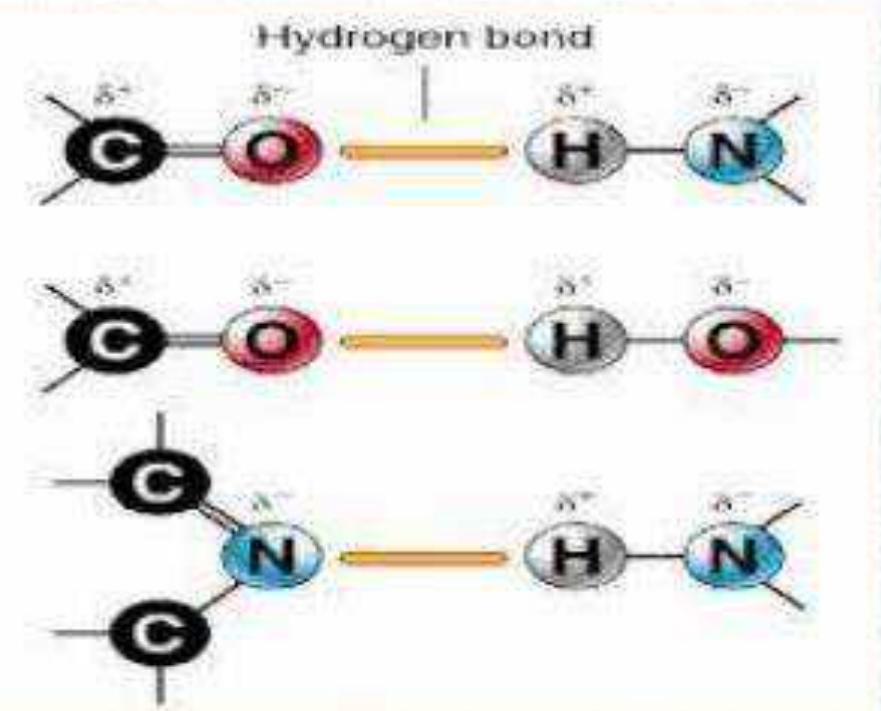
Tertiary structure - disulfide bond





Covalent bond between sulfur atoms on two cysteine amino acids.

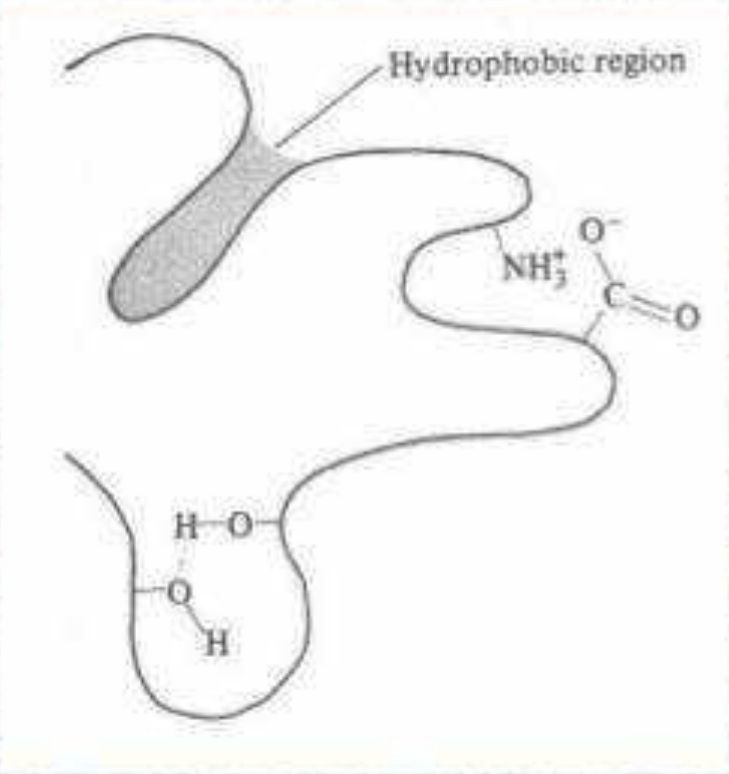
Tertiary structure - H bond



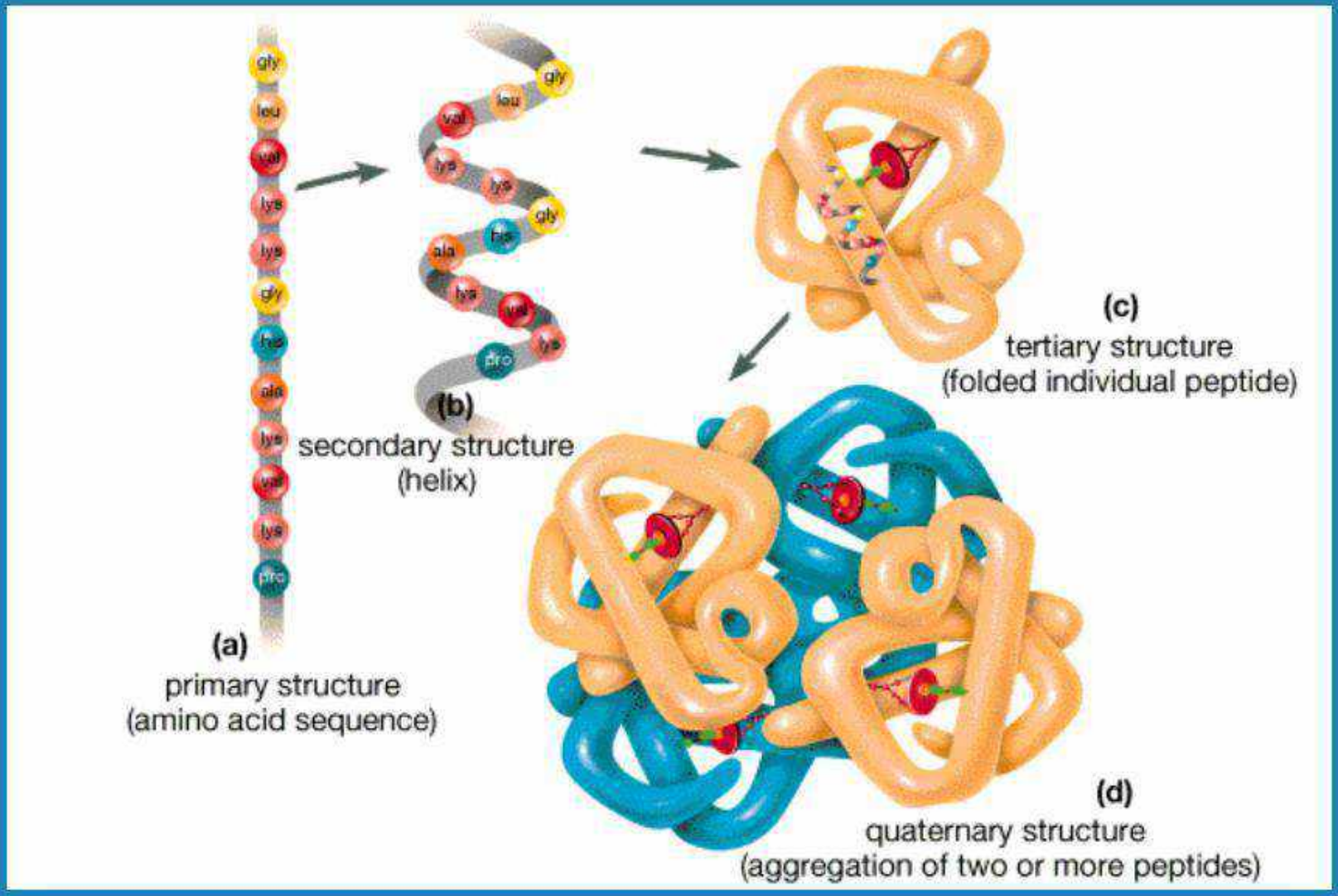
- H bonds are weak which allows to be broken and reformed easily.
- Allows structural change and produces 'functional'molecules

Tertiary structure - hydrophobic forces

- Close attraction of non-polar R groups through dispersion forces.
- They are non attractive interactions, but results from the inability of water to form hydrogen bonds with certain side chains.
- Very weak but collective interactions over large area stabilize structure.
- Repel polar and charged molecules/particles.

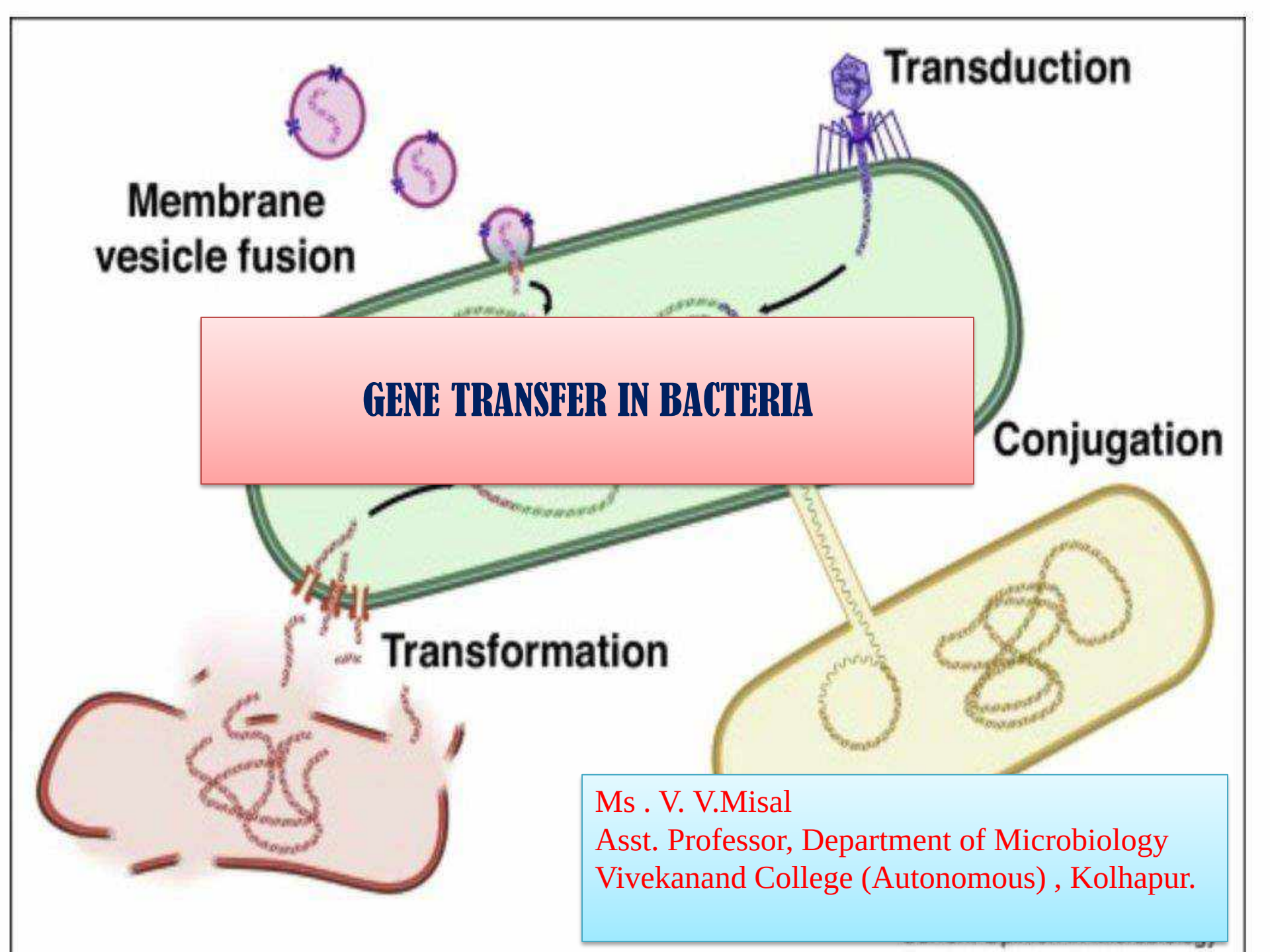


Four Levels of Protein Structure



Functions of Proteins

- **Structural** — **Collagen**; bones, tendons, cartilage
  Keratin; hair, skin, wool, nails, feathers
- **Movement** — **Myosin & Actin**; muscle contractions
- **Transport** — **Hemoglobin**; transports O_2
  Lipoproteins; transports lipids
- **Storage** — Casein; in milk. **Albumin**; in eggs
- **Hormone** — **Insulin**; regulates blood glucose
  Growth hormone; regulates growth
- **Protection** — **Immunoglobulins**; stimulate immunity
  Snake venom; **plant toxins**;
- **Enzymes** — **Sucrase**; catalyzes sucrose hydrolysis
  Pepsin; catalyzes protein hydrolysis



GENE TRANSFER IN BACTERIA

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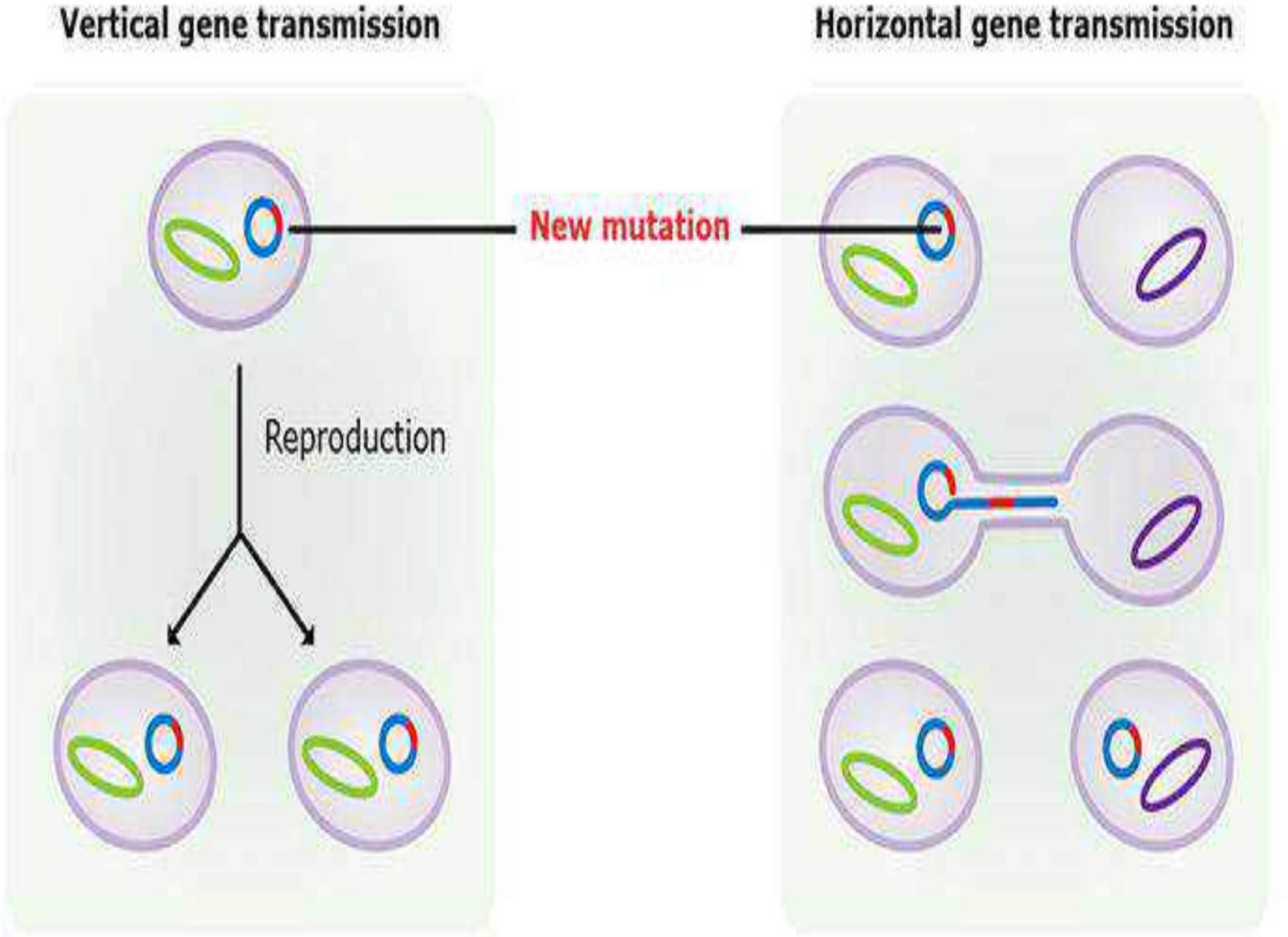
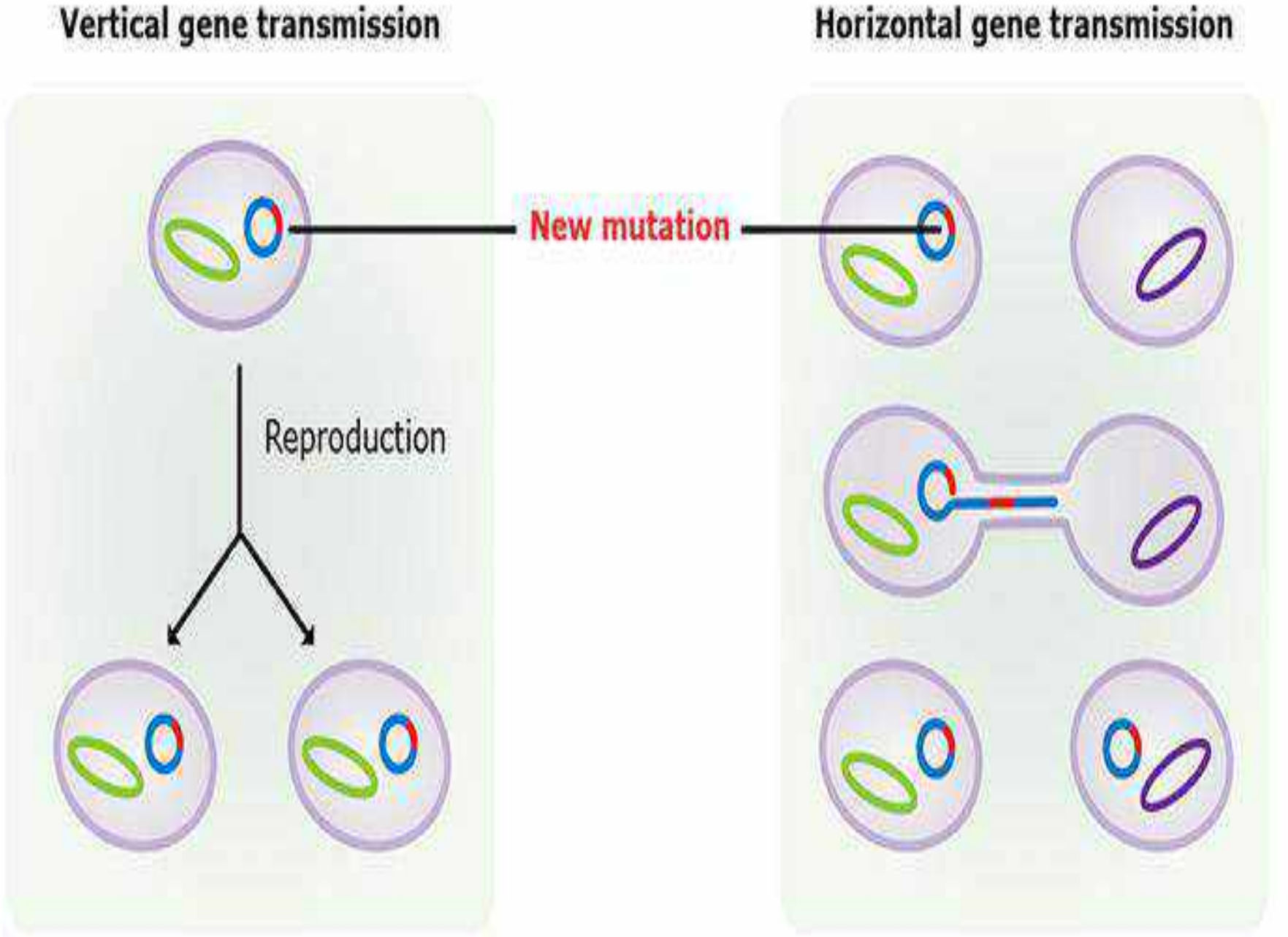
GENE TRANSFER

- It is defined simply as a technique to efficiently and stably introduce foreign genes into the genome of target cells.
- The insertion of unrelated, therapeutic genetic information in the form of DNA into target cells.


Vertical gene transmission
Horizontal gene transmission
New mutation
Reproduction


Vertical gene transmission
Horizontal gene transmission
New mutation
Reproduction

INTRODUCTION

- Gene transfer in which bacteria genotype can be changed by transfer of genetic material from one bacterium to another.
- In such instances, the transferred DNA either:

a) Recombine with genome of the recipient bacterium or

b) Is on the plasmid capable of replication in the recipient bacterium without recombination.

INTRODUCTION

- Gene transfer in which bacteria genotype can be changed by transfer of genetic material from one bacterium to another.
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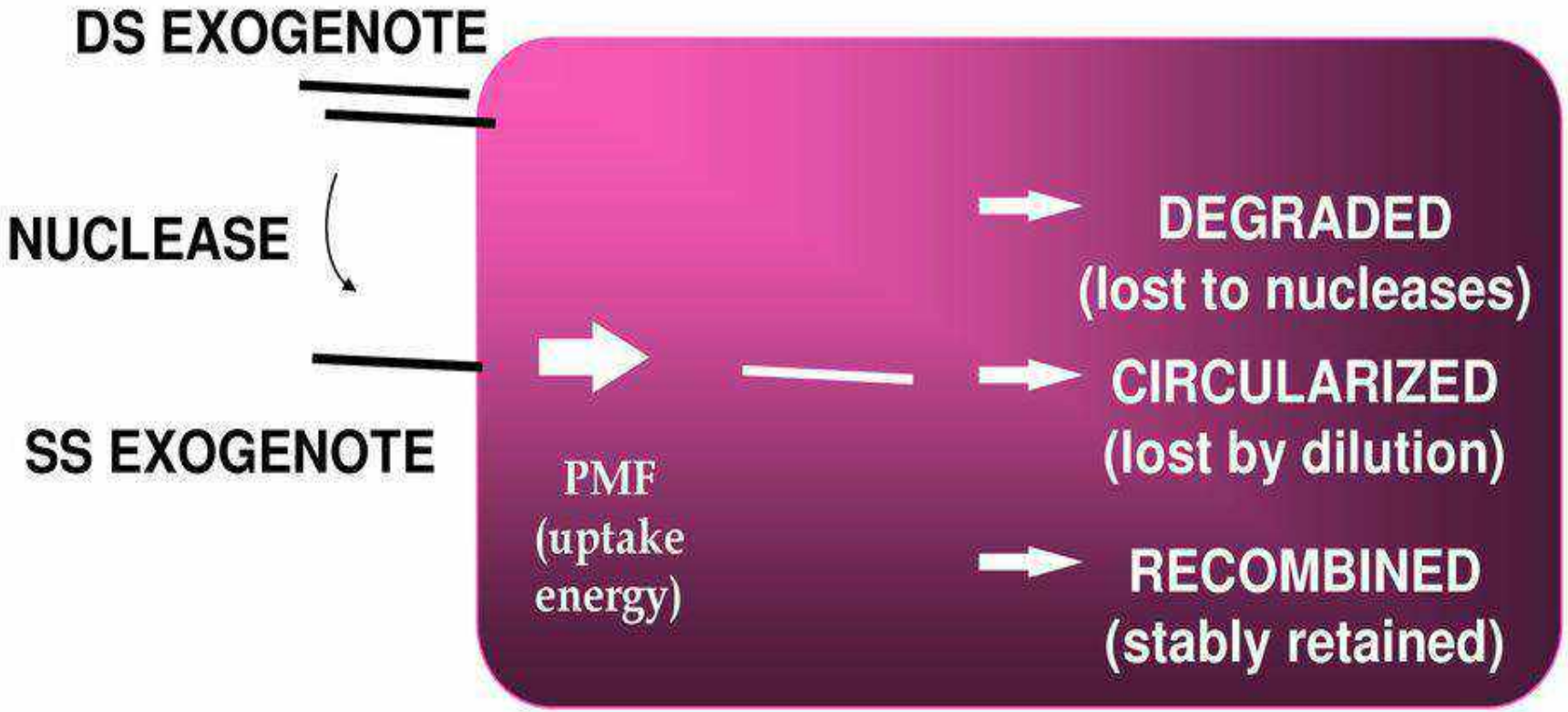
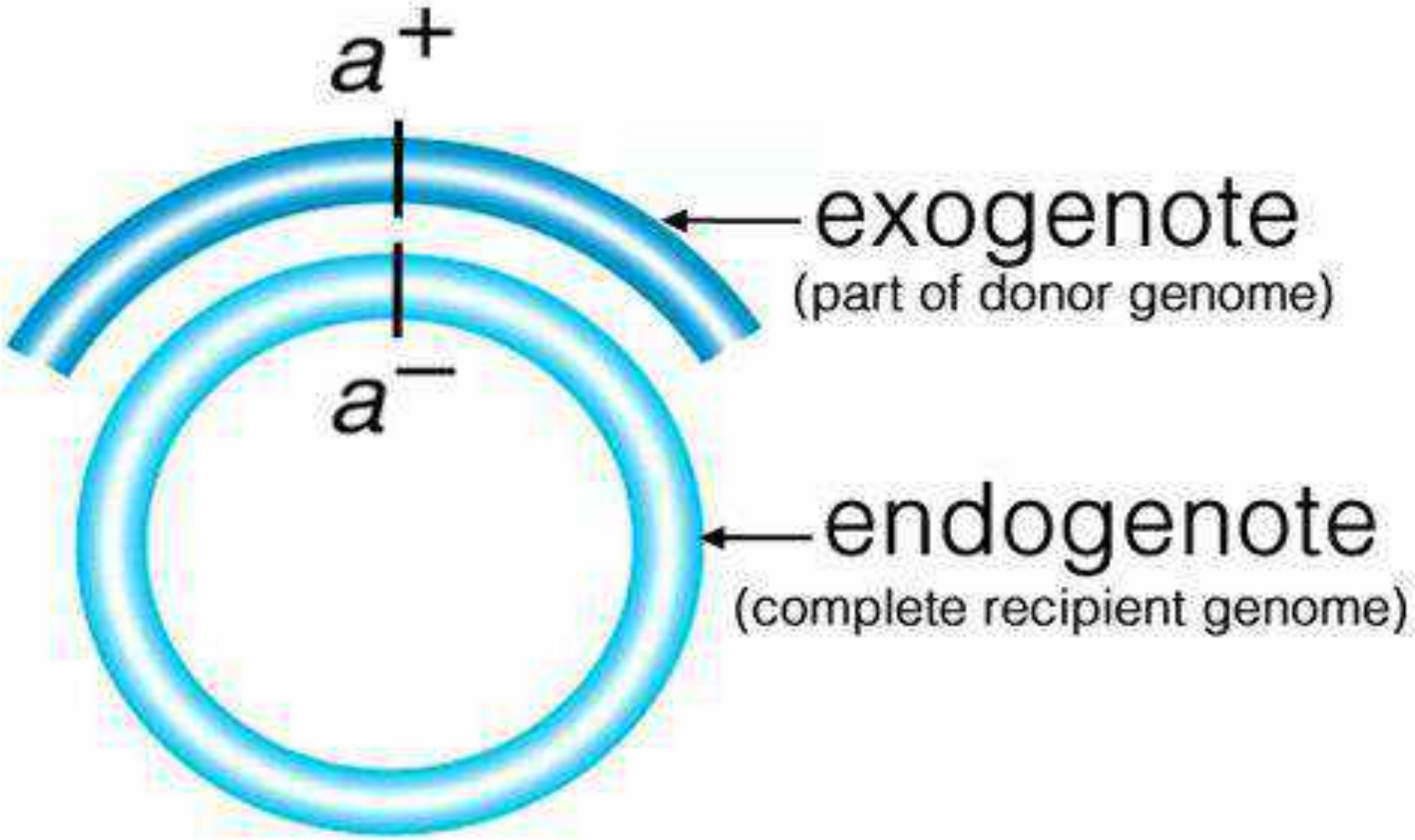
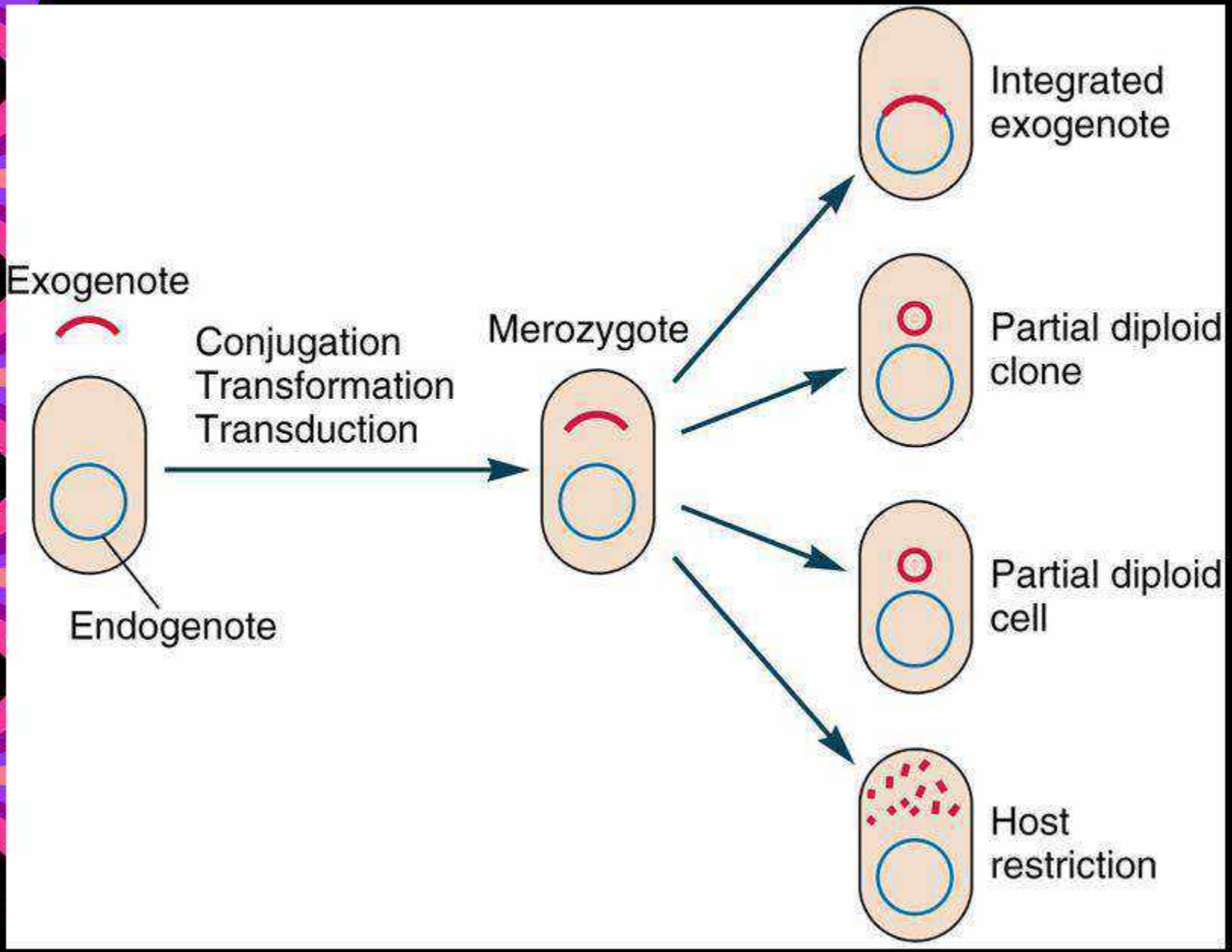
3 FATES OF THE EXOGENOTE

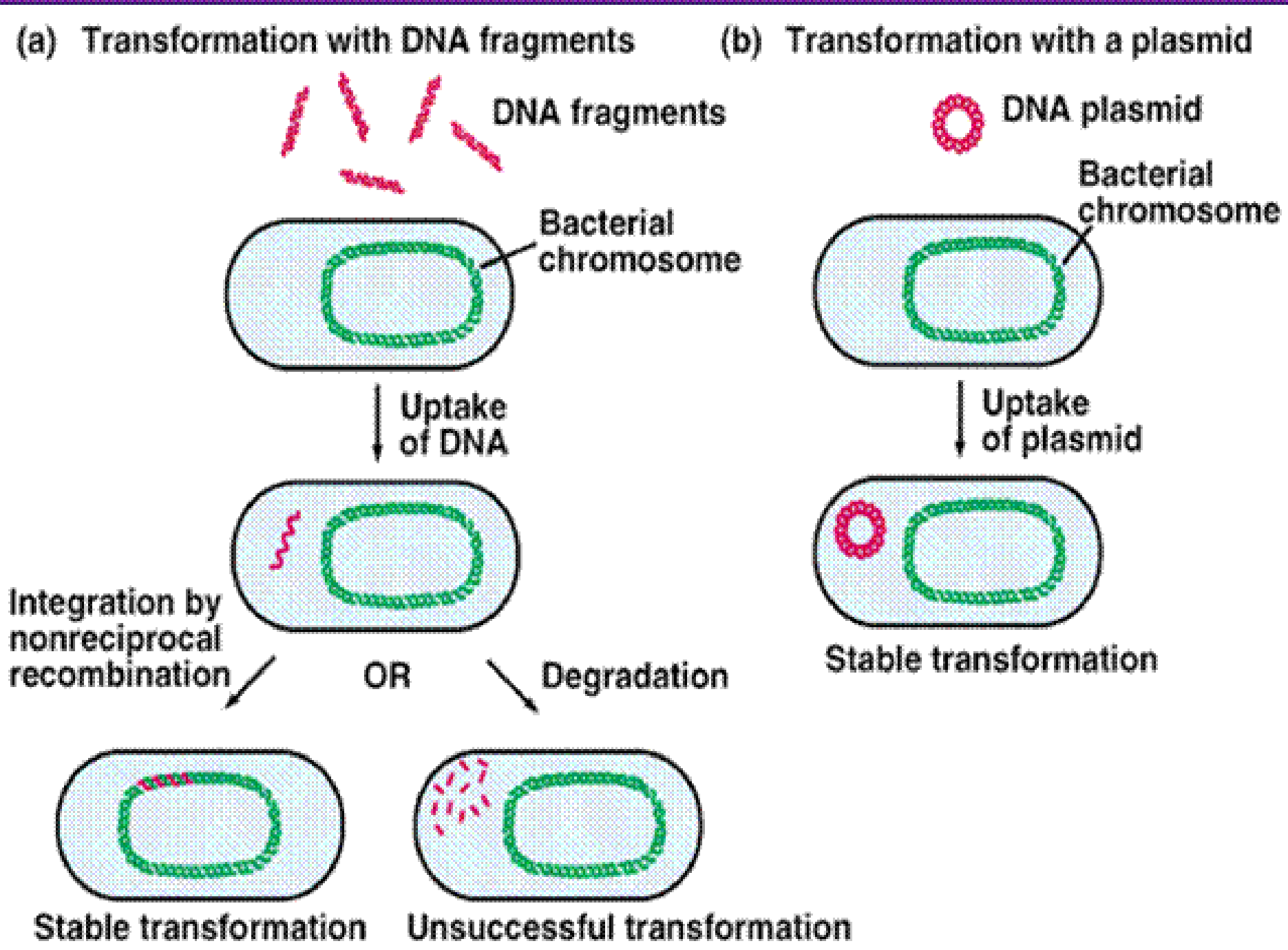


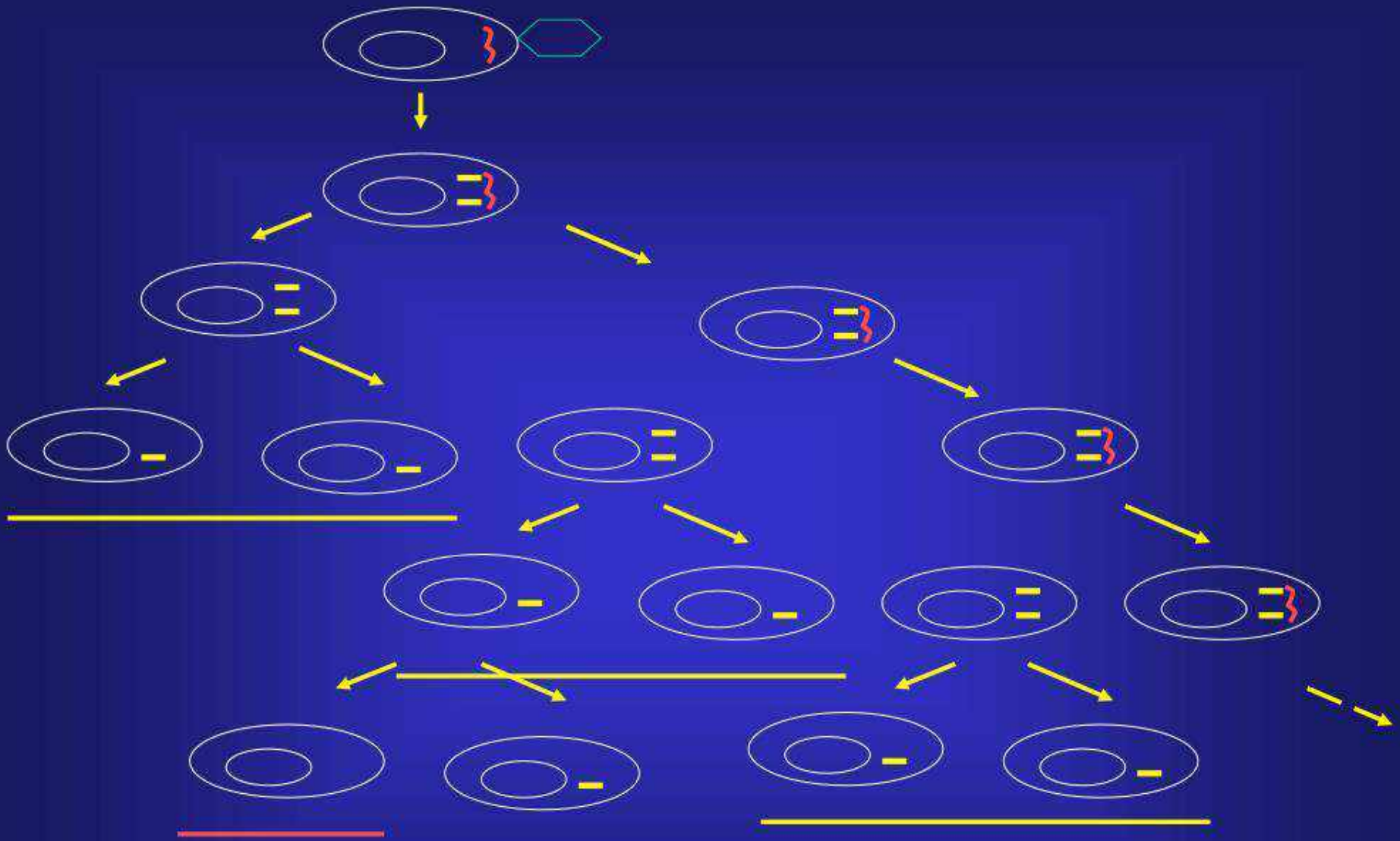


A merozygote is "diploid" at exogenote gene loci:
it (temporarily) has two alleles of those genes.

Fates of DNA


Exogenote
Conjugation
Transformation
Transduction
Endogenote
Merozygote
Integrated exogenote
Partial diploid clone
Partial diploid cell
Host restriction


(a) Transformation with DNA fragments
(b) Transformation with a plasmid
DNA fragments
DNA plasmid
Bacterial chromosome
Bacterial chromosome
Uptake of DNA
Uptake of plasmid
Integration by nonreciprocal recombination
OR
Degradation
Stable transformation
Stable transformation
Unsuccessful transformation



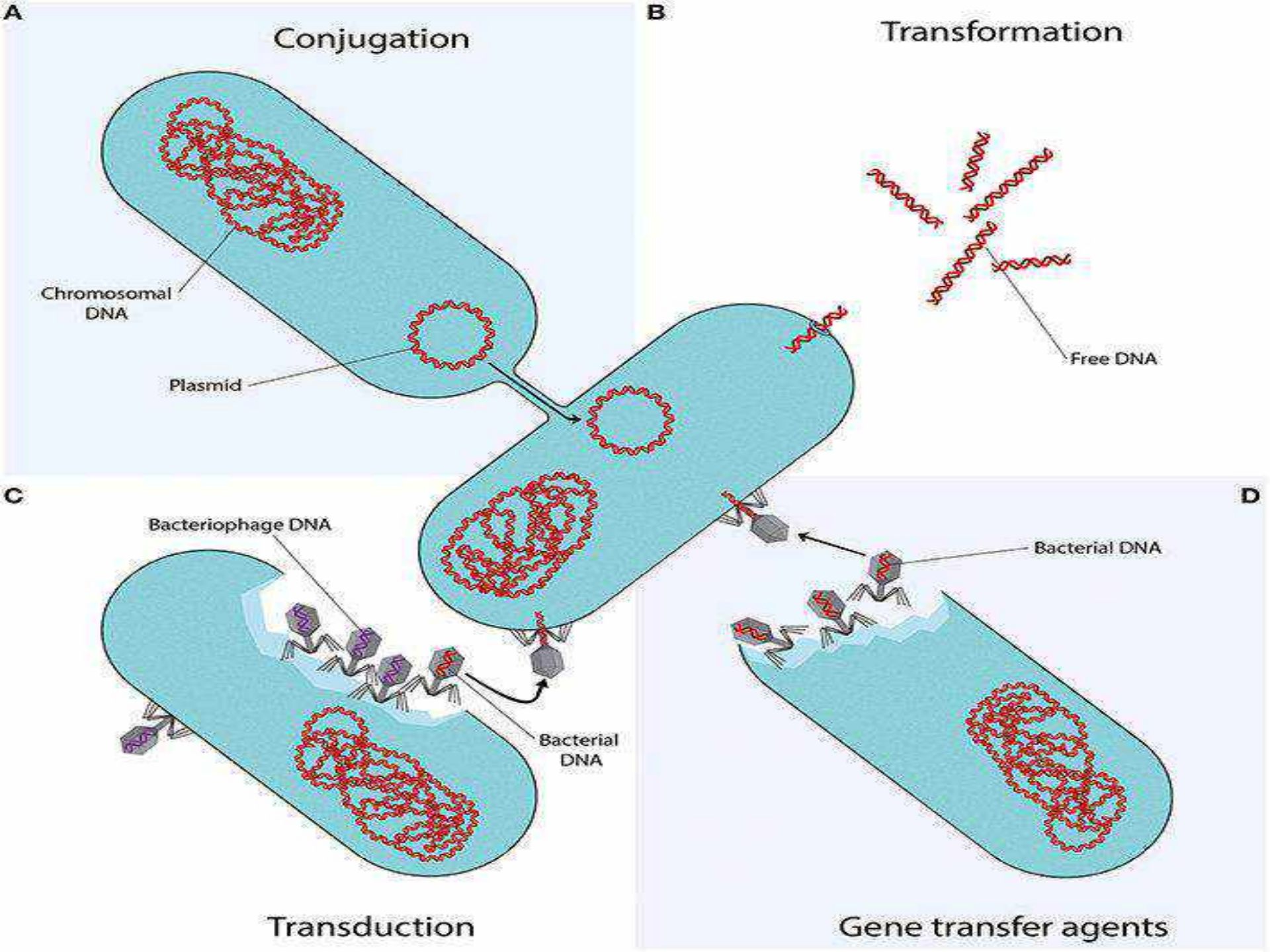
Abortive transduction

- Genetic recombination it occurs when sequence of DNA from two separate sources are integrated.
- The directed desirable gene transfer from one organism to another and the subsequent stable integration & expression of foreign gene into the genome is referred as genetic transformation.
- The transferred gene is known as transgene and the organism that develop after a successful gene transfer is known as transgenic.

MECHANISM OF GENE TRANSFER

There are four mechanisms of gene transfer and recombination between bacteria cells.

1. Conjugation
2. Transformation
3. Transduction
4. Cell- cell fusion


A
Conjugation
Chromosomal DNA
Plasmid
B
Transformation
Free DNA
C
Bacteriophage DNA
Bacterial DNA
Transduction
D
Bacterial DNA
Gene transfer agents

DNA TRANSFER BY ARTIFICIAL METHODS

Physical methods

1. Microinjection
2. Biolistics transformation

Chemical methods

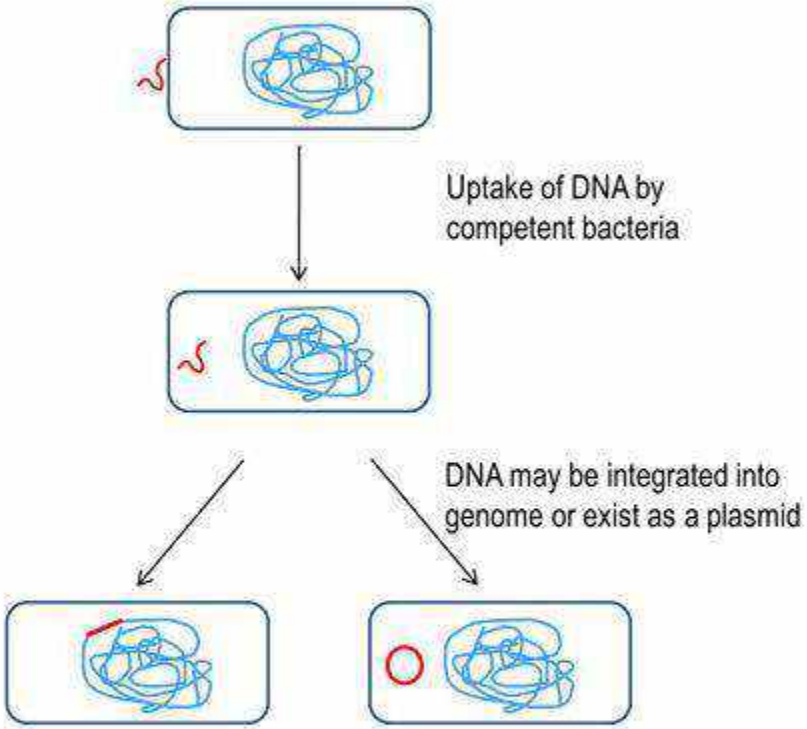
1. DNA transfer by calcium phosphate method
2. Liposome mediated transfer

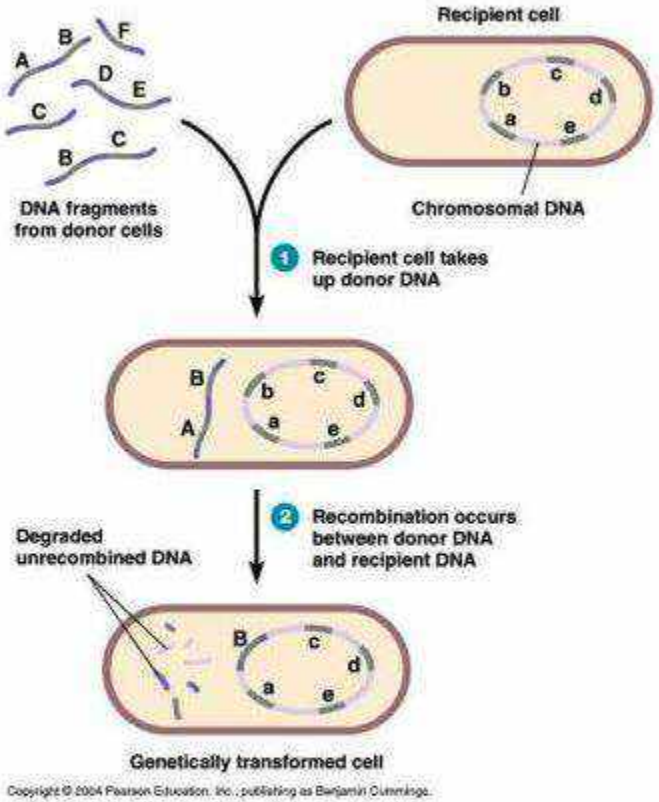
Electrical methods

1. Electroporation

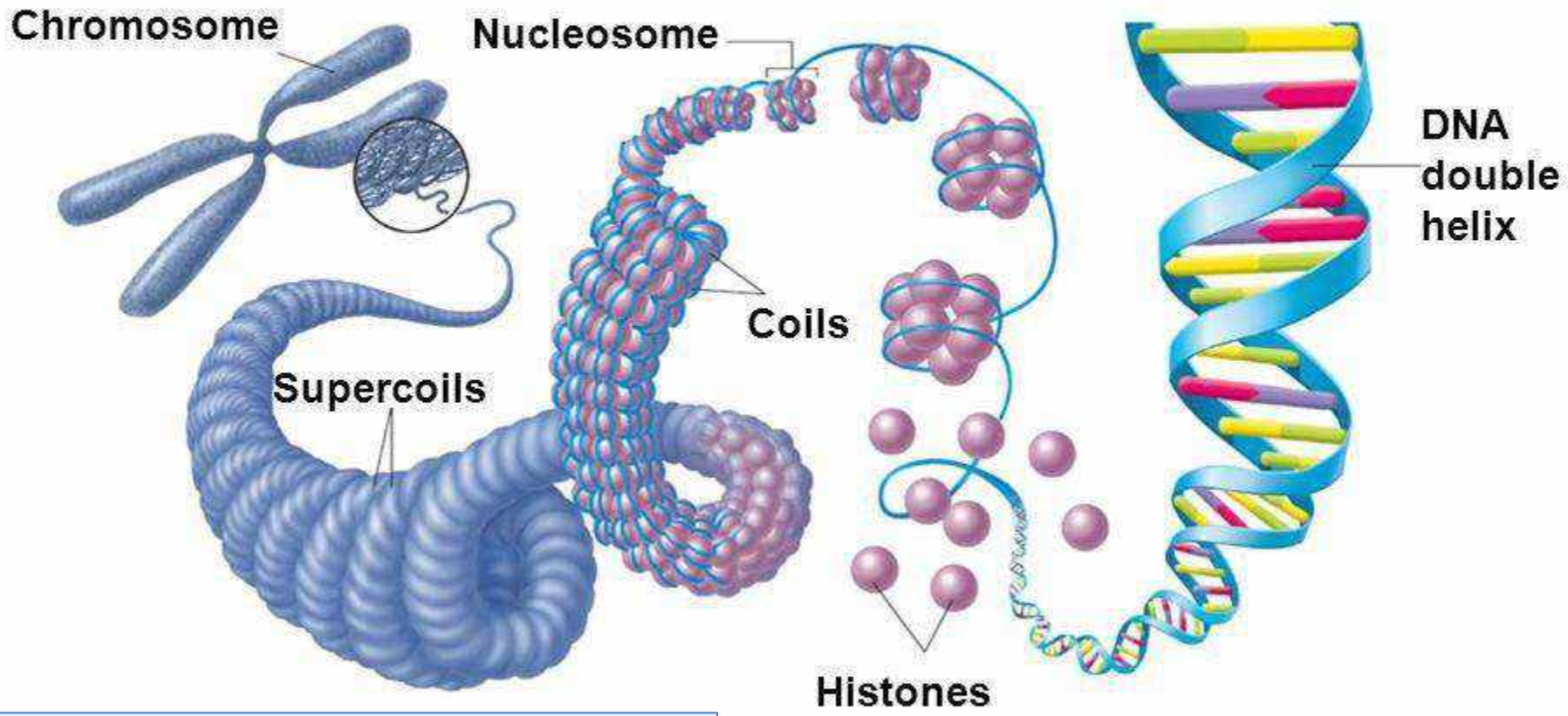
Transformation:

Update of DNA in the environment by bacteria





SRUCTURAL ORGANIZATION OF E.COLI CROMOSOME



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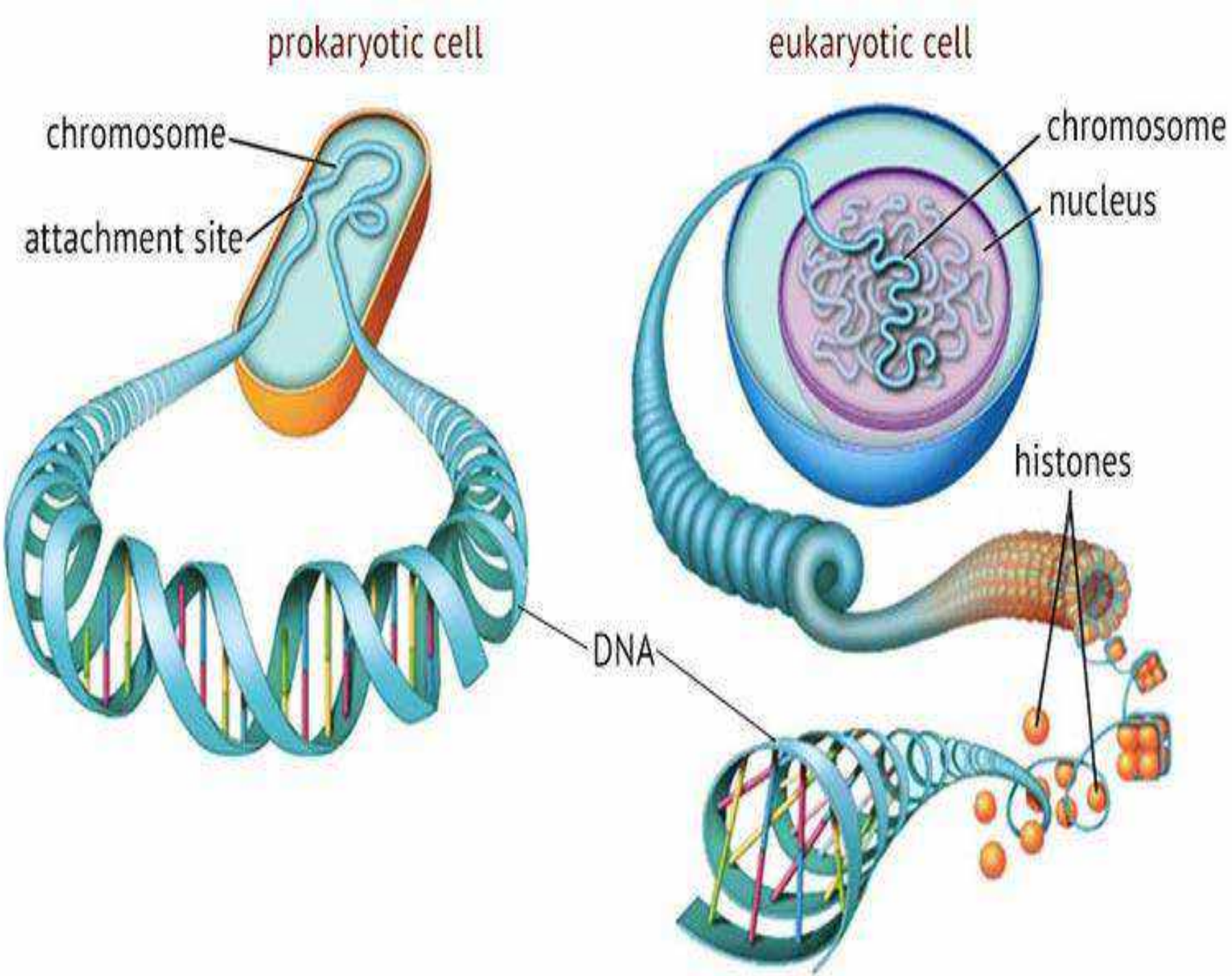
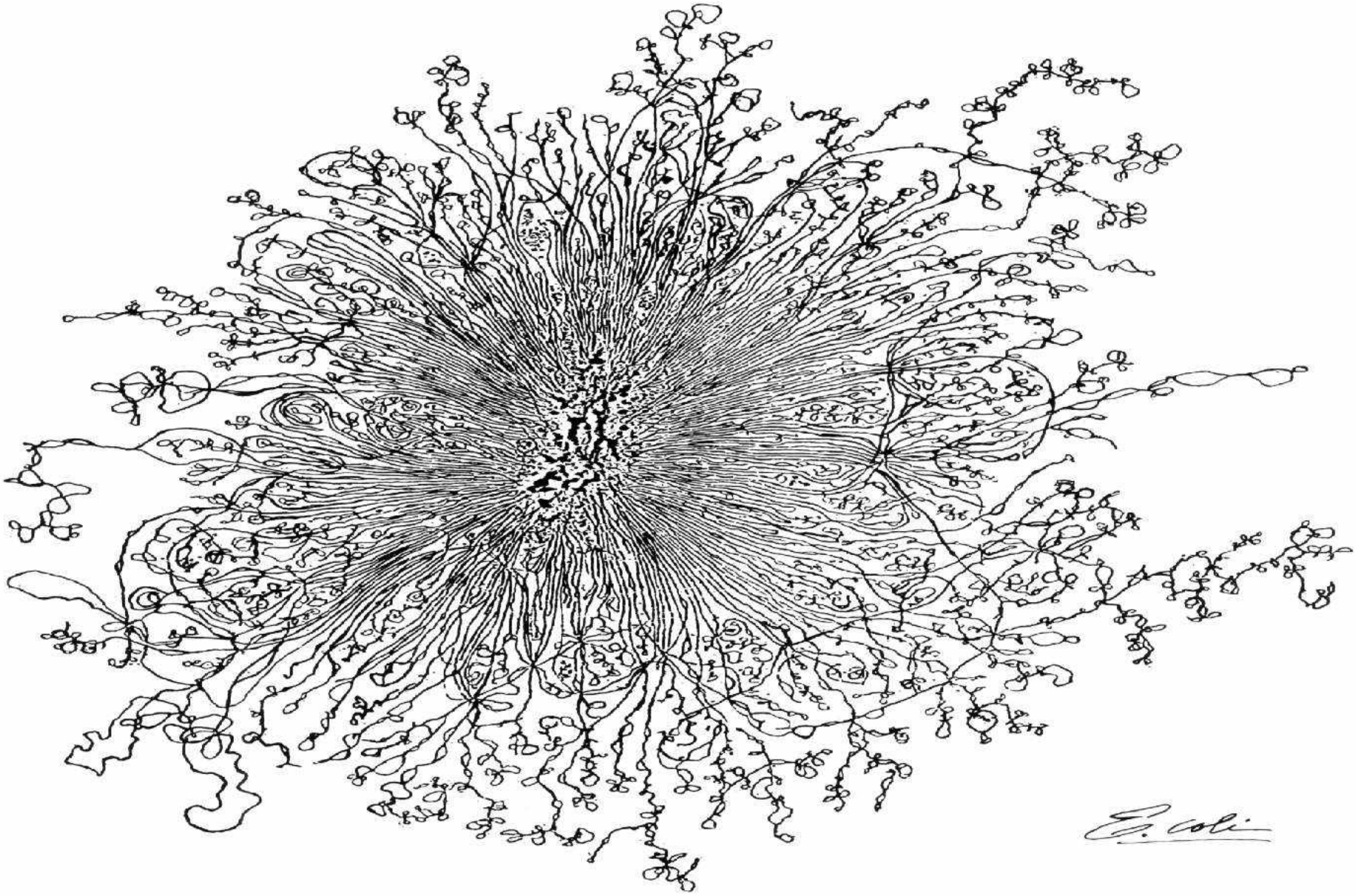

prokaryotic cell
eukaryotic cell
chromosome
attachment site
chromosome
nucleus
histones
DNA


E. coli

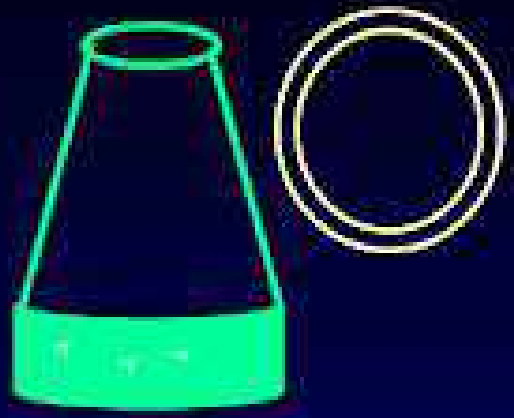
Cairn's Experiment

- The visualization of replicating chromosome was first accomplished by J. Cairns in 1963 using the technique called autoradiography.
- Autoradiography is a method of detecting and localizing radioactive isotopes in macromolecules by exposure to photographic emulsion that is sensitive to low energy radiation.
- Autoradiography is particularly useful in studying DNA metabolism because DNA can be specifically labeled by growing cells on [H^3]thymidine, the tritiated deoxyribonucleoside of thymidine.
- Thymidine is incorporated exclusively into DNA; it is not present in any other major component of the cell.

John Cairns

in media with low concentration of ^{3}H- thymidine

Grow cells for several generations
Small amounts of ^{3}H thymidine are incorporated into new DNA



All DNA is lightly labeled with radioactivity

Add a high concentration of ^{3}H- thymidine

Grow for brief period of time

Dense label at the replication fork where new DNA is being made

Cairns then isolated the chromosomes by lysing the cells very very gently and placed them on an electron micrograph (EM) grid which he exposed to X-ray film for two months.

❖ It is found that E.Coli chromosome possesses 4377 genesof which 4290 encodes for proteins and othes encodes for RNAs.

❖Each gene has approximate 600 pairs of nucleotides

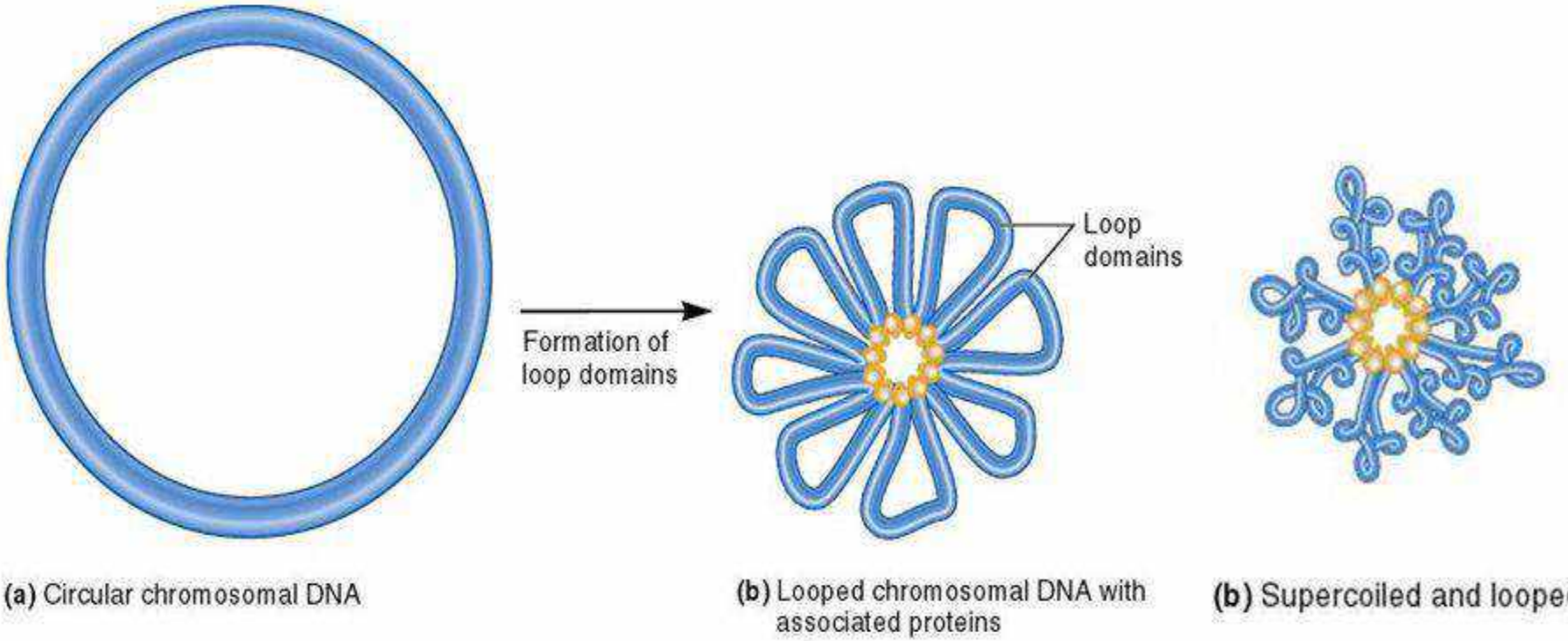
❖The dimentional study of *E.Coli* DNA and electron microscopic studies have shown that length of *E.Coli* DNA is more than 1 mm while the size of accommodating cell is 1-2 micro meter which indicates that DNA must be in packed or condensed form in the E.coli cell.

❖To study the organization of DNA in *E.Coli cell* , the attempt was done by A. Worcel and associates in 1970 .

❖They isolate intact nucleoid of *E.Coli free of* plasma membrane and chemical analysis of which shows 30% by weight RNA , 60- 70% by weight DNA and about 1 % by weight proteins(RNA polymerase)

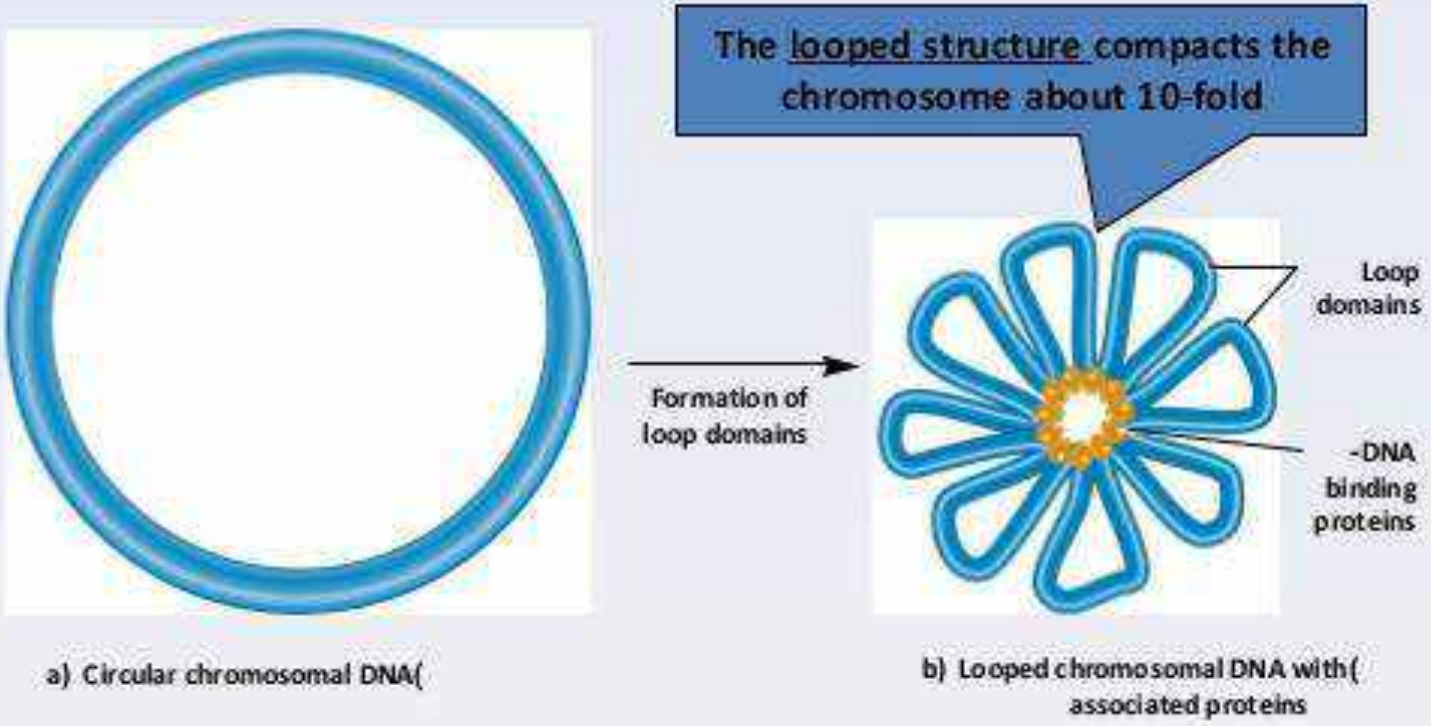
❖D.Pettijohn and R. Hetch have praposed a model called “ Folded fiber model”

Bacterial Chromosomes



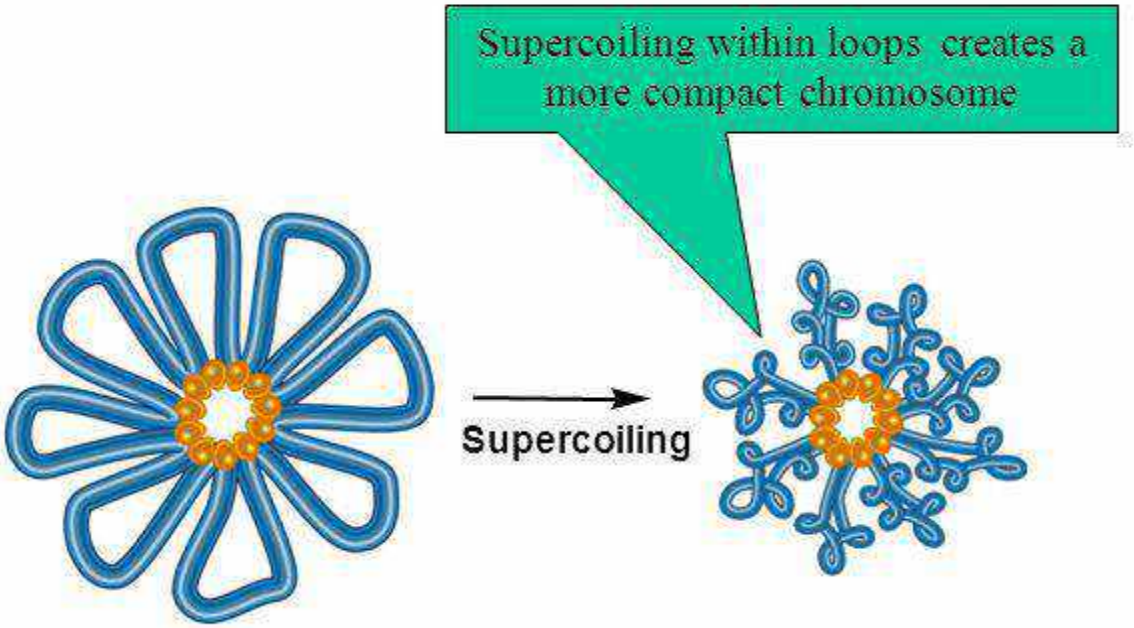
- Chromosomal DNA is compacted ~ 1000 fold to fit within cell

To fit within bacterial cell, the chromosome must be compacted ~1000-fold



Brooker, Fig 12.3

DNA supercoiling is a second important way to compact the bacterial chromosome

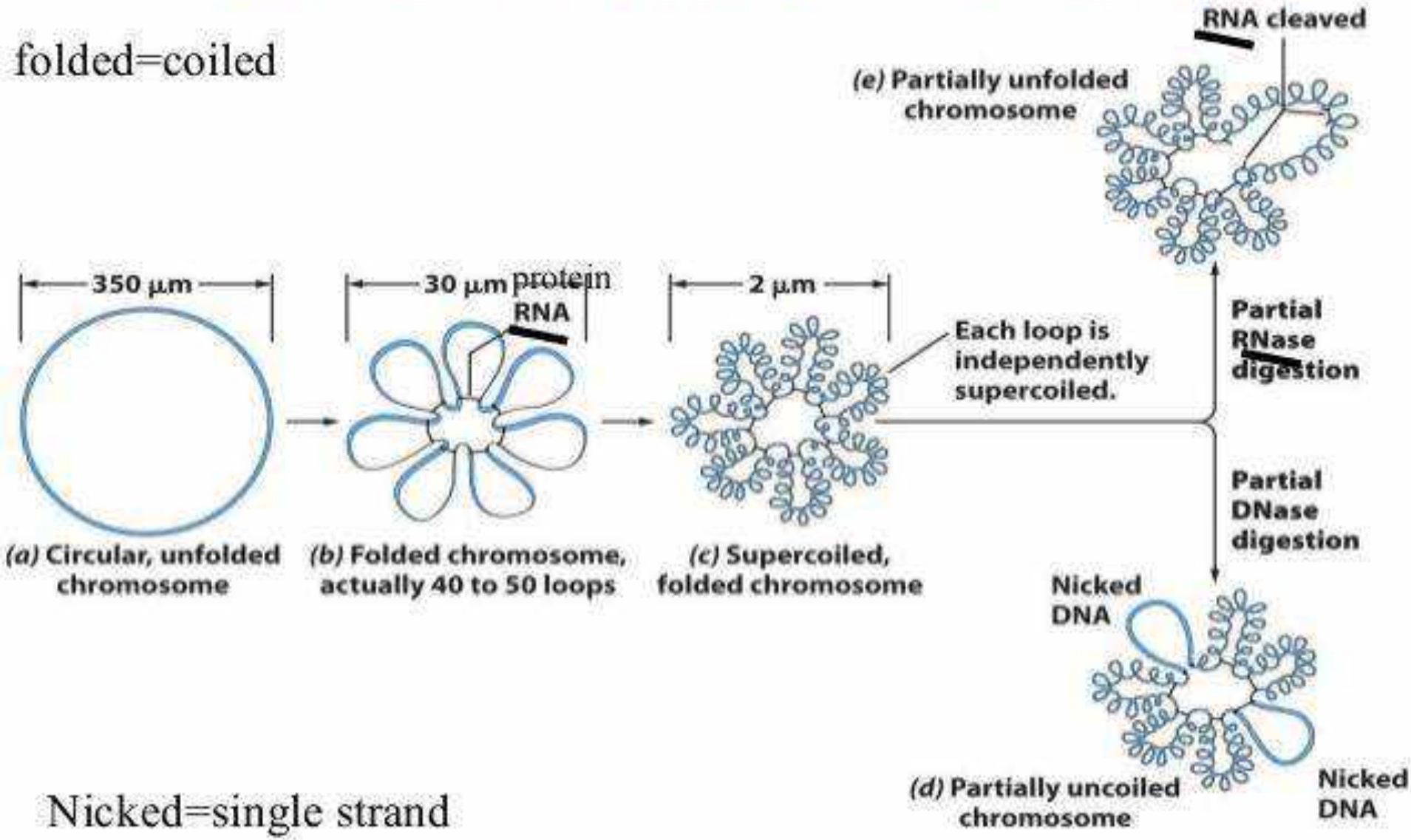


(b) Looped chromosomal DNA (c) Looped and supercoiled DNA

Brooker, Fig 12.3 -- illustration of DNA supercoiling

Model of *E. coli* Chromosome

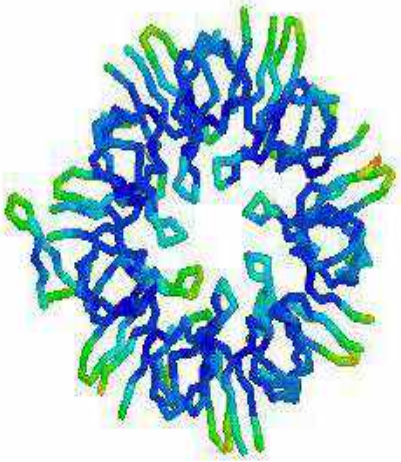
folded=coiled

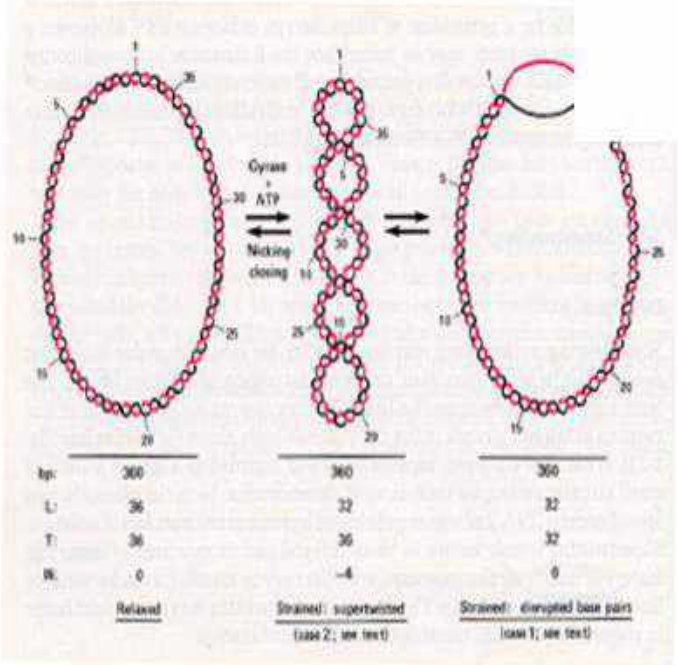


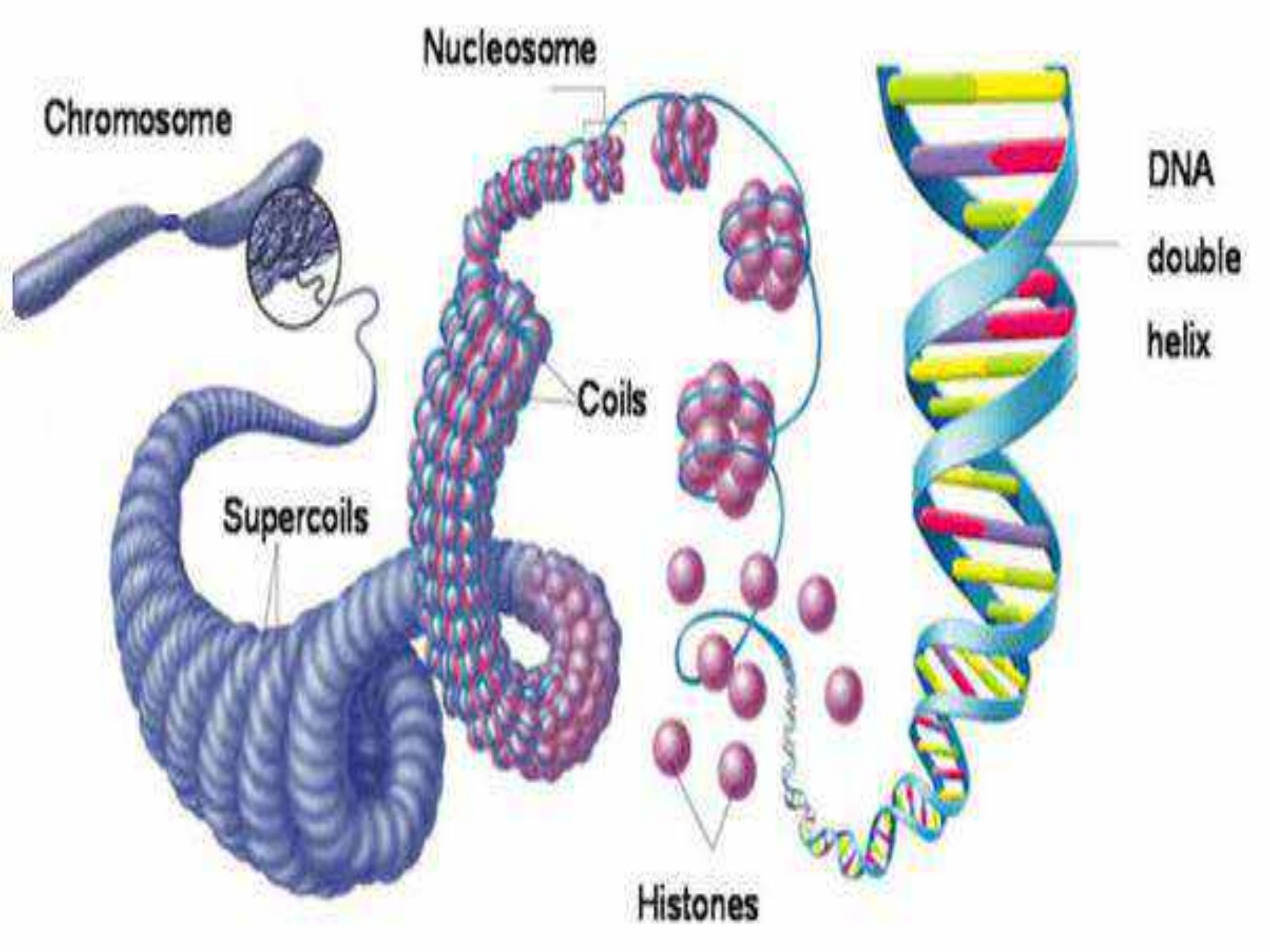
Nicked=single strand

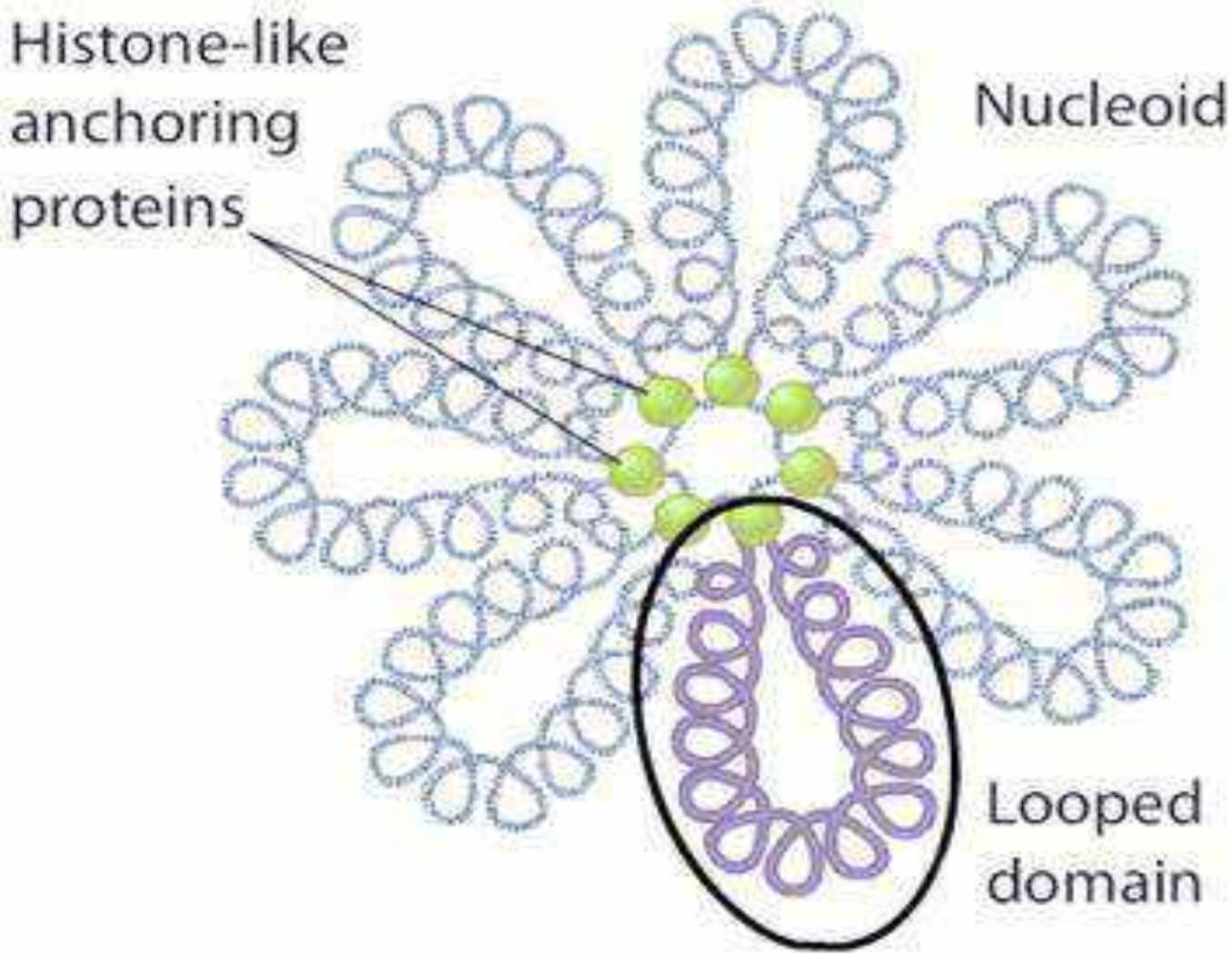
How does the DNA fit into the cell?

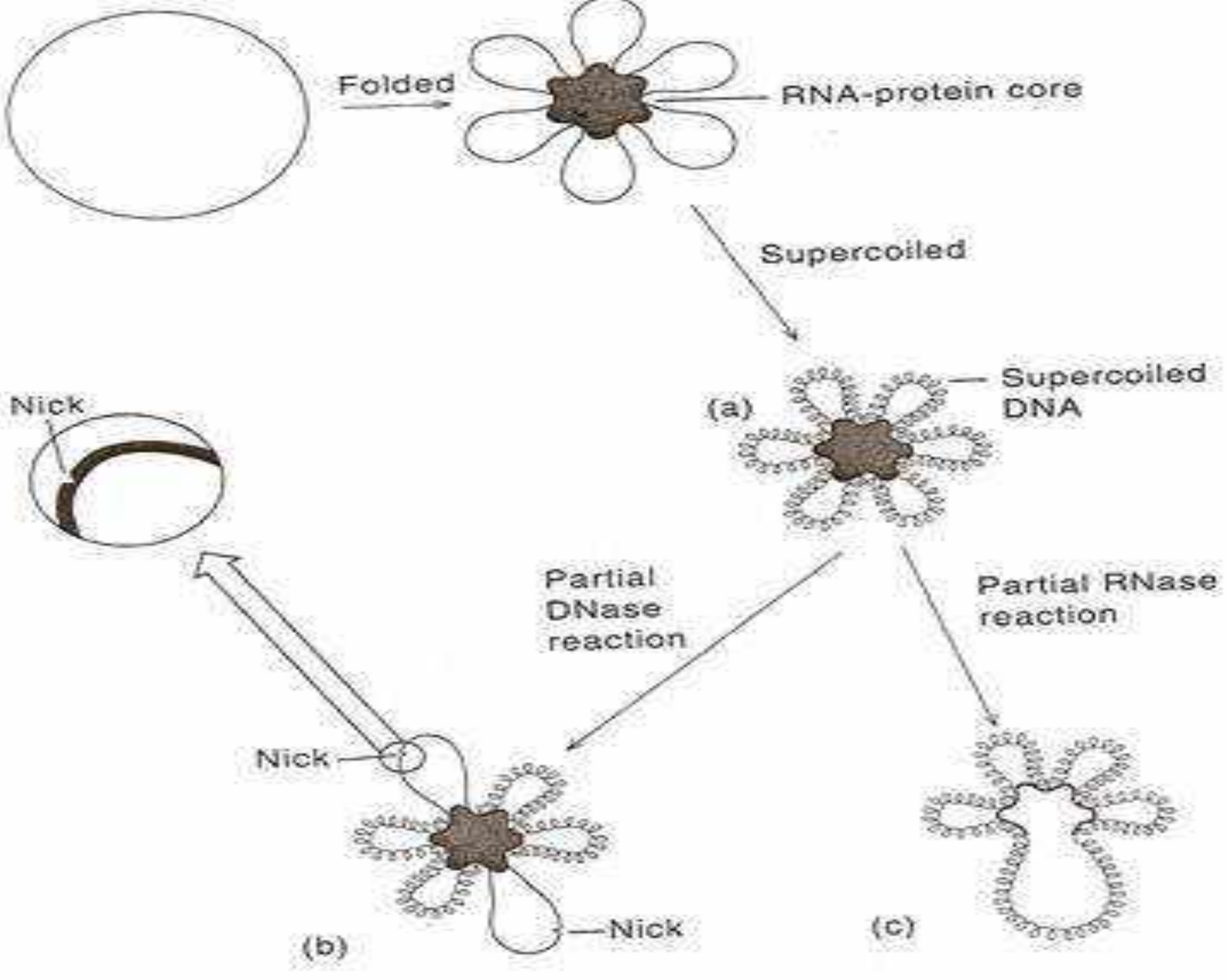
- DNA compacted 1000 fold
- Forms loop domains
- DNA supercoiling

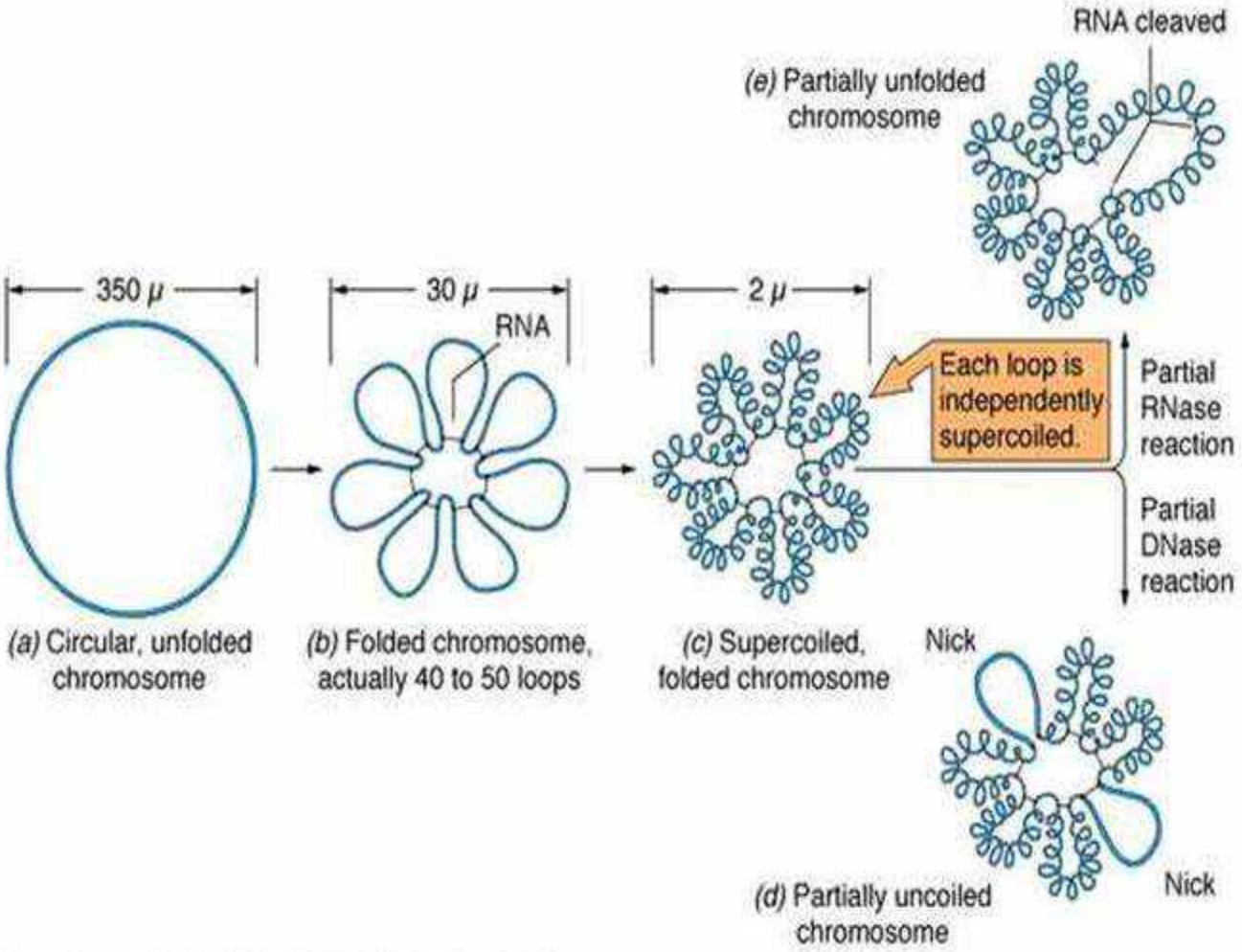





Nucleosome
Chromosome
DNA
double
helix
Coils
Supercoils
Histones


Histone-like
anchoring
proteins
Nucleoid
Looped
domain


Folded
RNA-protein core
Supercoiled
Supercoiled DNA
(a)
Nick
Partial DNase reaction
Partial RNase reaction
Nick
Nick
(b)
(c)

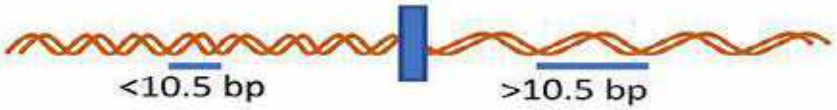


A

Relaxed state

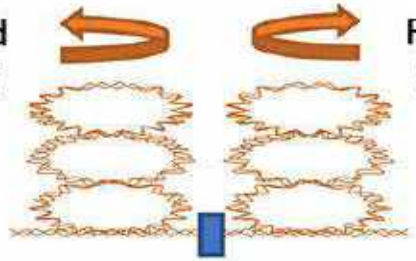
Twist

Overwound (+) Underwound (-)



Writhe

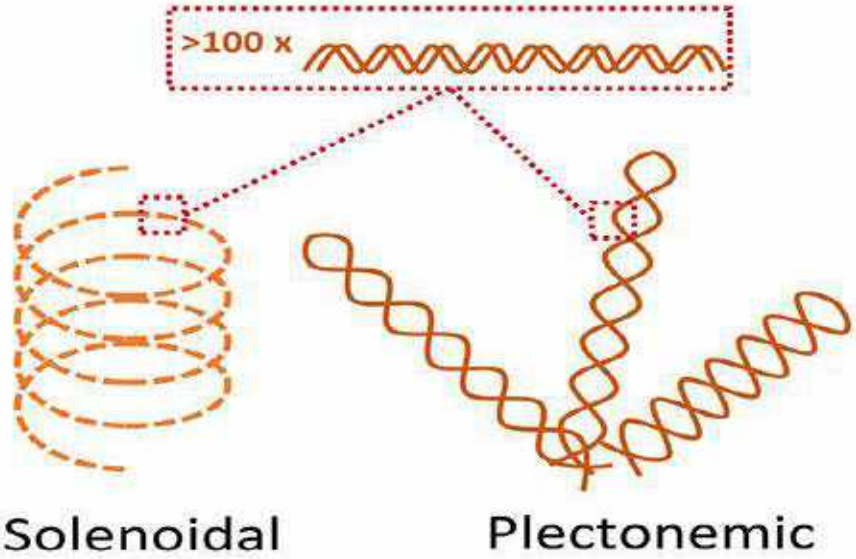
Left-handed
Overwound



Right-handed
Underwound

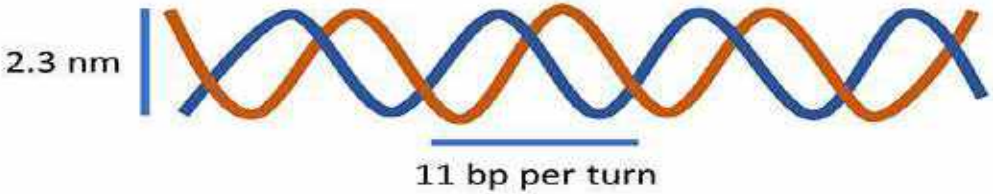
B

Supercoils



C

Alpha and Beta forms of DNA



A-form 2.6 Å per bp

B-form 3.4 Å per bp

Screening

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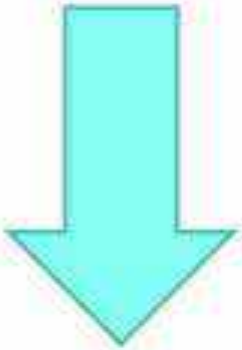
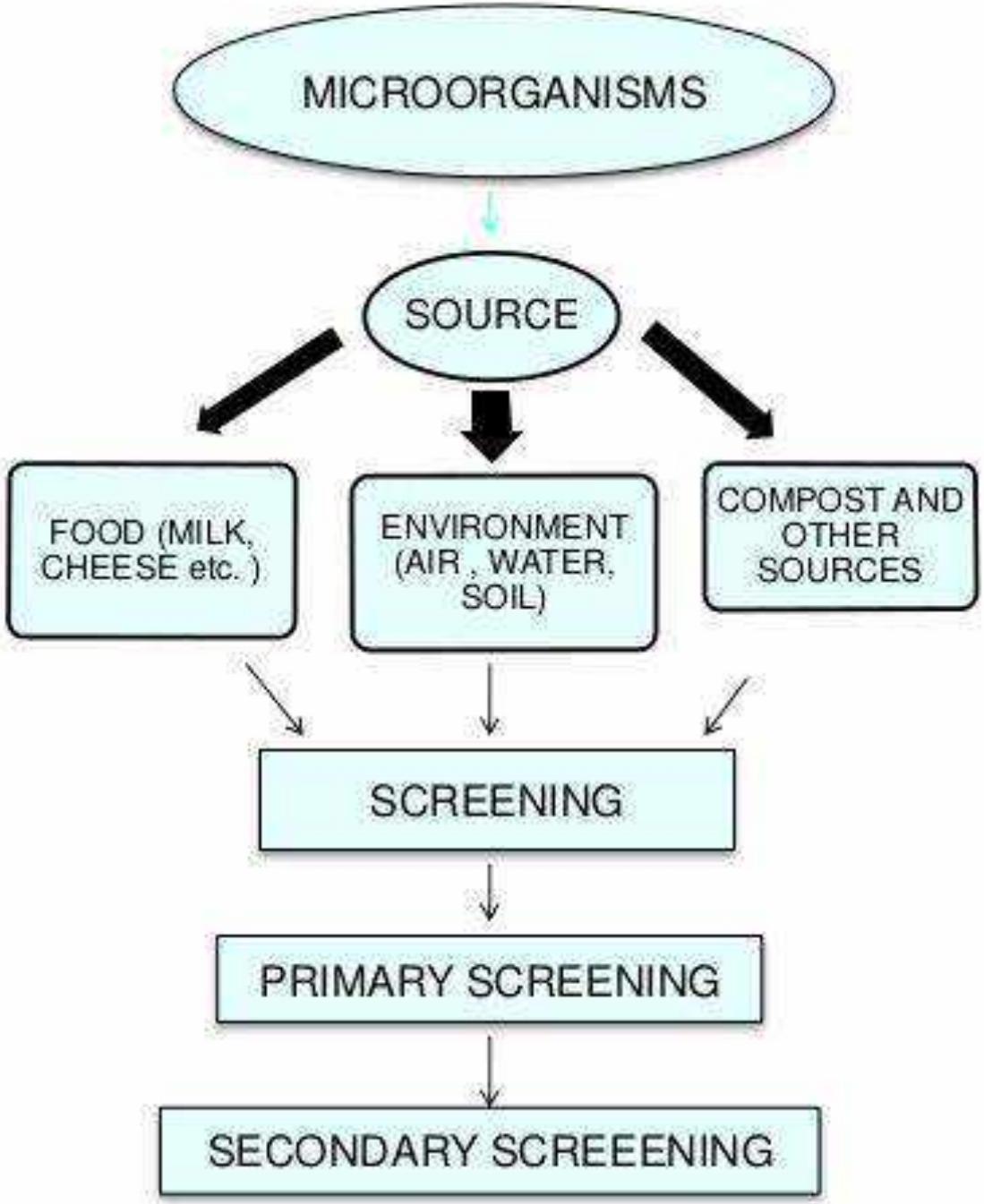
SCREENING



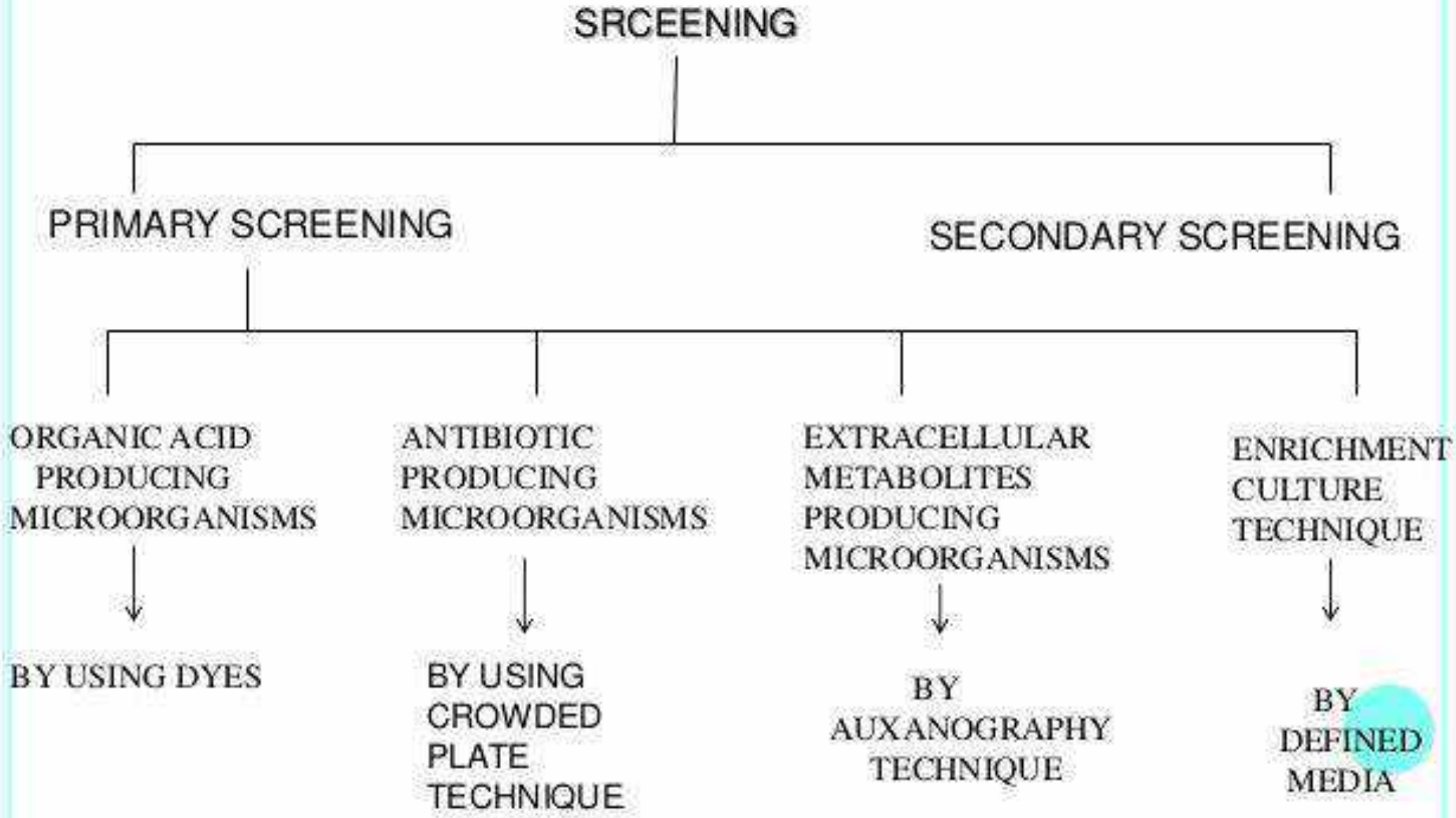
The procedure of isolation, detection , and separation of microorganisms of our interest from a mixed population by using highly selective procedures is called SCREENING


MICROORGANISMS
SOURCE
FOOD (MILK, CHEESE etc.)
ENVIRONMENT (AIR , WATER, SOIL)
COMPOST AND OTHER SOURCES
SCREENING
PRIMARY SCREENING
SECONDARY SCREEENING

IMPORTANT THINGS TO BE CONSIDERED WHILE SCREENING :-

- 1.) CHOICE OF SOURCE - Samples from screening is taken from soil, water, air, milk, compost etc.
- 2.) CHOICE OF SUBSTRATE -Nutrients and growth factors should be supplied for growth of desired microorganism.
- 3.) CHOICE OF DETECTION - Proper isolation and detection of desired microorganisms is important

TYPES OF SCREENING



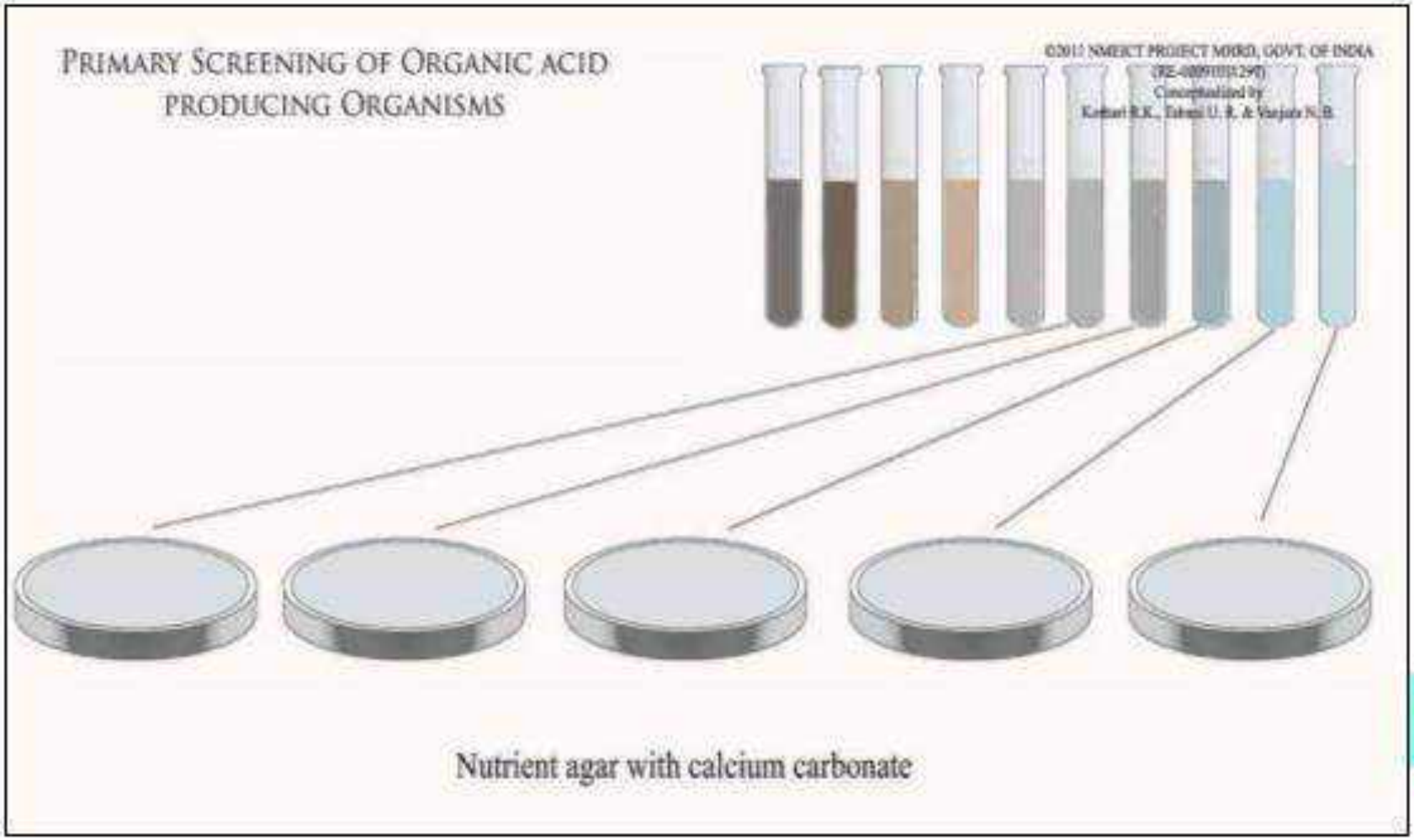
PRIMARY SCREENING

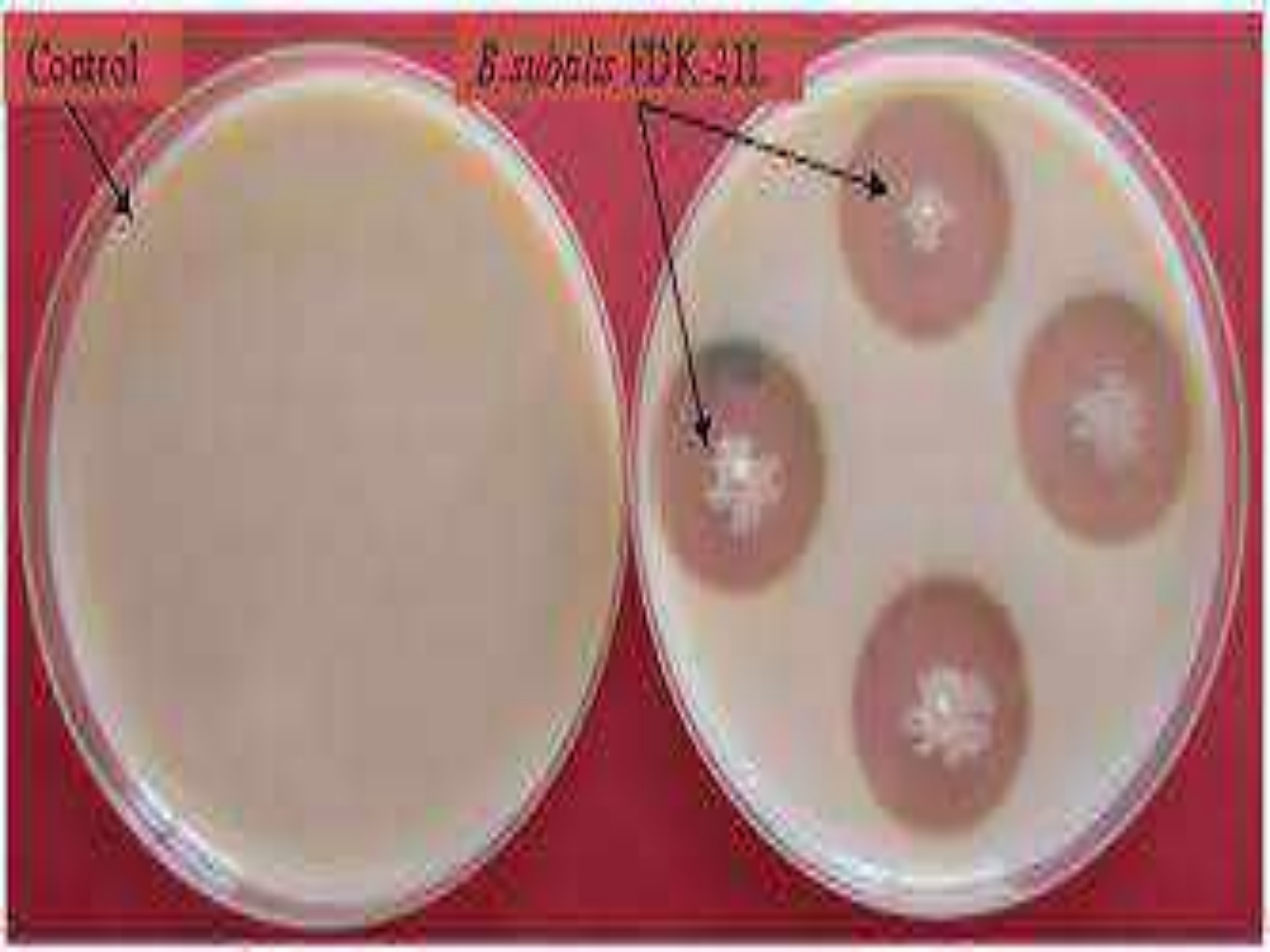
- It's a process for detection and isolation of microorganisms of our interest.
- Determines which microorganisms are able to produce a compounds.
- Does not provide much idea about the production or yield potential of microorganisms.
- It separate out only a few microorganisms, only few have commercial value while discards the valueless microorganisms .

1) PRIMARY SCREENING OF ORGANIC ACID PRODUCING MICROORGANISMS

- The ph indicating **dyes** may be used for detecting microorganism that are capable of producing organic acids.
- These dyes undergo color changes according to its ph.
- Dyes such as **Neutral red, Bromothymol blue** are added to the poorly buffered nutrient agar media .
- Colonies are subcultured to make stock culture.
- Further testing is needed since inorganic acids, bases are also metabolic products of microbial growth.

- Incorporation of $CaCO_3$ in medium is also used to screen organic acid producing microbes on basis of formation of clear zone of dissolved $CaCO_3$ around the colony.

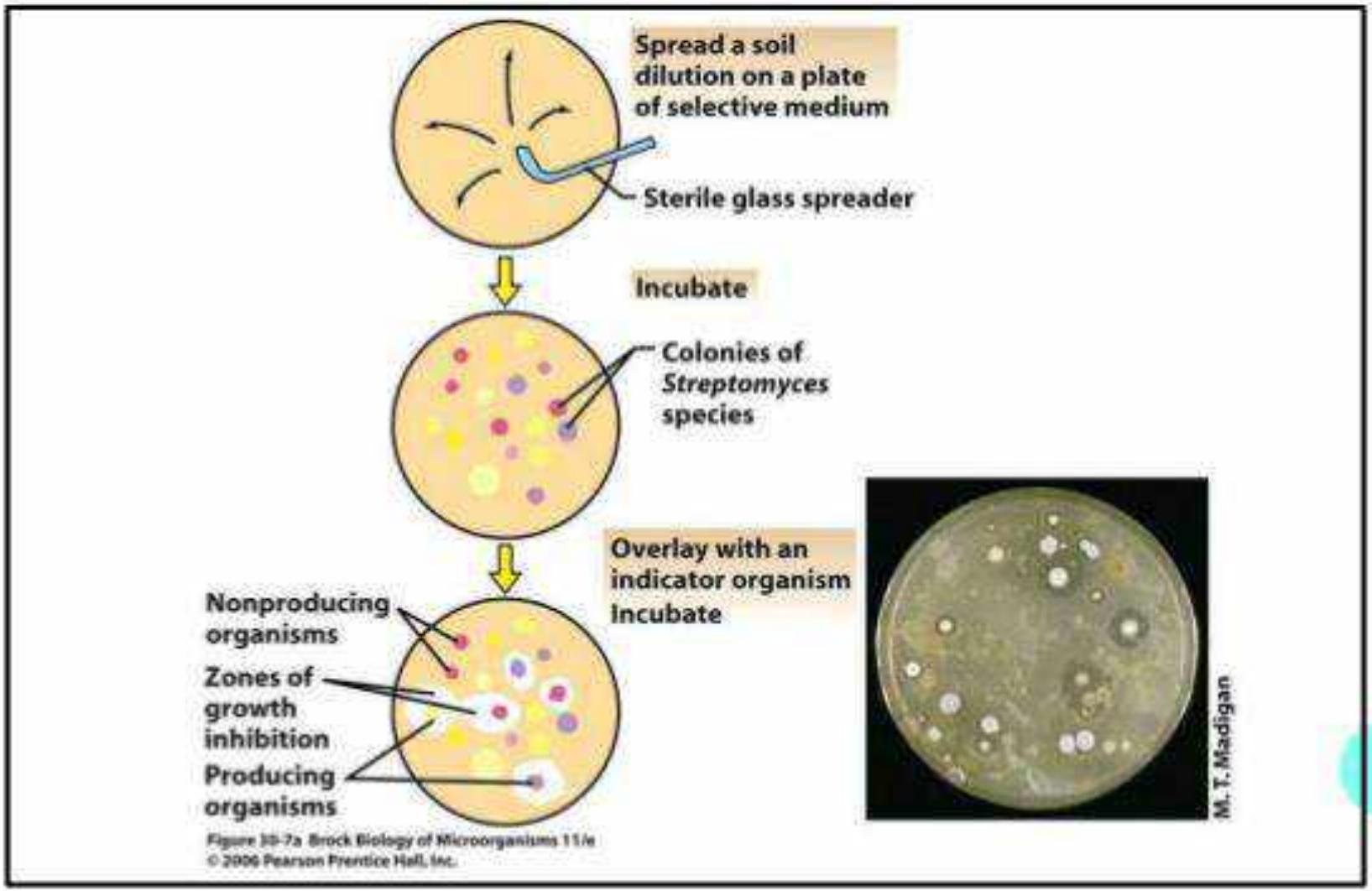



Control
B. subtilis VBK-21

2) PRIMARY SCREENING OF ANTIBIOTIC PRODUCING MICROORGANISMS

- Crowded plate technique is used for screening of antibiotic producing microorganisms.
- Does not give information about the sensitivity of antibiotics towards other microorganisms.
- Dilutions are made and then pouring and spreading of soil samples that give 300 to 400 or more colonies per plate.
- Colonies showing antibiotic activity are indicated by zone of inhibition around the colony .
- Such colonies are sub cultured and purified by streak before making stock cultures.

❖The purified cultures are then tested to find the Microbial Inhibition Spectrum.



3) PRIMARY SCREENING EXTRACELLULAR METABOLITE PRODUCING MICROORGANISM

- Auxanography technique is employed for detecting microorganisms able to produce growth factors , vitamins , amino acids etc. extracellularly.
- The 2 major steps are:-

A.)Preparation of first plate

- A filter paper strip is put across the bottom of petri dish.
- The nutrient agar is prepared and poured on the paper disc
- and allowed to solidify.
- Soil sample is diluted and proper dilutions are inoculated.

B.) Preparation of second plate

- A minimal media lacking the growth factors is prepared and seeded with the test organism.
- The seeded medium is poured onto fresh petri plate and the plate is allowed to set.

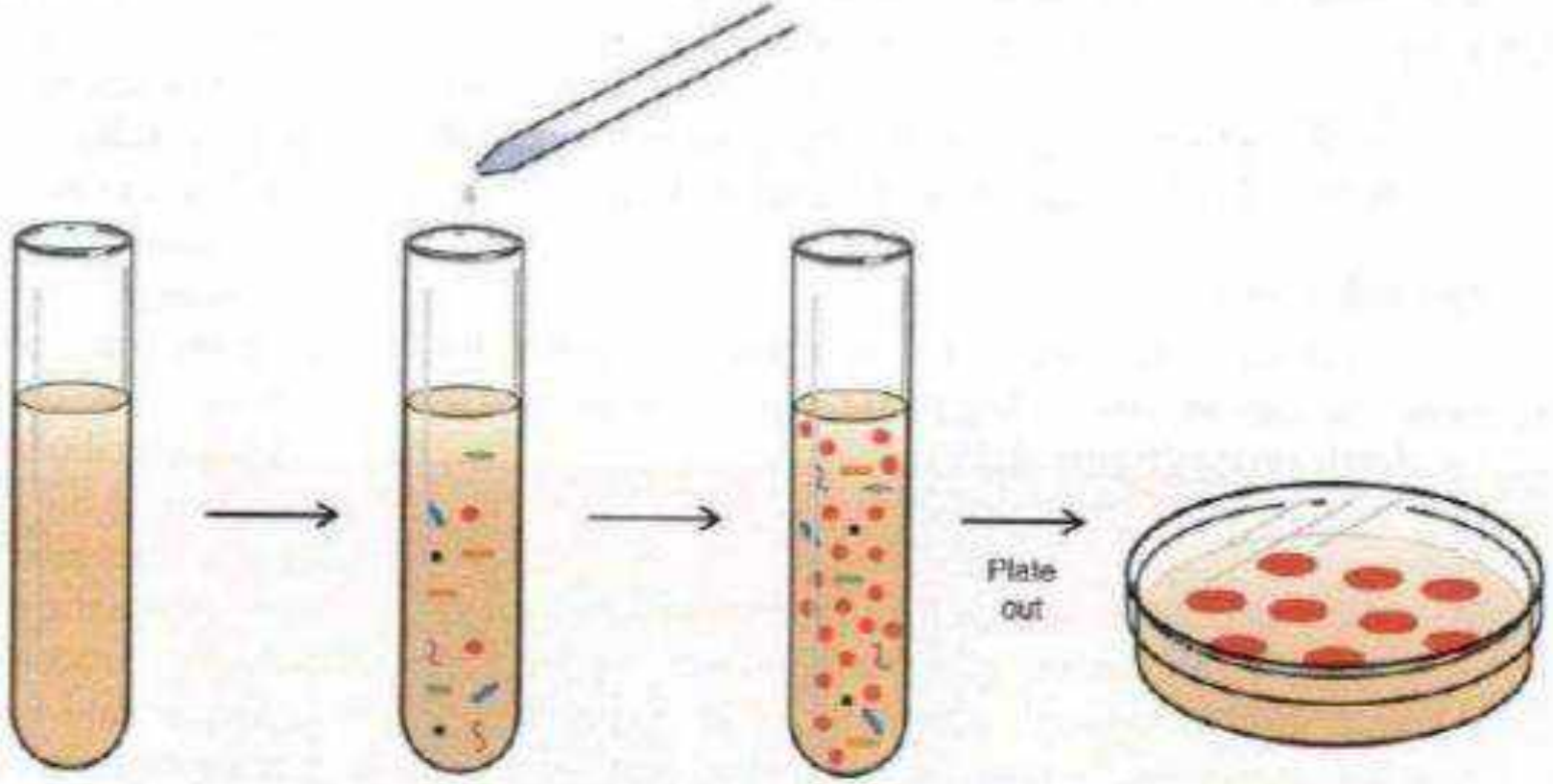
- The agar in first plate is then lifted and placed on the second plate without inverting.
- The growth factors produced on agar can diffuse into the lower layer containing test organism.
- The zones of stimulated growth of test organism around colonies is an indication that organism produce growth factor extracellularly.

4) ENRICHMENT CULTURE TECHNIQUE

- This was designed by Beijerinck to isolate the desired microorganism from heterogeneous microbial population.
- It consists of following steps :
  - a.) Nutrient broth is inoculated with microbial source material and incubated.
  - b.) A small portion of all inoculums is plated onto the solid medium and well isolated colonies are obtained.
  - c.) Suspected colonies from the plate are sub cultured on fresh media and subjected for further testing.

Enrichment cultures

Isolating an organism from natural sources



Medium contains select nutrient sources chosen because few bacteria, other than the organism of interest, can use them.

Sample that contains a wide variety of organisms, including the organism of interest, is added to the medium.

Organism of interest can multiply, whereas most others cannot.

Enriched sample is plated onto appropriate agar medium. A pure culture is obtained by selecting a single colony of the organism of interest.

SECONDARY SCREENING

It's a systematic screening programme intended to isolate industrially important or useful microorganisms .

SOME IMPORTANT POINTS ASSOCIATED WITH SECONDARY SCREENING ARE:-

- It is useful in sorting of microorganisms that have real commercial value. The microorganisms having poor applicability in fermentation process are discarded.
- Provides the information whether the product formed by microorganisms is new or not. This may be accomplished by paper , thin layer, chromatographic technique.

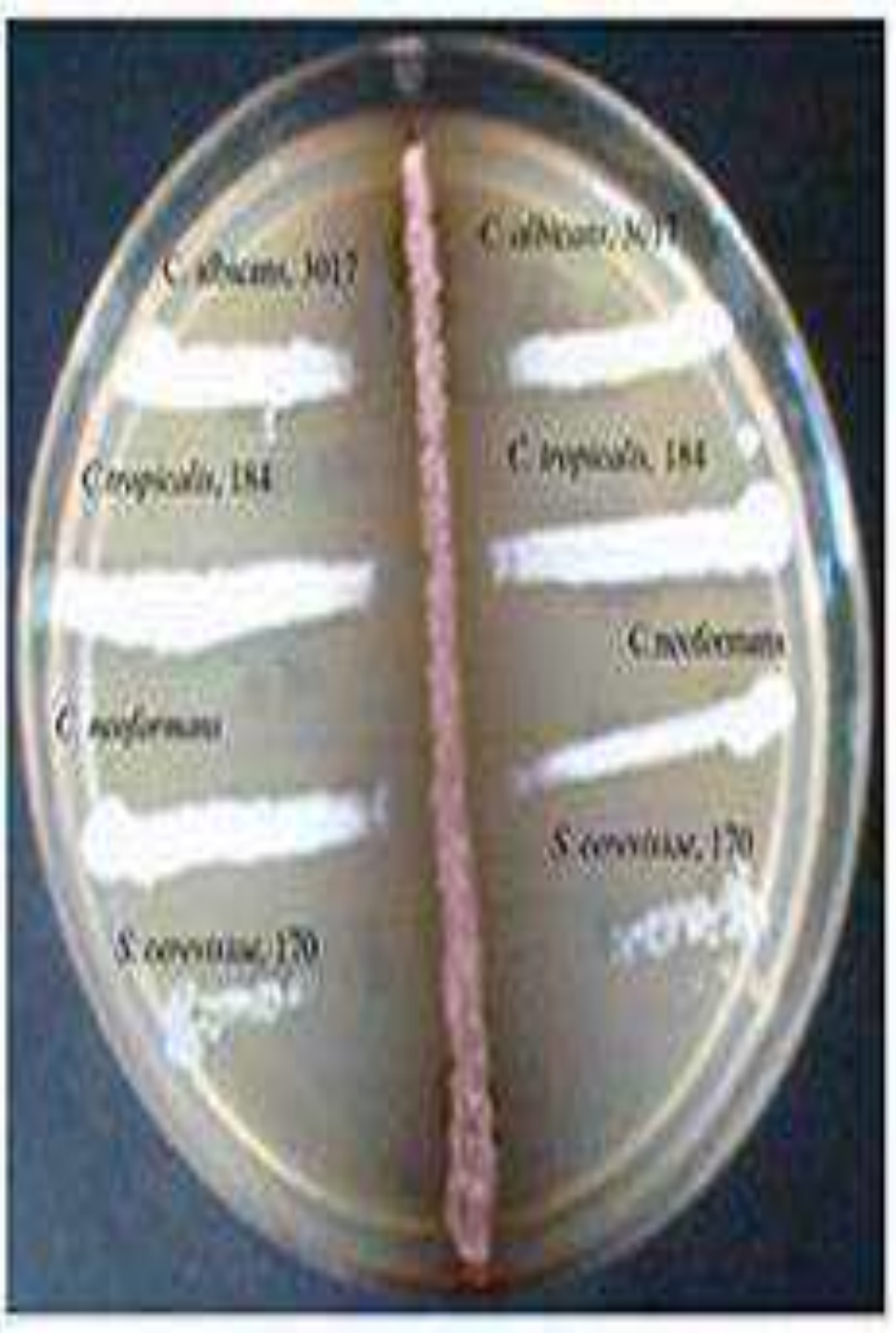
- It should show whether the product possess physical properties such as UV light absorption or fluorescence or chemical properties that can be employed to detect the compound during use of paper chromatography.
- It is conducted on agar plates, in flasks or in small fermentor containing liquid media.
- It gives an idea about the economic position of the fermentation process involving the use of a newly discovered culture.
- It helps in providing information regarding the product yield potentials of different isolates.
- It determines the optimum conditions for growth or accumulation of a product associated with a particular culture.

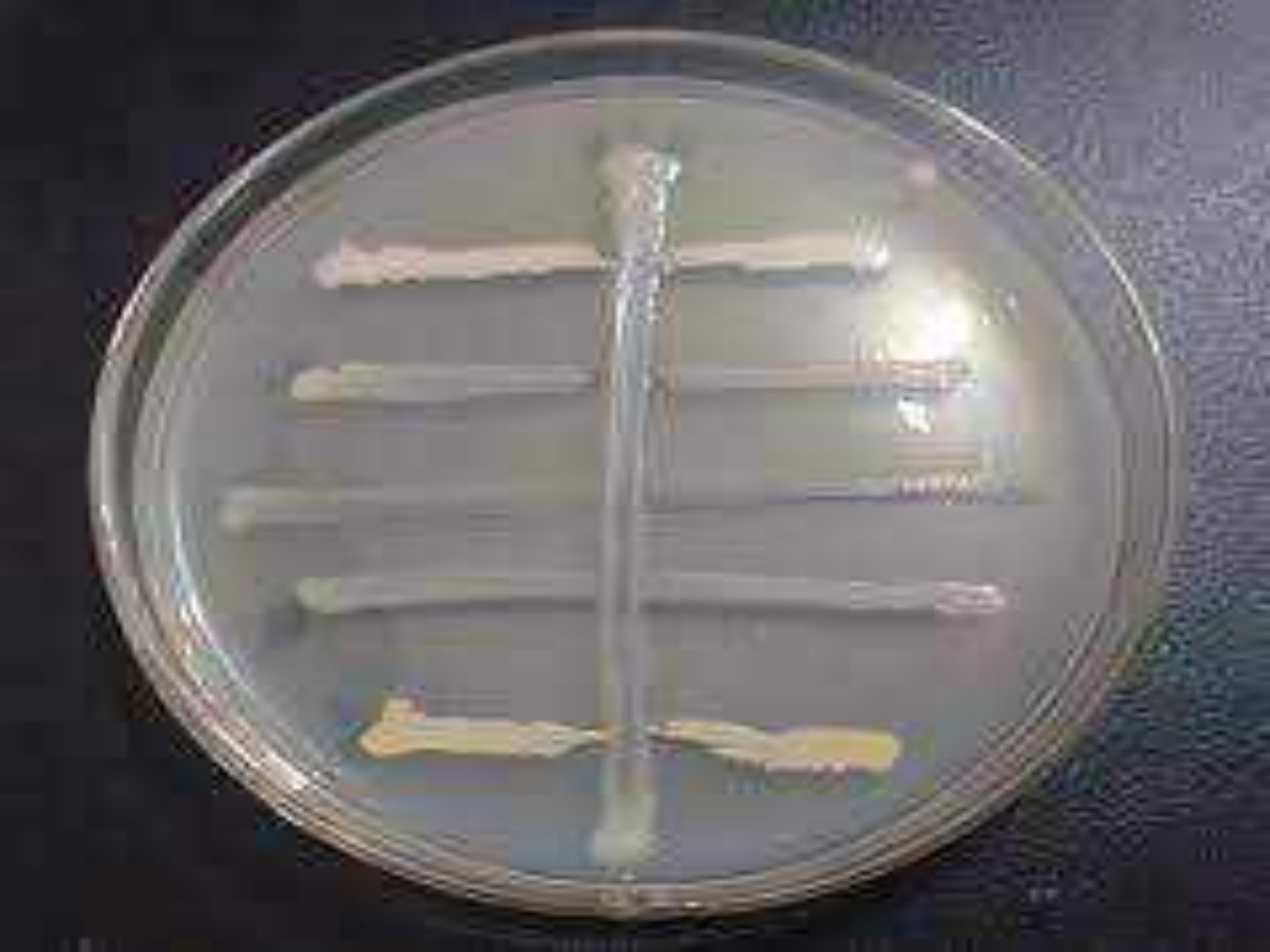
- Chemical, physical and biological properties of a product are also determined during secondary screening. Moreover, it reveals whether a product produced in the culture broth occurs in more than one chemical form.
- It detects gross genetic instability in microbial cultures. This type of information is very important, since microorganisms tending to undergo mutation or alteration is some way may lose their capability for maximum accumulation of the fermentation products.
- It tells about the chemical stability of the fermentation product.
- It can be qualitative or quantitative in its approach.

EXAMPLE OF SECONDARY SCREENING – ANTIBIOTIC PRODUCNING STREPTOMYCES SPECIES

1. Streptomyces isolates are streaked as a narrow band on nutrient agar plates are incubated .
2. Test organisms are then streaked from the edge of plates without touching streptomyceal isolate and then the plates are then incubated .
3. At the end of incubation, growth inhibitory zones for each organism are measured in millimeters .
4. Such organisms are again subjected for further testing by growing the culture in sterilized liquid media and incubated at constant temperature in a mechanical shaker.


S. aureus, 96
E. coli, 1304
P. aeruginosa, 741


C. albicans, 3017
C. tropicalis, 184
C. neoformans
S. cerevisiae, 170



Fermentor

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Fermentor

Design,Parts and Their Function

- De Becze and Liebmann (1944) used the first large scale fermentor (above 20 lit capacity) for a production of yeast.
- British scientist – Cain Weizmann developed a fermentor for the production of acetone during first world war.
- The first pilot plant fermentor for penicillin production was erected in India at HAL,Pune in 1950.

BASIC DESIGN OF A FERMENTOR

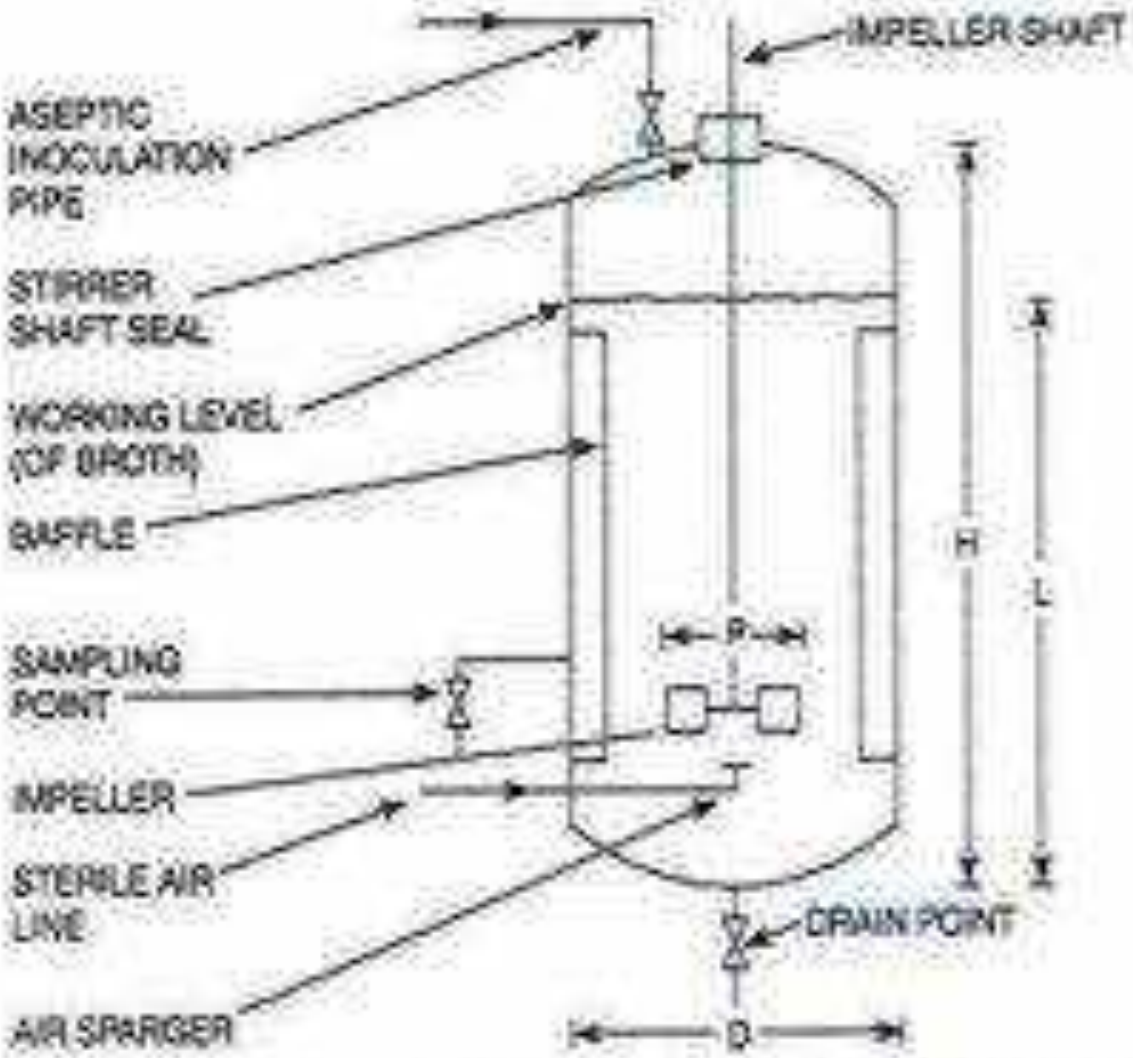
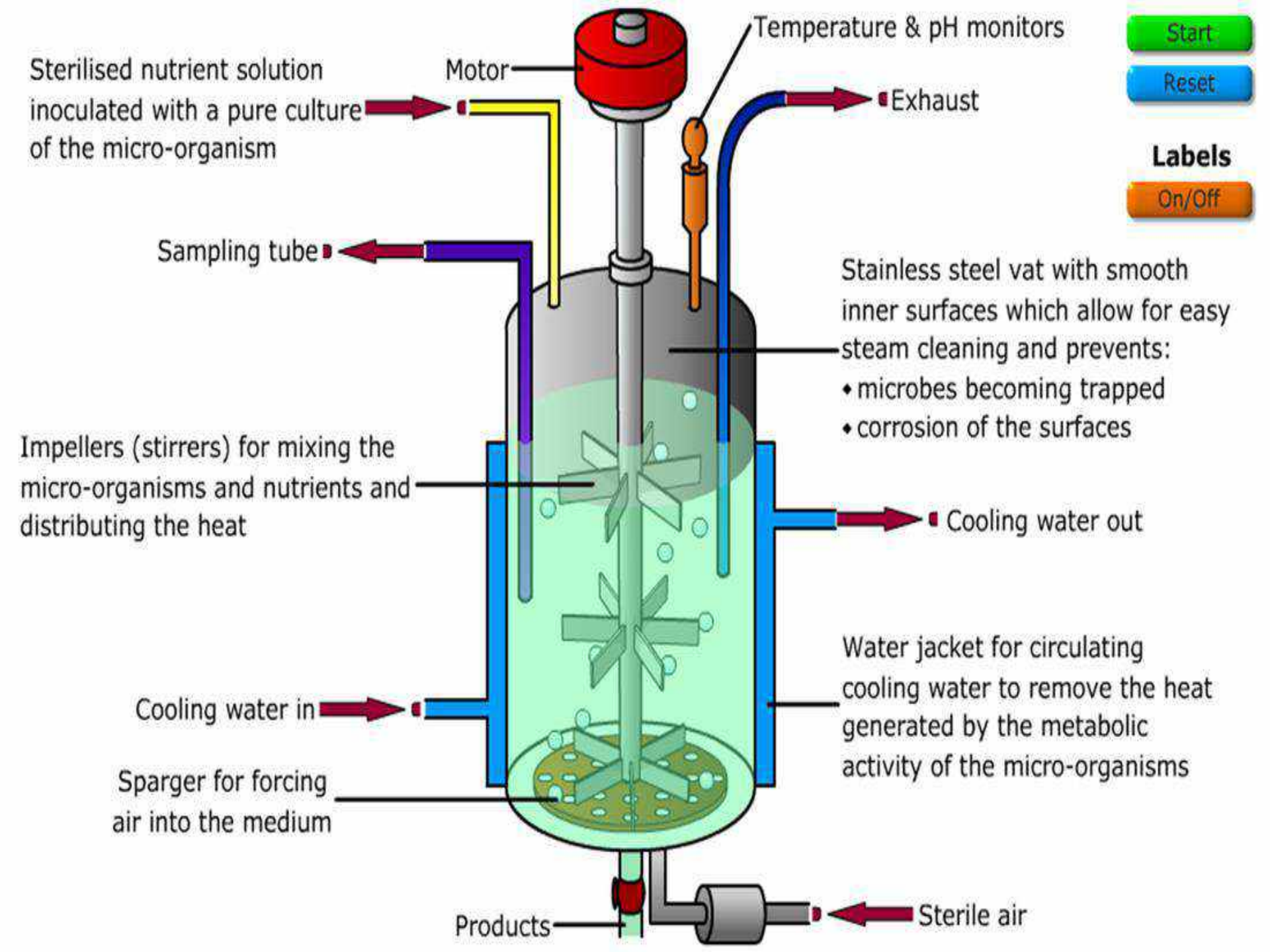


FIG. 14.3. Diagram of a fermenter with one multi-bladed impeller. H, fermenter height; L, liquid height; D, tank diameter; P, impeller diameter.


Start
Reset
Labels
On/Off
Temperature & pH monitors
Motor
Exhaust
Sterilised nutrient solution inoculated with a pure culture of the micro-organism
Sampling tube
Stainless steel vat with smooth inner surfaces which allow for easy steam cleaning and prevents:
• microbes becoming trapped
• corrosion of the surfaces
Impellers (stirrers) for mixing the micro-organisms and nutrients and distributing the heat
Cooling water out
Water jacket for circulating cooling water to remove the heat generated by the metabolic activity of the micro-organisms
Cooling water in
Sparger for forcing air into the medium
Products
Sterile air

IDEAL FERMENTOR PROPERTIES

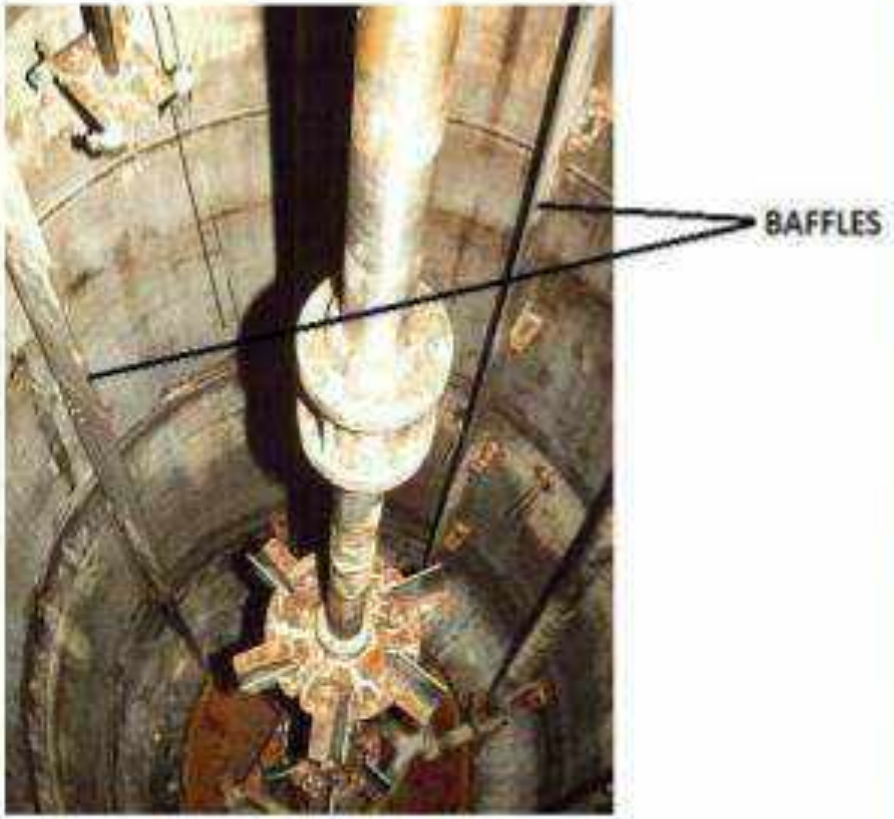
- Supports maximum growth of the organism
- Aseptical operation
- Adequate aeration and agitation
- Low power consuming
- Tempurature control system
- pH control system
- Sampling facilities

- Provision for control of contaminants
- Provision for intermittent addition of antifoams
- Inoculum introduction facility
- Mechanism for biomass/ product removal
- Setting for rapid incorporation of sterile air
- Withstands pressure
- Ease of manipulation

- Minimum evaporation loss
- Minimum use of labour
- Range of processes
- Smooth internal surfaces
- Similar in geometry to both smaller & larger vessels in pilot plant
- Cheapest material usuage
- Adequate service provisions

BAFFLES

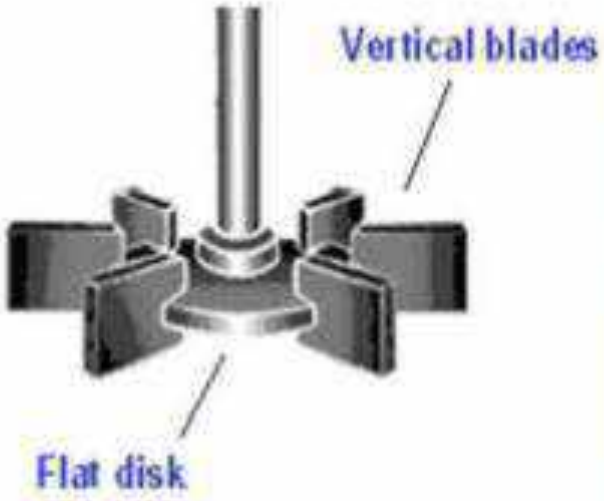
- To minimize fluid swirling and vortex formation
- Metal strips roughly 1/10th vessel diameter and attached radially to the wall
- 6-8 baffles are used in industrial scale bioreactors.
- Baffling tends to increase transmittable power, to improve mixing and aeration



AGITATOR (IMPELLER)

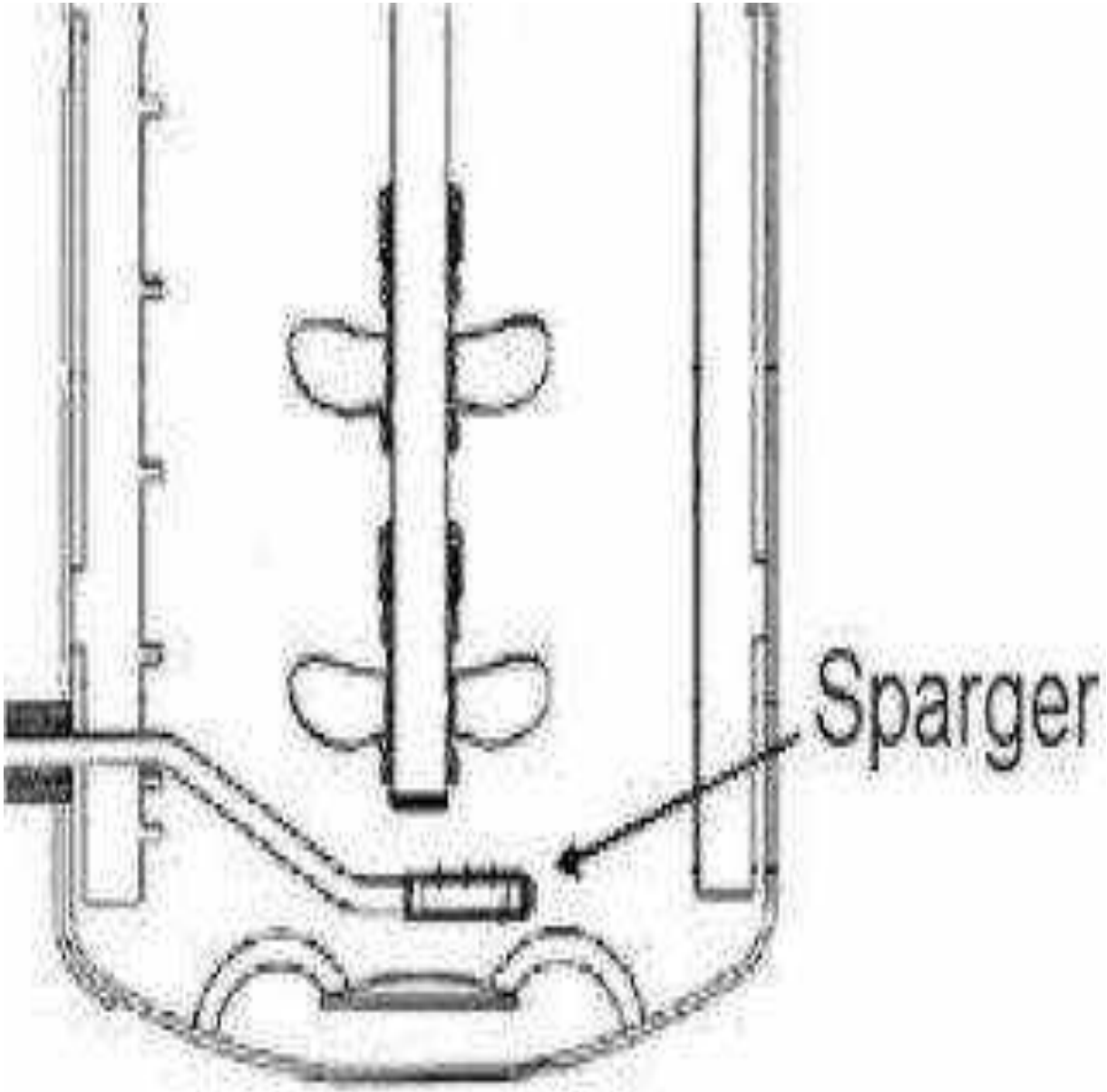
- Mounted on the shaft at a specific distance off the tank bottom
- Bulk fluid and gas phase mixing
- Oxygen transfer
- Heat transfer
- Suspension of solid particles
- Maintain a uniform environment throughout the vessel contents

Rushton turbine of 1/3rd the bioreactor diameter is considered the optimum design for use in many fermentation processes.



AERATION SYSTEM

- Syn : sparger
- A device that introduce air into medium
- Has a pipe with minute holes (1/64 - 1/32 inch or large)
- Hole – allows air under P to escape into medium
- For mycelial growth – ¼ inch holes
- Impeller blades disperses air released through sparger into medium


Sparger

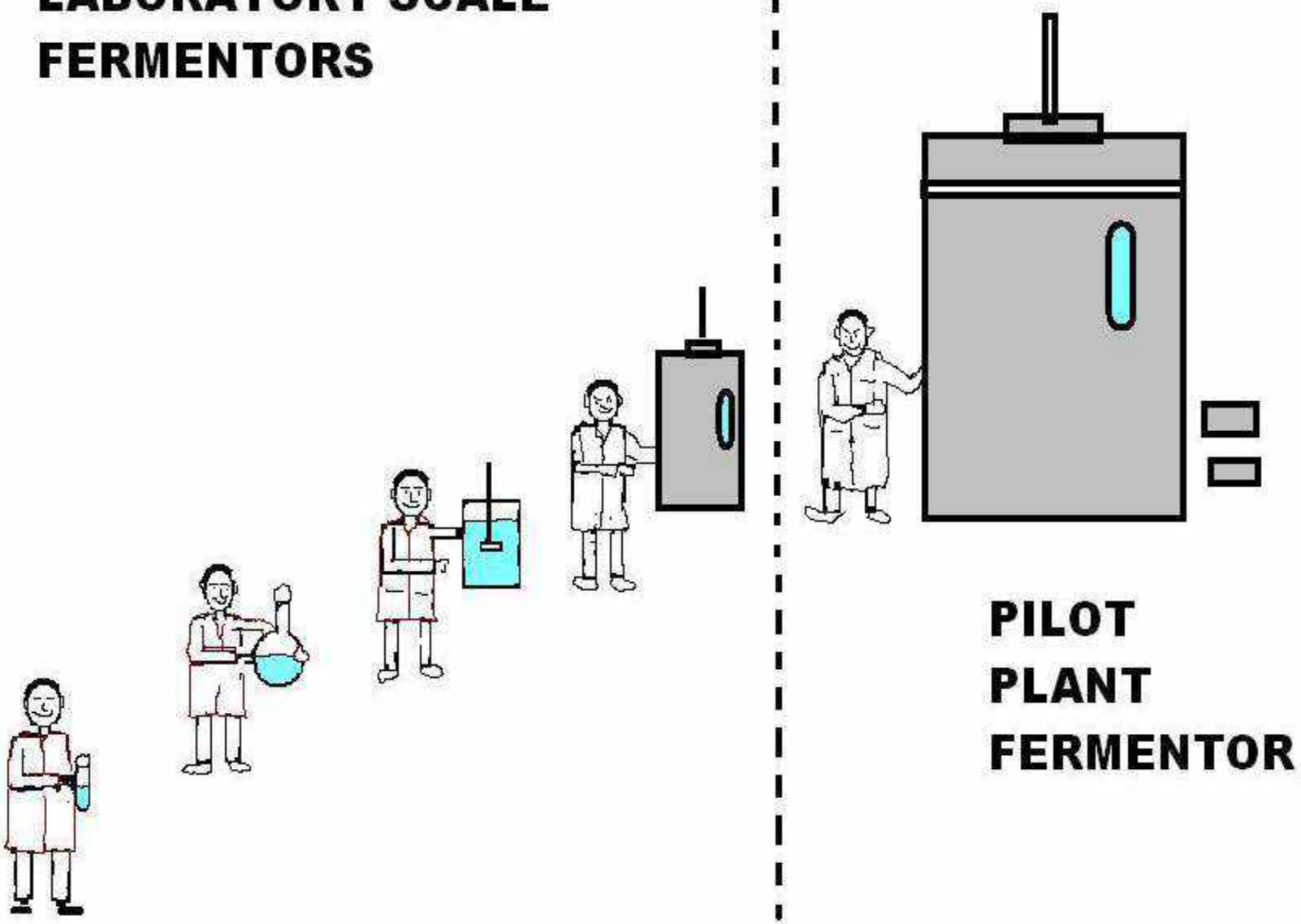
Types of fermentor

1. Small laboratory fermentor –
   Capacity- 1-2 lit maximum 12-15 lit
   Use- In research and development of fermentation process.

2. Pilot scale fermentor-
   Capacity -100-500 lit and maximum upto 10000 lit
   Use- To optimize the fermentation conditions.

3. Large scale or Industrial fermentor-
   Capacity- 20000-50000 liters
   Use - for Actual production .

LABORATORY SCALE FERMENTORS



PILOT PLANT FERMENTOR

MONOCLONAL ANTIBODIES

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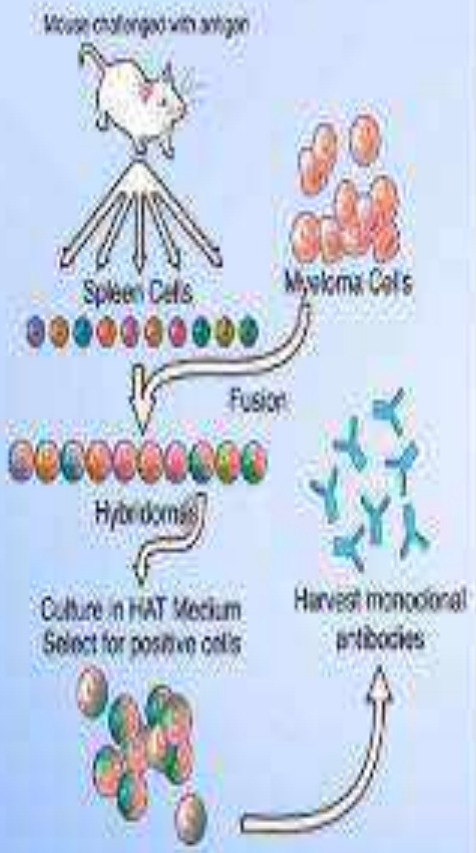
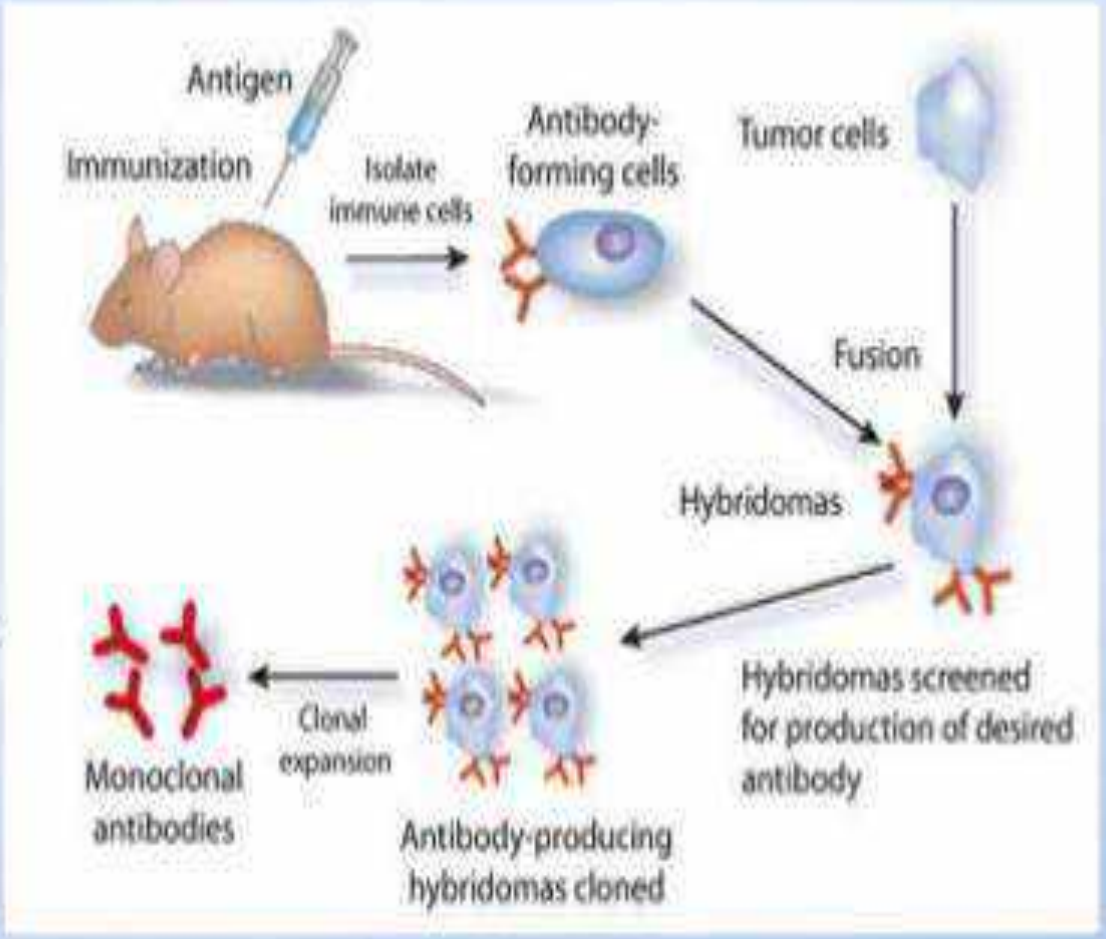




Preparation of Monoclonal Antibodies

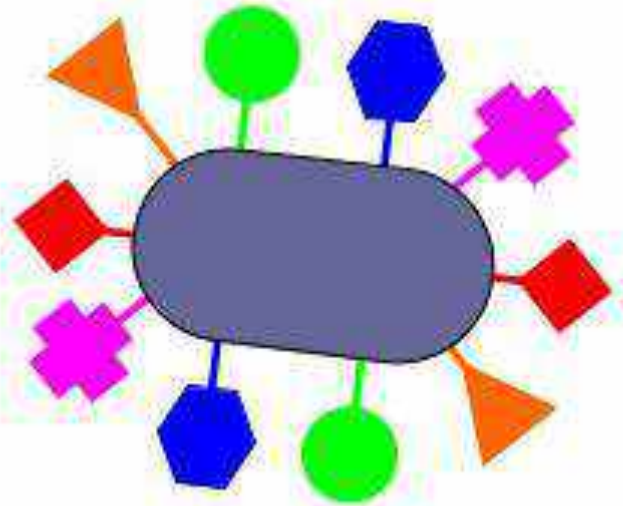
	Polyclonal antibodies	Monoclonal Antibodies
Produced by:	Many B cell clones	A single B cell clone
Bind to:	Multiple epitopes of all antigens used in the immunization	A single epitope of a single antigen
Antibody class:	A mixture of different Ab classes (isotypes)	All of a single Ab class
Ag-binding sites:	A mixture of Abs with different antigen-binding sites	All Abs have the same anti binding site
Potential for cross-reactivity:	High	Low

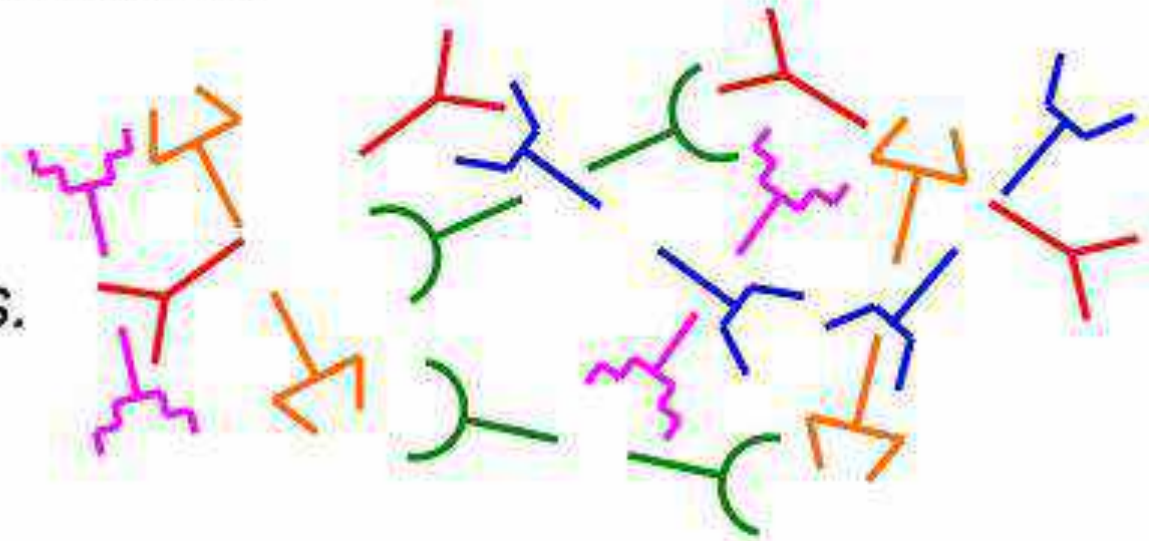
Most microbes have more than one antigen on their surface, so…

…they stimulate more than one type of lymphocyte…

…resulting in the production of *many different* antibodies.

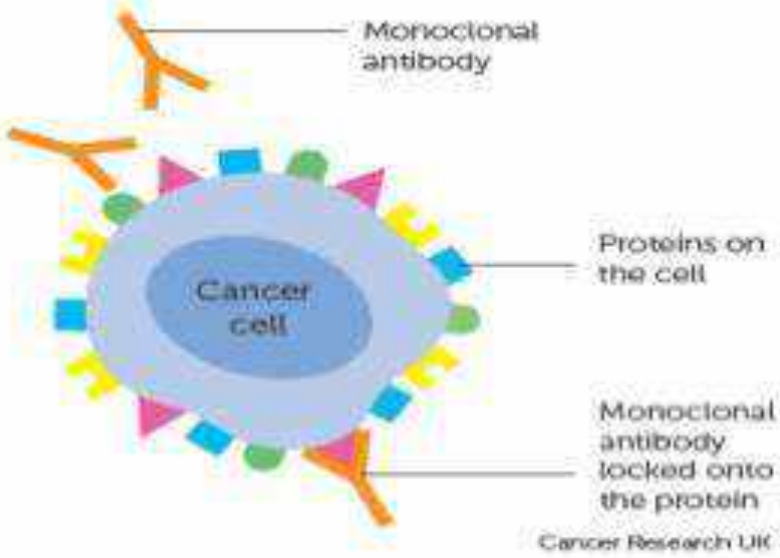
These are called *polyclonal antibodies.*



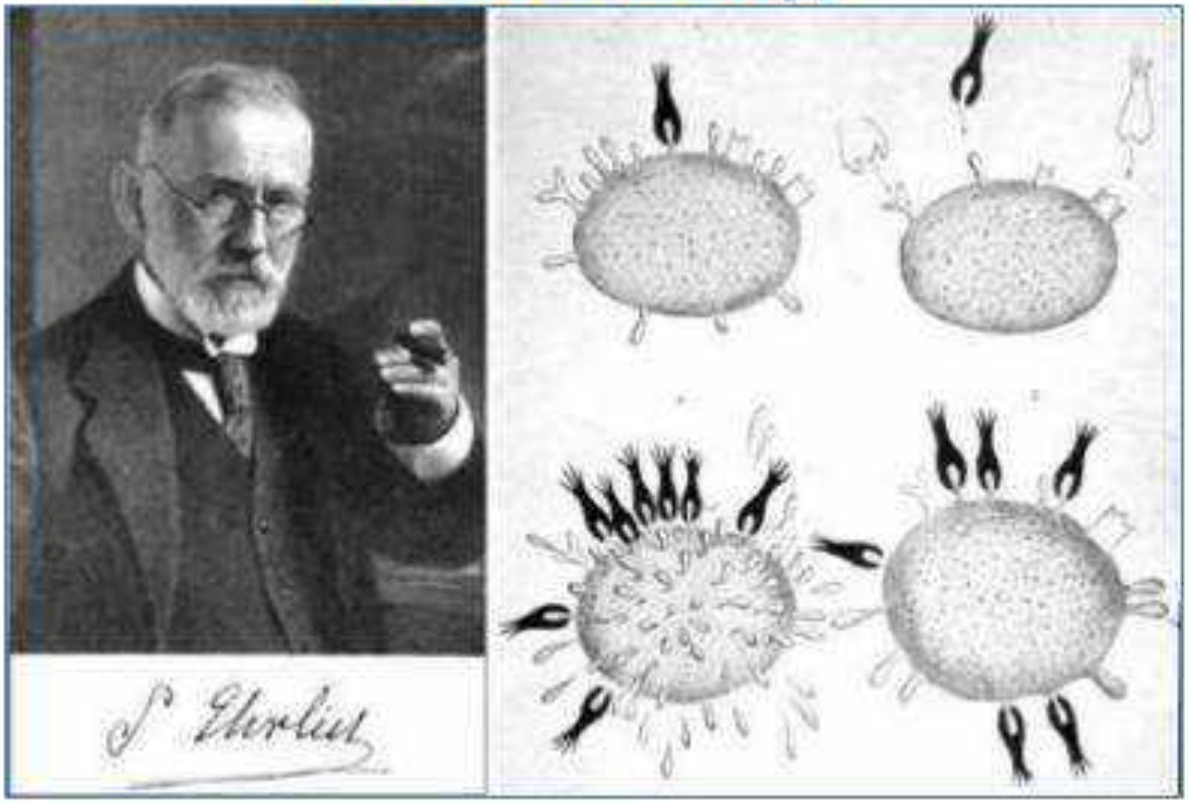


MONOCLONAL ANTIBODIES

- These are homogenous preparations of antibodies (or fragments of antibodies) in which every antibody in the product is identical in its protein sequence, and thus every antibody is expected to have the same antigen recognition site, affinity, biologic interactions, and downstream biologic effects.



Discovery



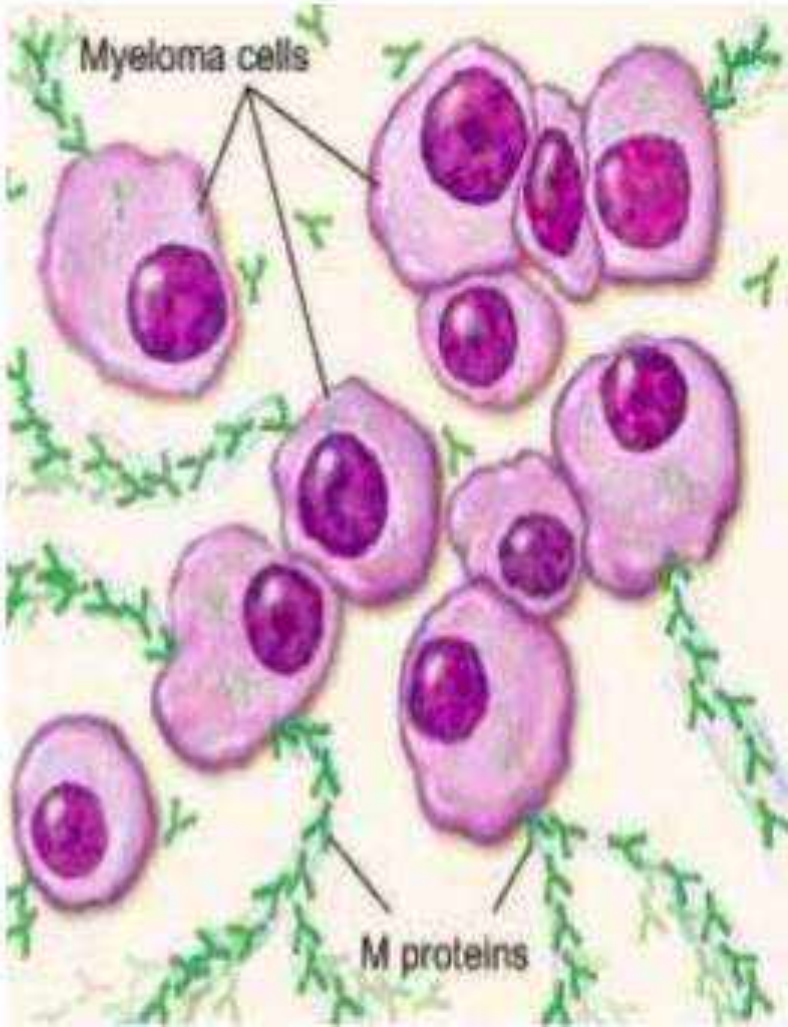
The idea of a "magic bullet" was first proposed by Paul Ehrlich, who, at the beginning of the 20th century, postulated that, a compound can be made that selectively targeted a disease-causing agent.

Monoclonal Antibodies: History and Development

- **Paul Enrlich** at the beginning of 20th century coined the term "magic bullets" and postulated that, if a compound could be made that selectively targets a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity.
- In the 1970s, the **B-cell cancer multiple myeloma** was known. It was understood that these cancerous B-cells all produce a single type of antibody (a paraprotein).

In the 1970s, the B-cell cancer - multiple myeloma was known.

- It was understood that these cancerous B-cells all produce a single type of antibody (a paraprotein).
- This was used to study the structure of antibodies, but it was not yet possible to produce identical antibodies specific to a given antigen



Niels K. Jerne

Georges J.F. Köhler

César Milstein

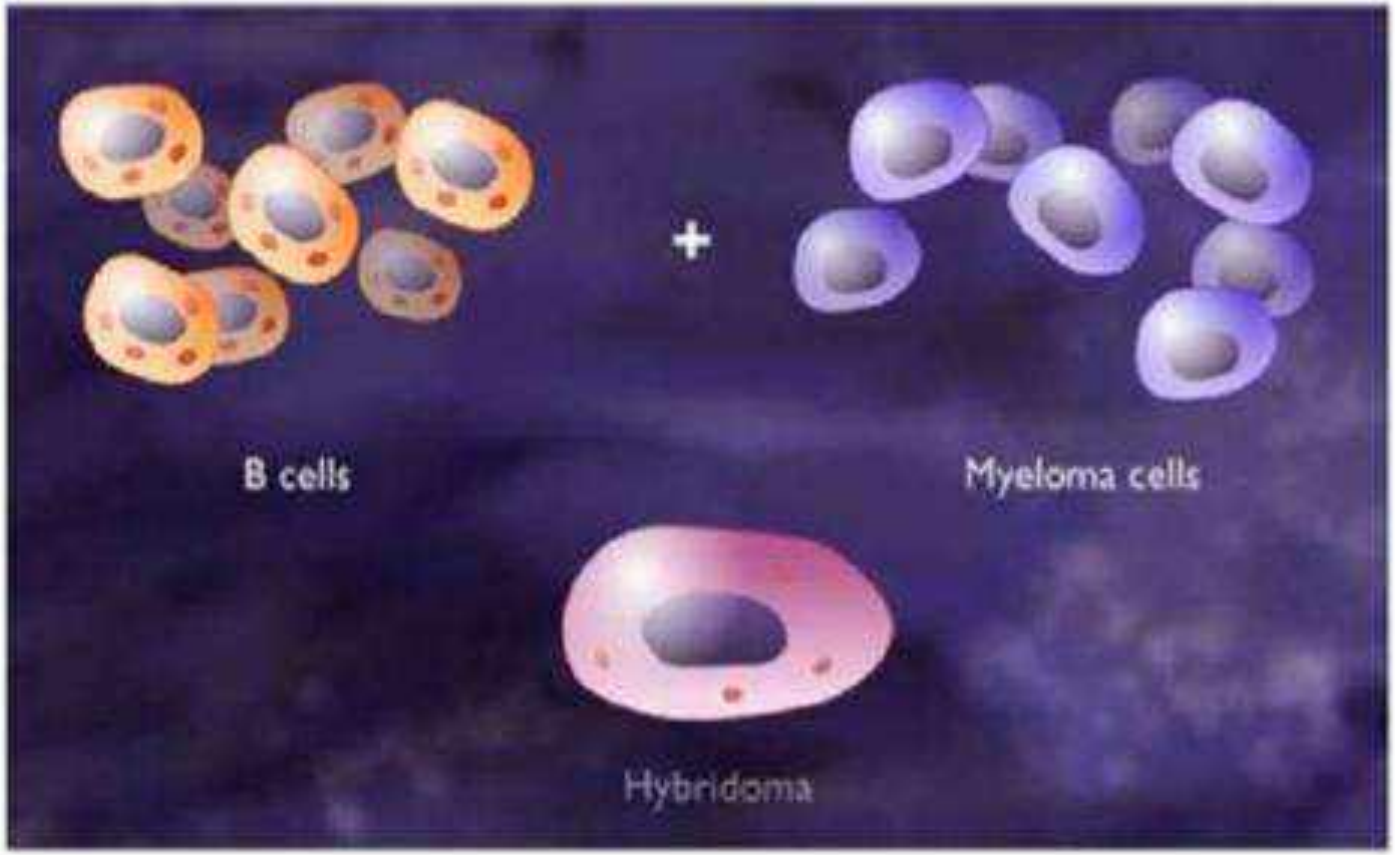
Somatic cell hybridization - 1975

- In 1975, **Kohler and Milstein** provided the most outstanding proof of the clonal selection theory by fusion of normal and malignant cells (Hybridoma technology) for which they received Nobel prize in 1984.
- In 1986, first monoclonal antibody was licenced by FDA. Orthoclone OKT3 (muromonab-CD3) which was approved for use in preventing kidney transplant rejection.

Georges J.F. Köhler

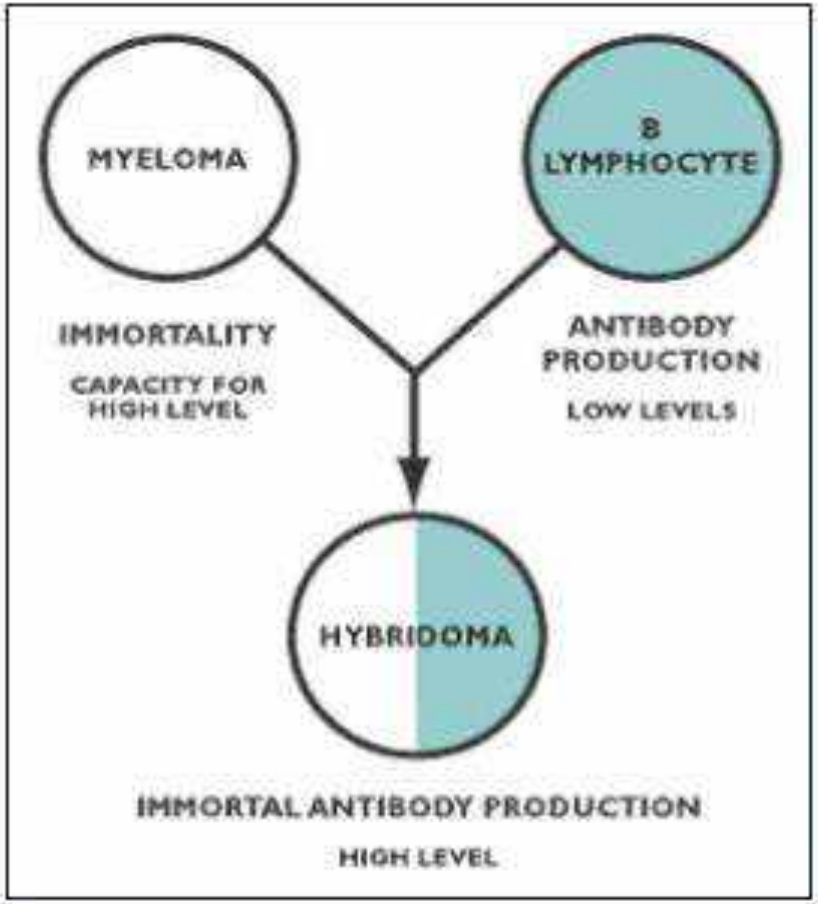
César Milstein

- The advent of hybridoma technology by Kohler and Milstein made it possible to produce large quantities of antibodies with high purity and monospecificity for a single binding region (epitope) on an antigen .



Production of monoclonal antibodies

- By HYBRIDOMA TECHNIQUE



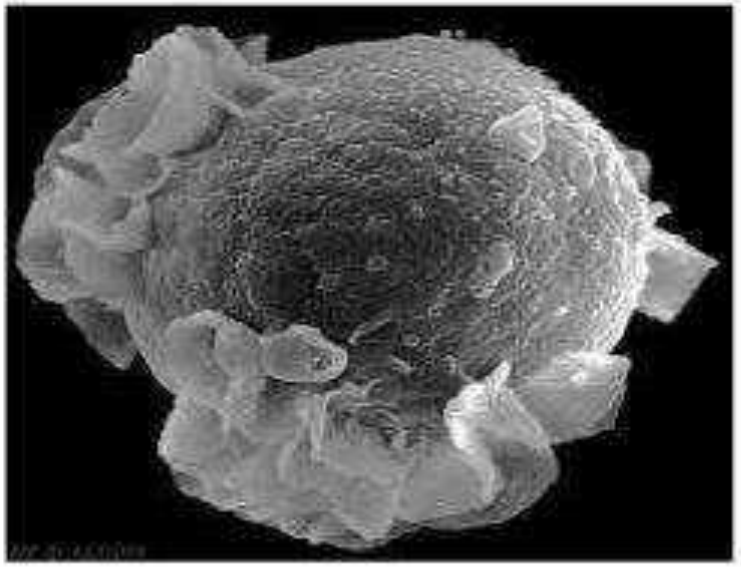
MYELOMA CELL

- These cells do not have th eenzyme hypoxanthine guanine-phosphoribosyl transferase (HGPRT).
- This enzyme enables cells to synthesize purines using an extracellular source of **hypoxanthine** as a precursor.
- Ordinarily, the absence of HGPRT is not a problem for the cell because cells have an alternate (**de novo**)pathway that they can use to synthesize purines.
- However, when cells are exposed to **aminopterin (a folic acid analog)**, they are unable to use de novo pathway and are now fully dependent on HGPRT for survival.

B cell

Bcell has the enzyme **HGPRT**
But B cells die soon

- They do not have the capacity to grow indefinitely because of their **limited life span**



Scanning Electron Microscopic view of a Bcell

THE PROCEDURE

CELLS FUSED :

- Spleen cells from a mouse that has been immunized with the desired antigen
- Myeloma cells.

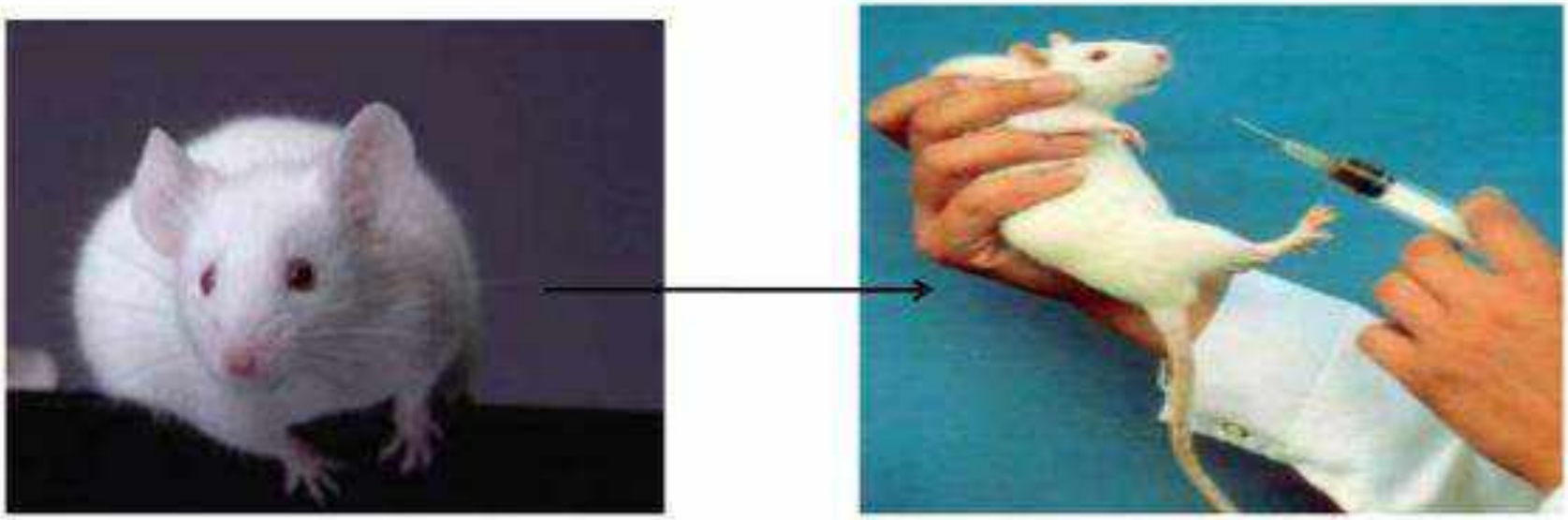
FUSION AGENT:

- Polyethylene glycol

MEDIUM:

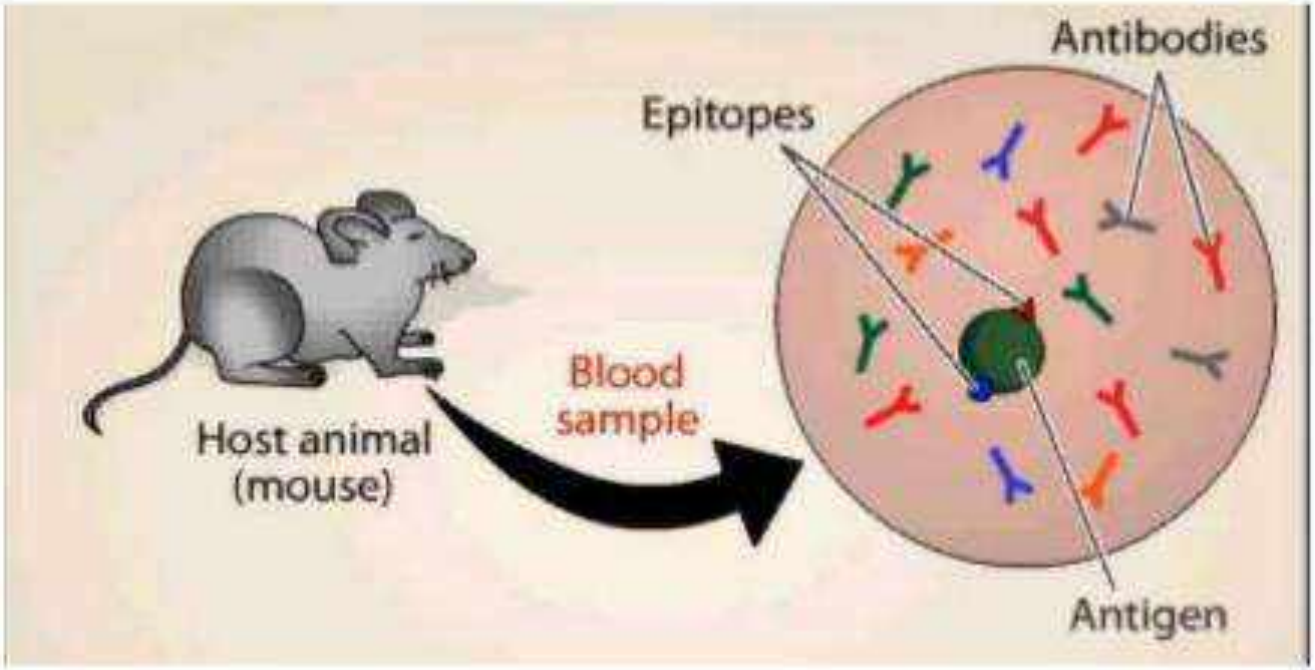
- HAT Medium {Hypoxathine-Aminopterin- Thymidine}

Step 1. Immunization of mouse



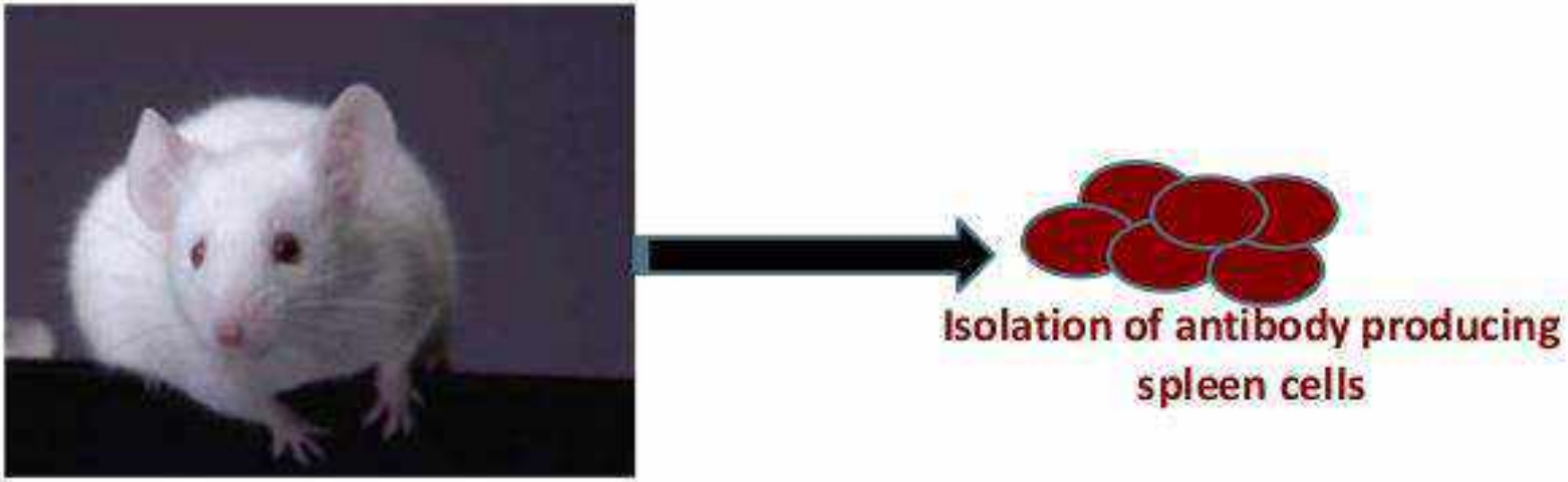
Mice are immunized every 2-3 weeks with an antigen that is prepared for injection

Step 2: Screening of Mice for Antibody Production



- Blood samples are obtained from mice for measurement of serum antibodies whose titer is determined with various techniques, such as enzyme-linked immunosorbent assay (ELISA) and flow cytometry.

Step 3 : Isolation of antibody producing spleen cells

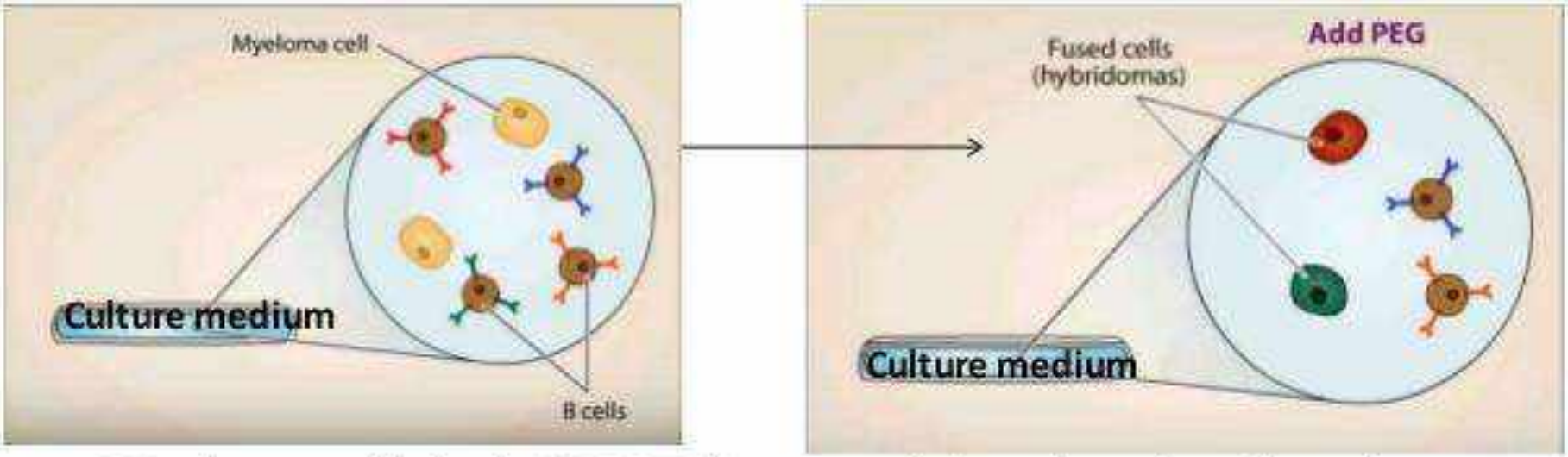


•When the antibody titer is high enough, mice are commonly boosted by injecting antigen without adjuvant intra peritoneally or intravenously (via the tail veins) 3 days before fusion but 2 weeks after the previous immunization.

•If the titer is too low, mice can be boosted until an adequate response is achieved, as determined by repeated blood sampling.

•Then the mice are euthanized and their spleens removed for in vitro hybridoma cell production.

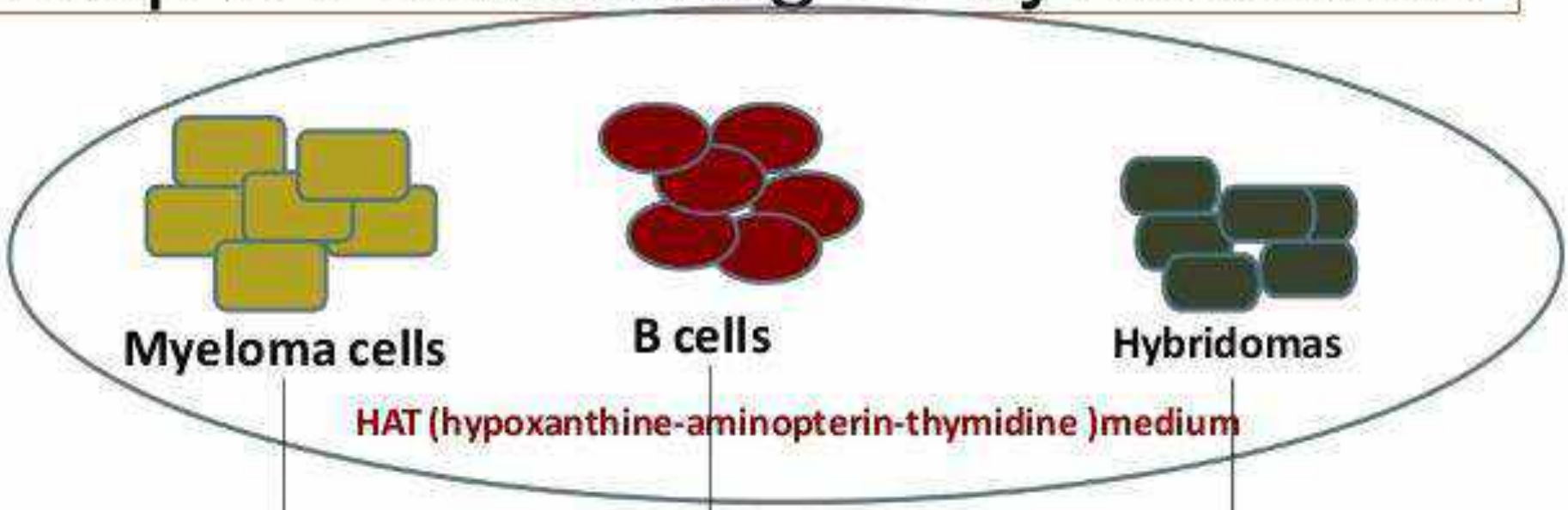
Step 4 : Production of hybridomas



- Myeloma cells lack HPGRT (hypoxanthine phospho ribosyl transferase) enzyme,which is responsible for synthesis of nucleotides.

- The cells are then screened in HAT (hypoxanthine-aminopterin-thymidine) medium which blocks the pathway for nucleotide synthesis.

Step 5 : Screening of hybridomas



Nucleotide synthesis pathway blocked

Nucleotide synthesis pathway blocked

Nucleotide synthesis pathway blocked

Nucleotide synthesis pathway blocked

HPGRT gene present

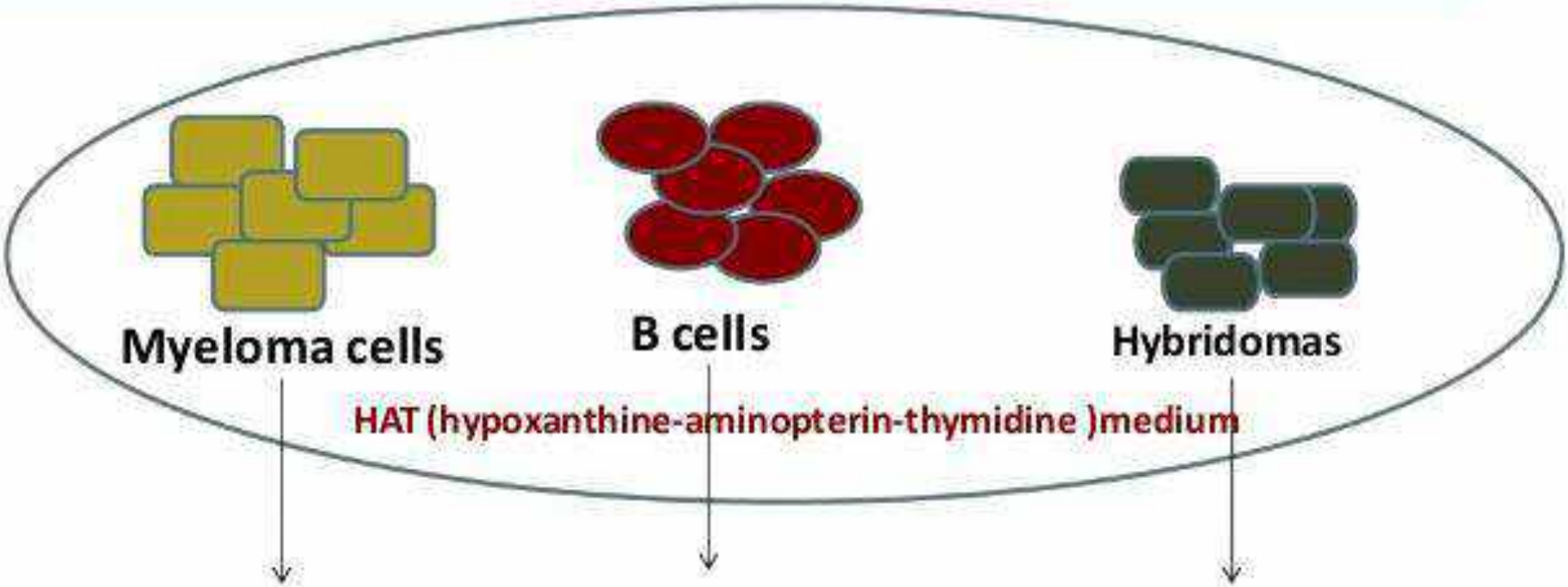
HPGRT gene present

Nucleotides fail to get synthesized

Nucleotides get synthesized

Nucleotides get synthesized

Step 5 : Screening of hybridomas

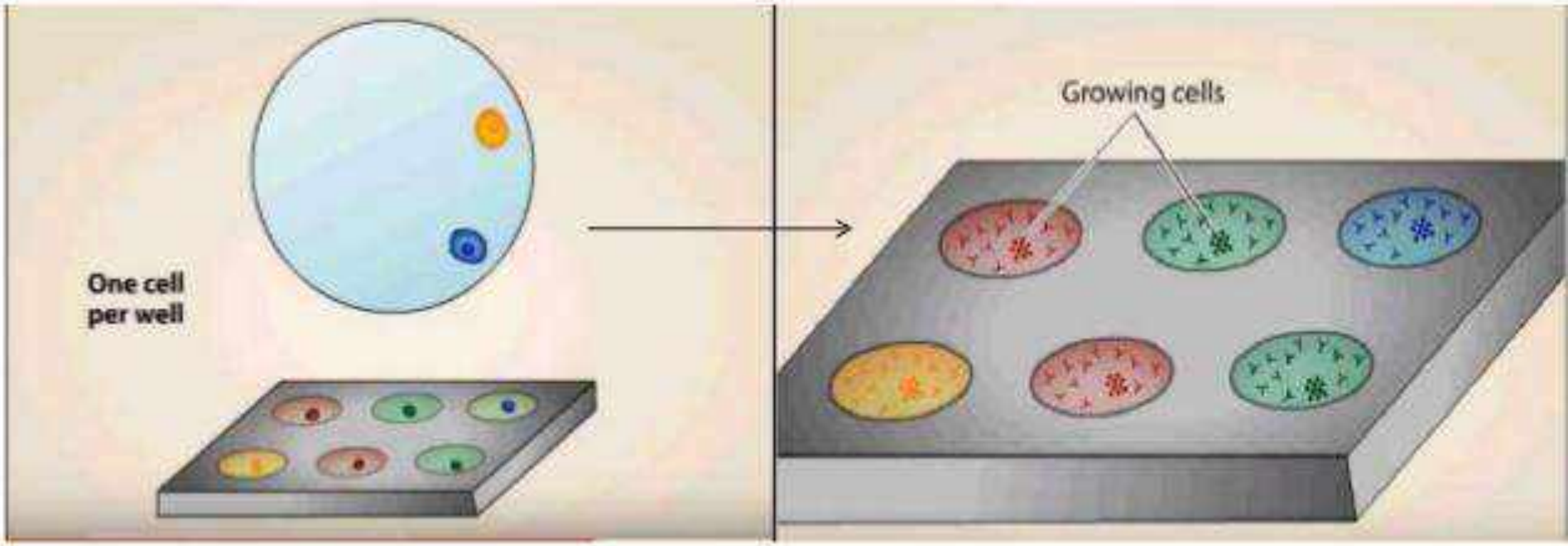


Cells die due to lack of nucleotide synthesis

Cells die due to short life span

Cells survive

Step 6 : Culturing Hybridoma Cells – Monoclones Production

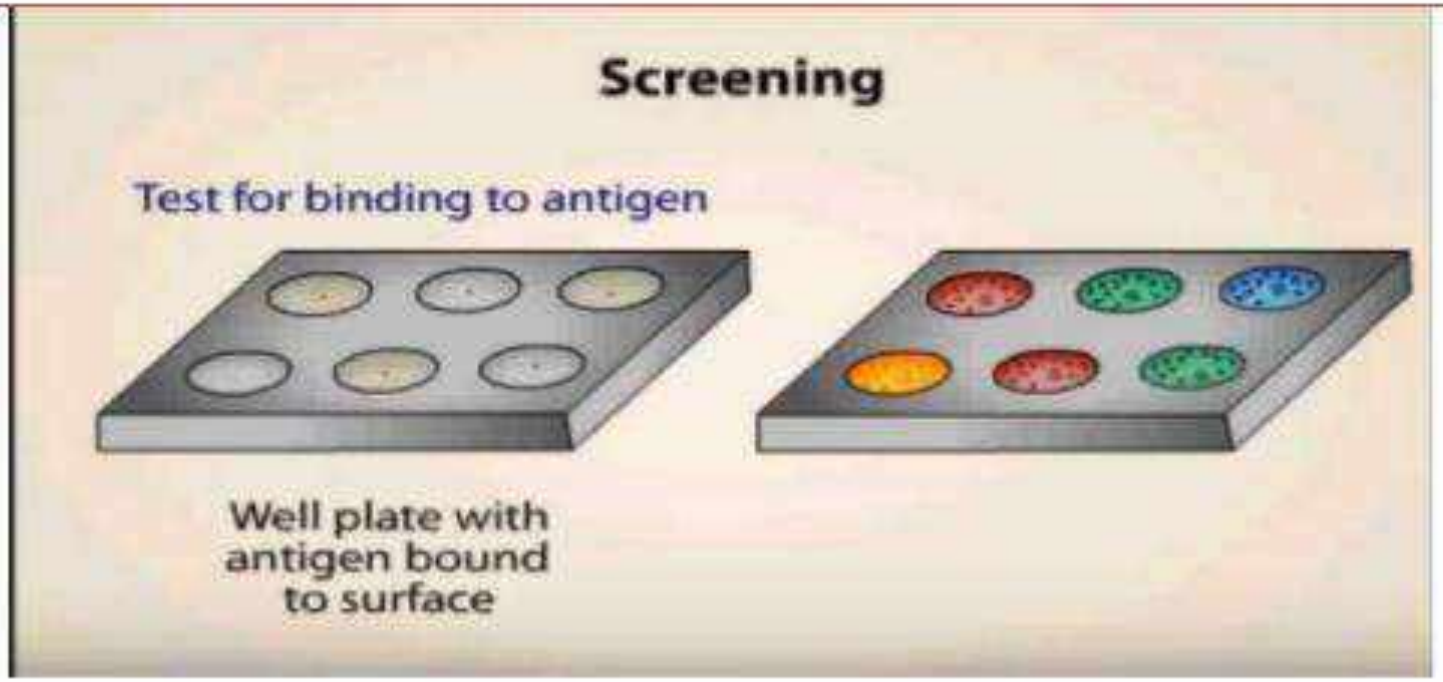


Hybridomas are separated and individually cultured : 1 cell per well

These cells are called as clonal culture. Because each cell in the well is derived from singe cell and are therefore identical.

After few weeks, when growing cultures can be seen, further screening can be done for desired antibody.

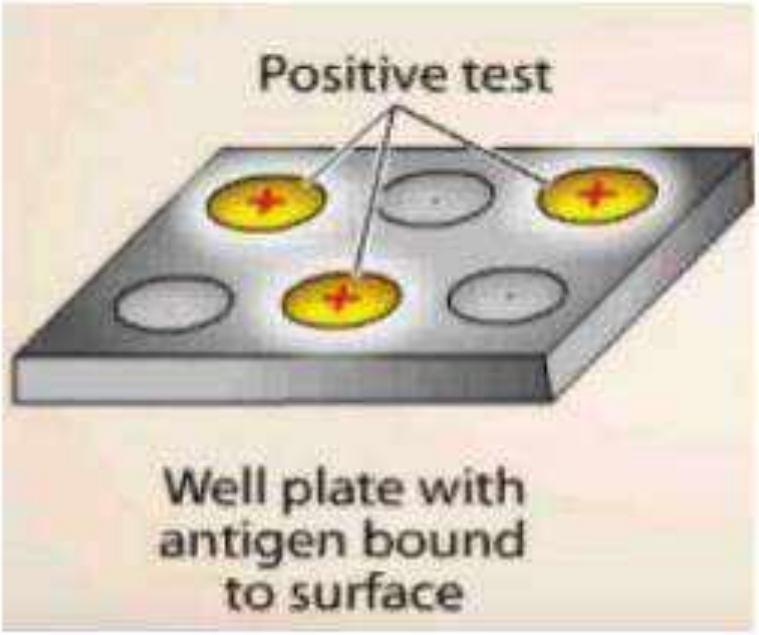
Step 7 : Screening for desired antibodies

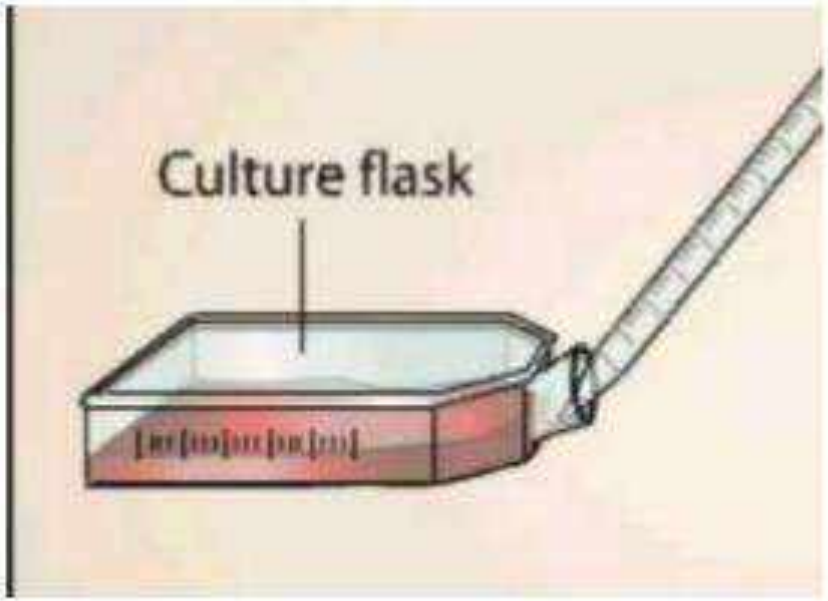


- Antigens are immobilized in the wells and the antibodies are transferred (one per well) so that they bind to the complementary antigen.

- Different antibodies react to different epitopes on the same antigen

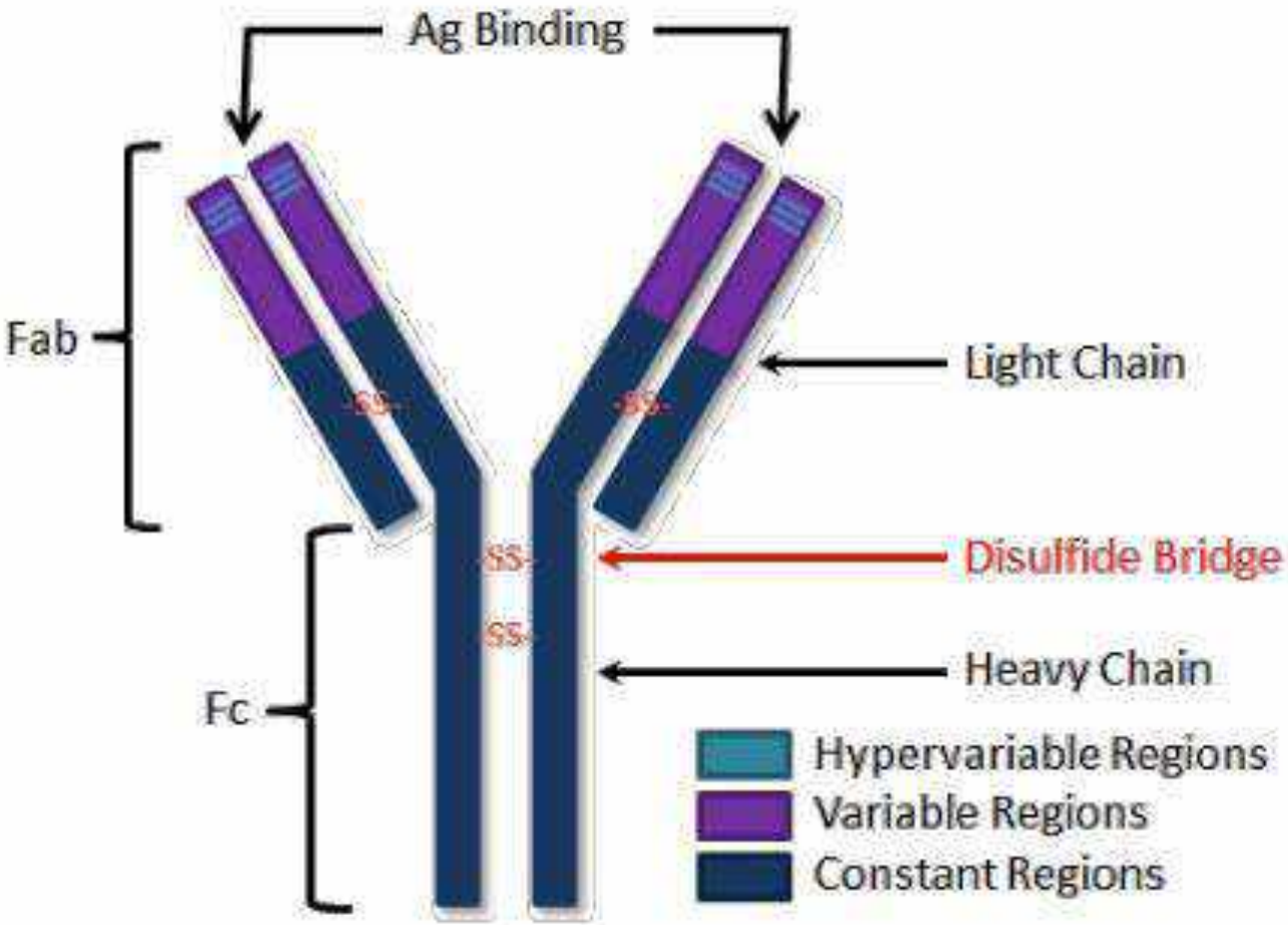
Step 8 : Selection and culture of screened antibodies



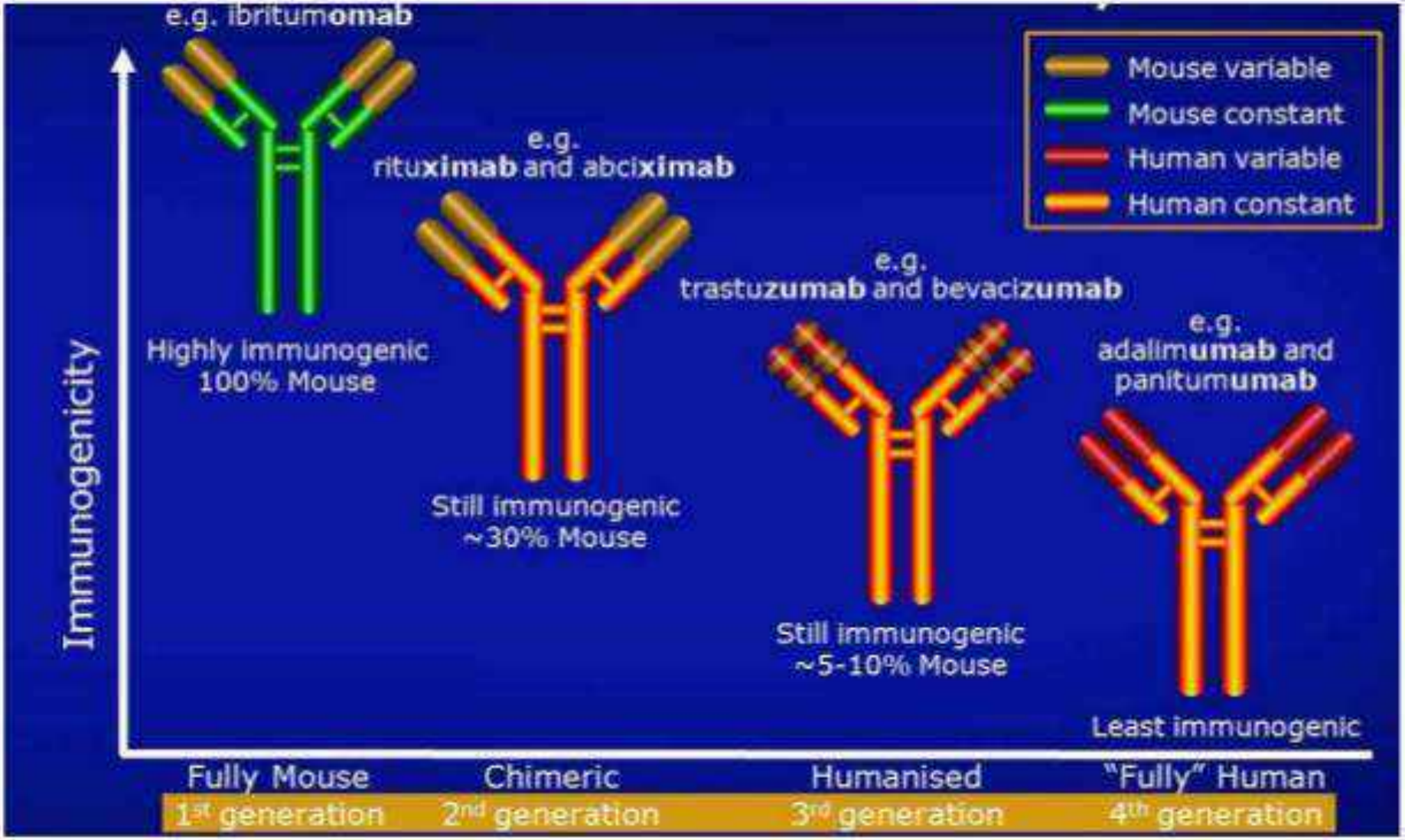


Finally, the desired antibodies are grown in mass culture and are frozen for storage.

Immunoglobulin-G (IgG) molecules are most commonly employed as the working backbones of current therapeutic monoclonal antibodies,



EVOLUTION OF MONOCLONAL ANTIBODIES

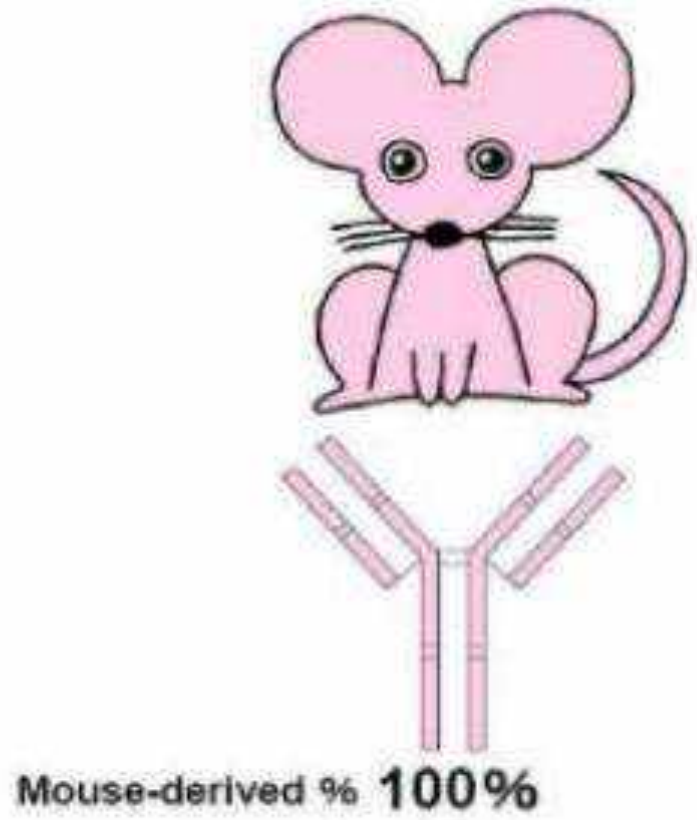


Murine antibody

- Whole of the antibody is of murine origin

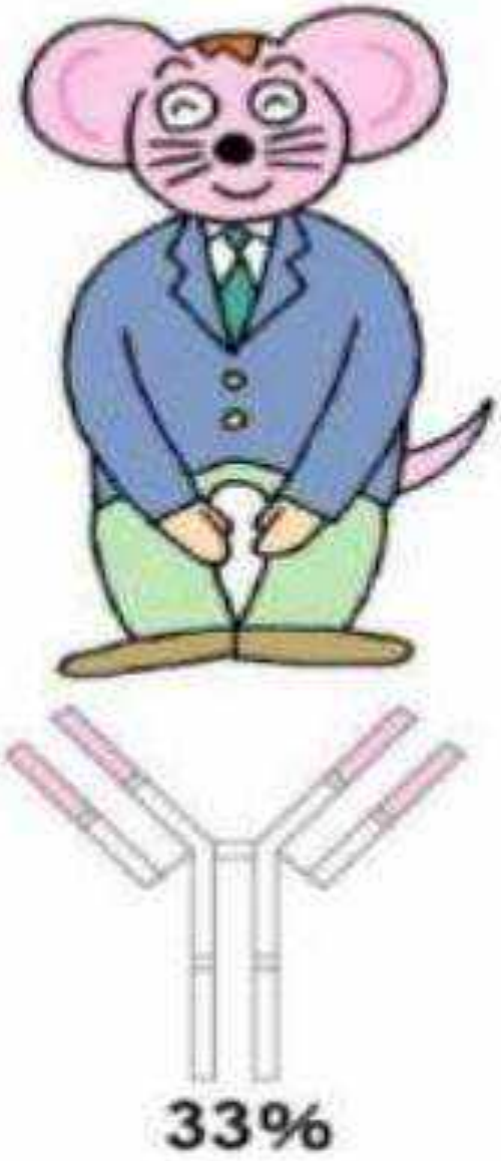
Major problems associated with murine antibodies include

- Reduced stimulation of cytotoxicity
- Allergic reactions
- Anaphylactic shock



Chimeric Antibodies

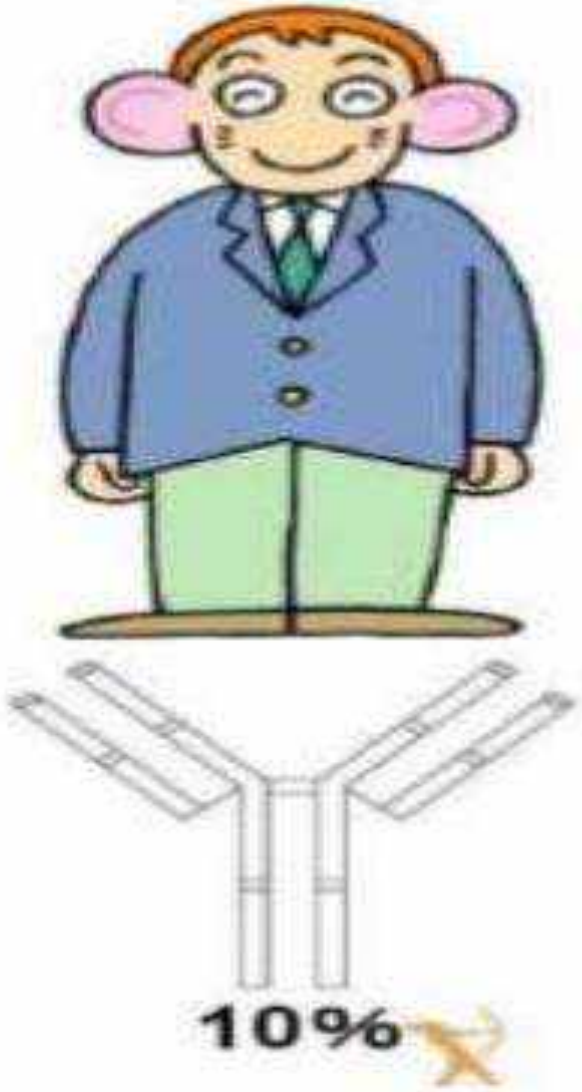
- Chimeric antibodies are those in which the Fc part of an immunoglobulin is of a human sequence
- Antibodies are approximately 65% human.
- This reduces immunogenicity and thus increases serum half-life.



HUMANISED MAB

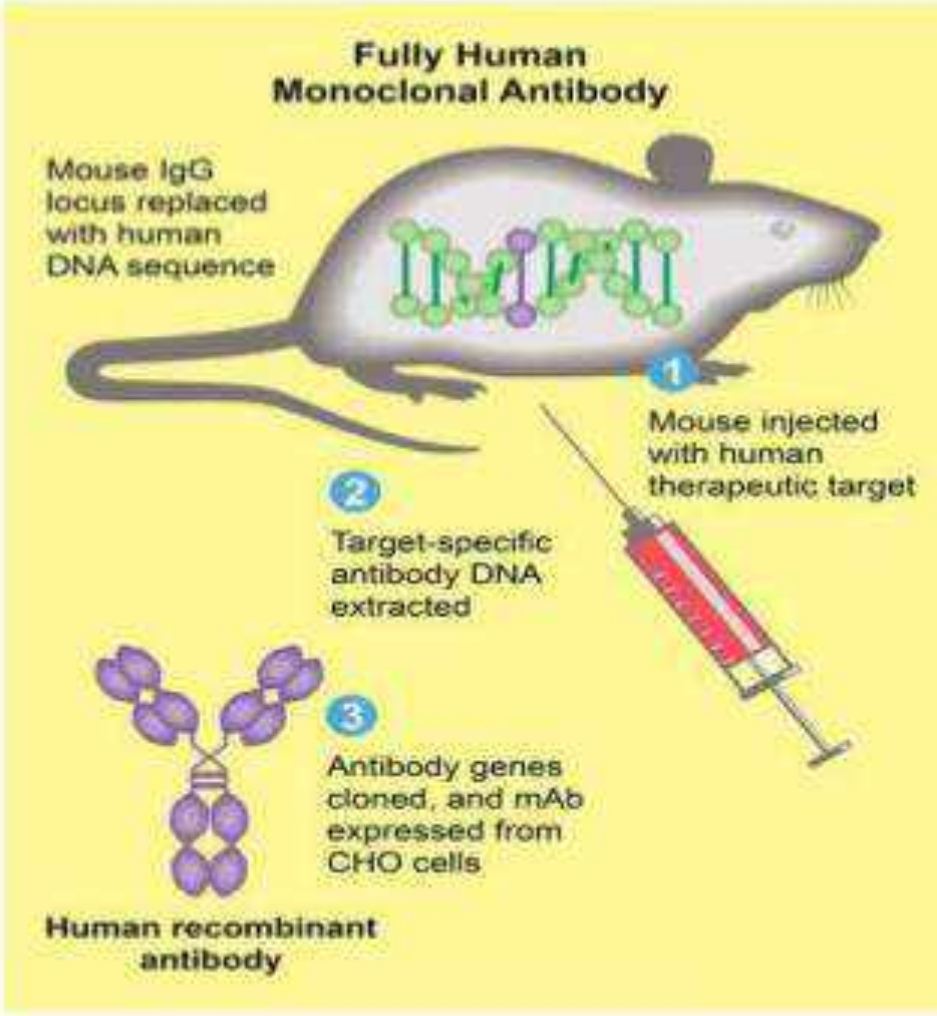
- Humanised antibodies contain segments from sources in the complementary determining regions (CDR) interspersed among human derived segments in constant regions

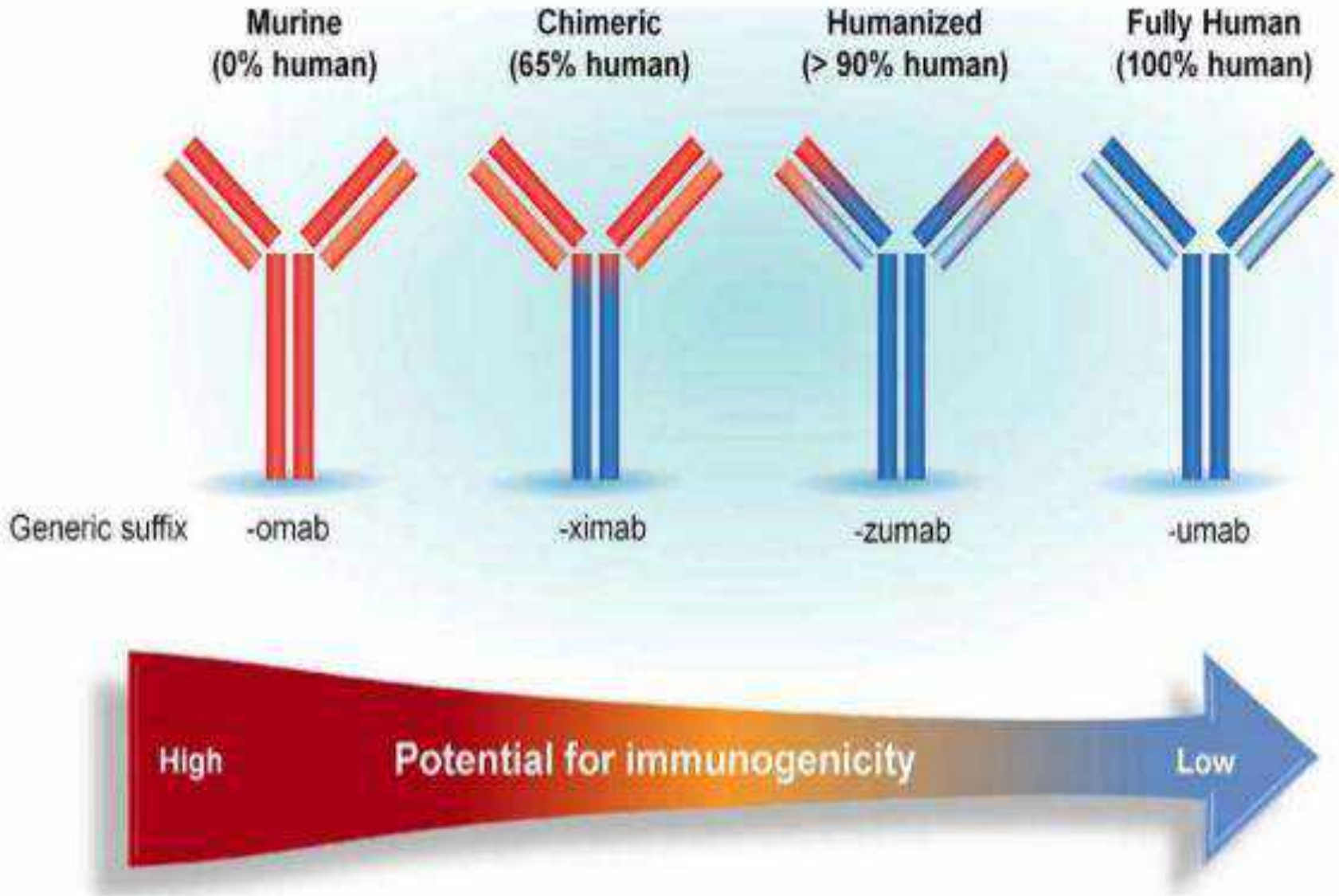
- This results in a molecule of approximately 95% human origin



Human Monoclonal Antibody

- Human monoclonal antibodies are produced by transferring human immunoglobulin genes into the murine genome, after which the transgenic mouse is vaccinated against the desired antigen, leading to the production of monoclonal antibodies




Murine
(0% human)
Chimeric
(65% human)
Humanized
(> 90% human)
Fully Human
(100% human)
Generic suffix
-omab
-ximab
-zumab
-umab
High
Potential for immunogenicity
Low

NOMENCLATURE

Every monoclonal antibody has the following components in its name :

Variable-Target Substem-Source Substem-Stem-Additional words

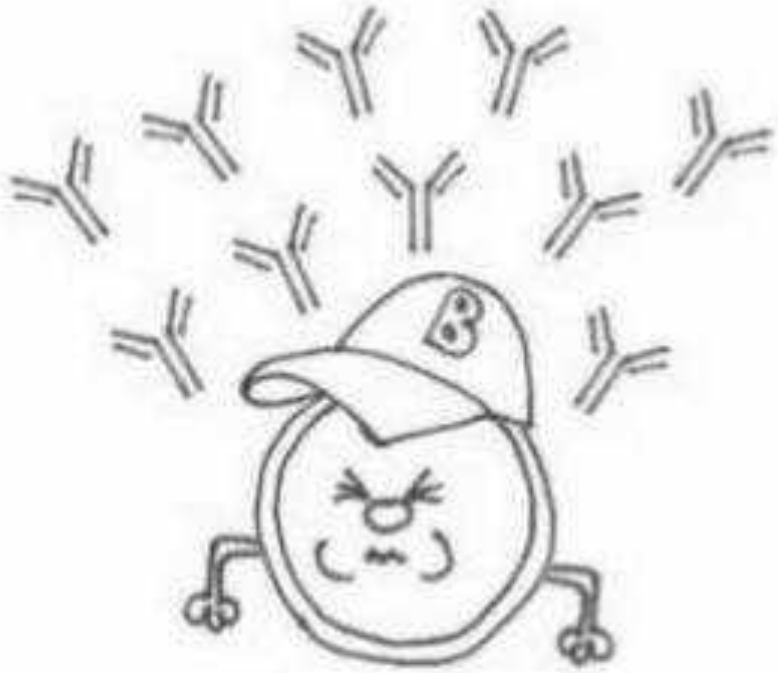
Ex: Alacizumab pegol is

Ala –ci-zu-mab-pegol

ANTIBODIES

- It is a specialized glycoprotein, produced from activated B cells (plasma cells) in response to an antigen that triggers its production .

- Also called 'Immunoglobulins' because they immunologically react with the antigen



Type II Hypersensitivity –

Blood Transfusion reactions

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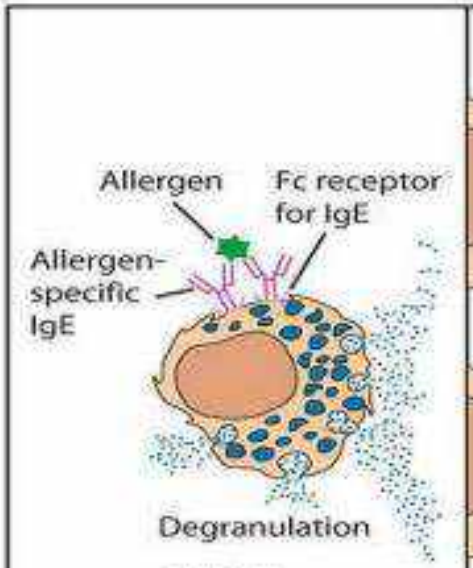
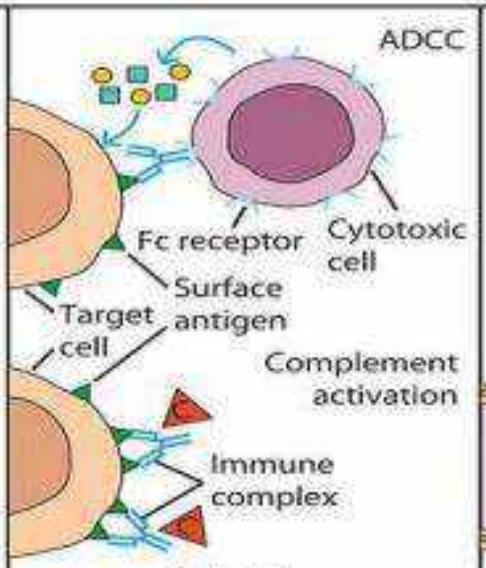
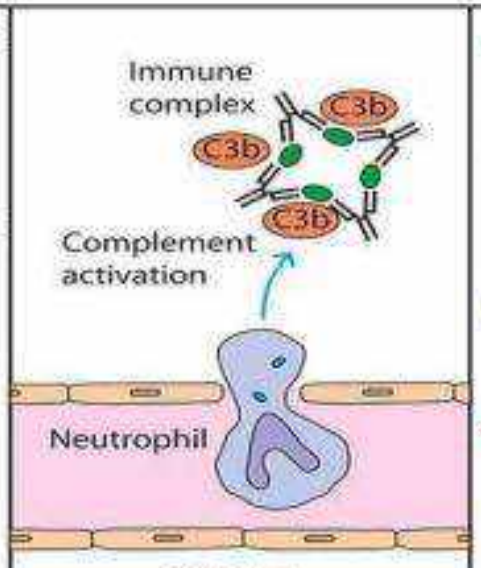
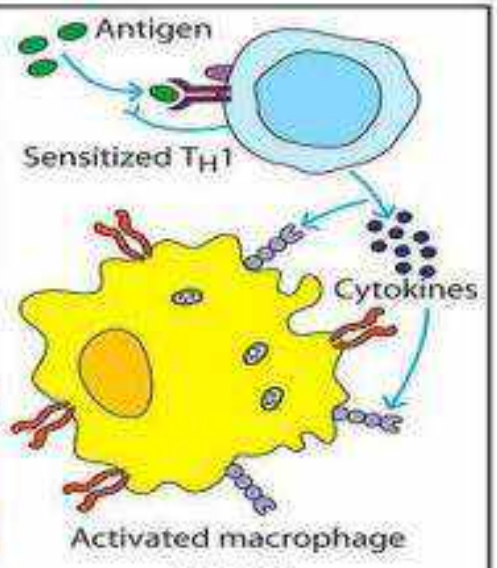
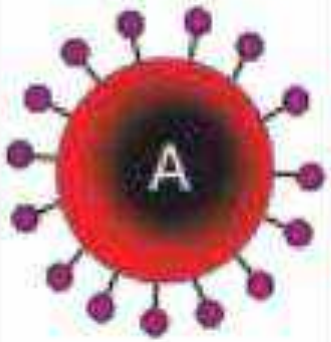
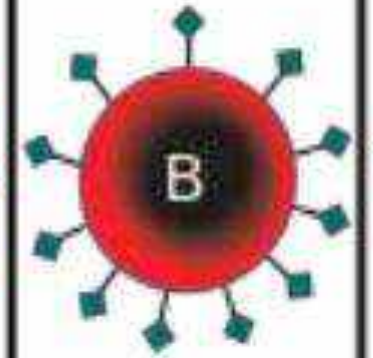
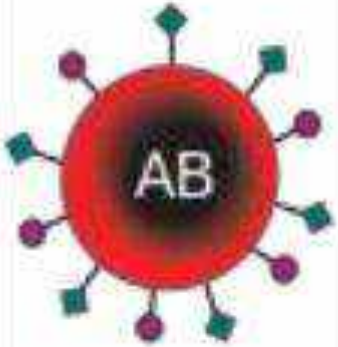
Vivekanand College (Autonomous) , Kolhapur.

Hypersensitivity Reactions

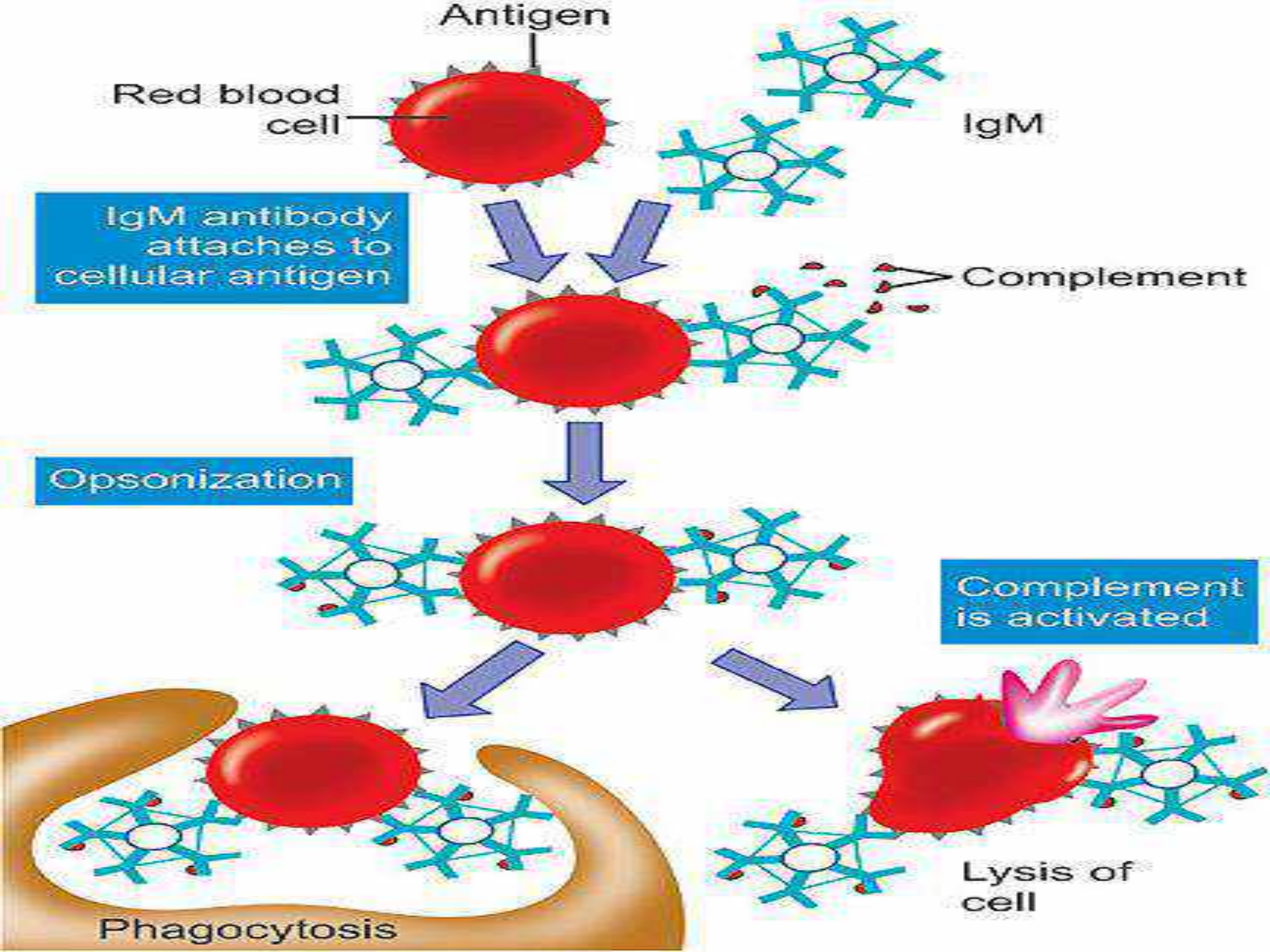
 **Type I**	 **Type II**	 **Type III**	 **Type IV**
IgE-Mediated Hypersensitivity	IgG-Mediated Cytotoxic Hypersensitivity	Immune Complex-Mediated Hypersensitivity	Cell-Mediated Hypersensitivity
Ag induces crosslinking of IgE bound to mast cells and basophils with release of vasoactive mediators	Ab directed against cell surface antigens meditates cell destruction via complement activation or ADCC	Ag-Ab complexes deposited in various tissues induce complement activation and an ensuing inflammatory response mediated by massive infiltration of neutrophils	Sensitized T_H1 cells release cytokines that activate macrophages or T_C cells which mediate direct cellular damage
Typical manifestations include systemic anaphylaxis and localized anaphylaxis such as hay fever, asthma, hives, food allergies, and eczema	Typical manifestations include blood transfusion reactions, erythroblastosis fetalis, and autoimmune hemolytic anemia	Typical manifestations include localized Arthus reaction and generalized reactions such as serum sickness, necrotizing vasculitis, glomerulnephritis, rheumatoid arthritis, and systemic lupus erythematosus	Typical manifestations include contact dermatitis, tubercular lesions and graft rejection

TYPE II (CYTOTOXIC) HYPERSENSITIVITY

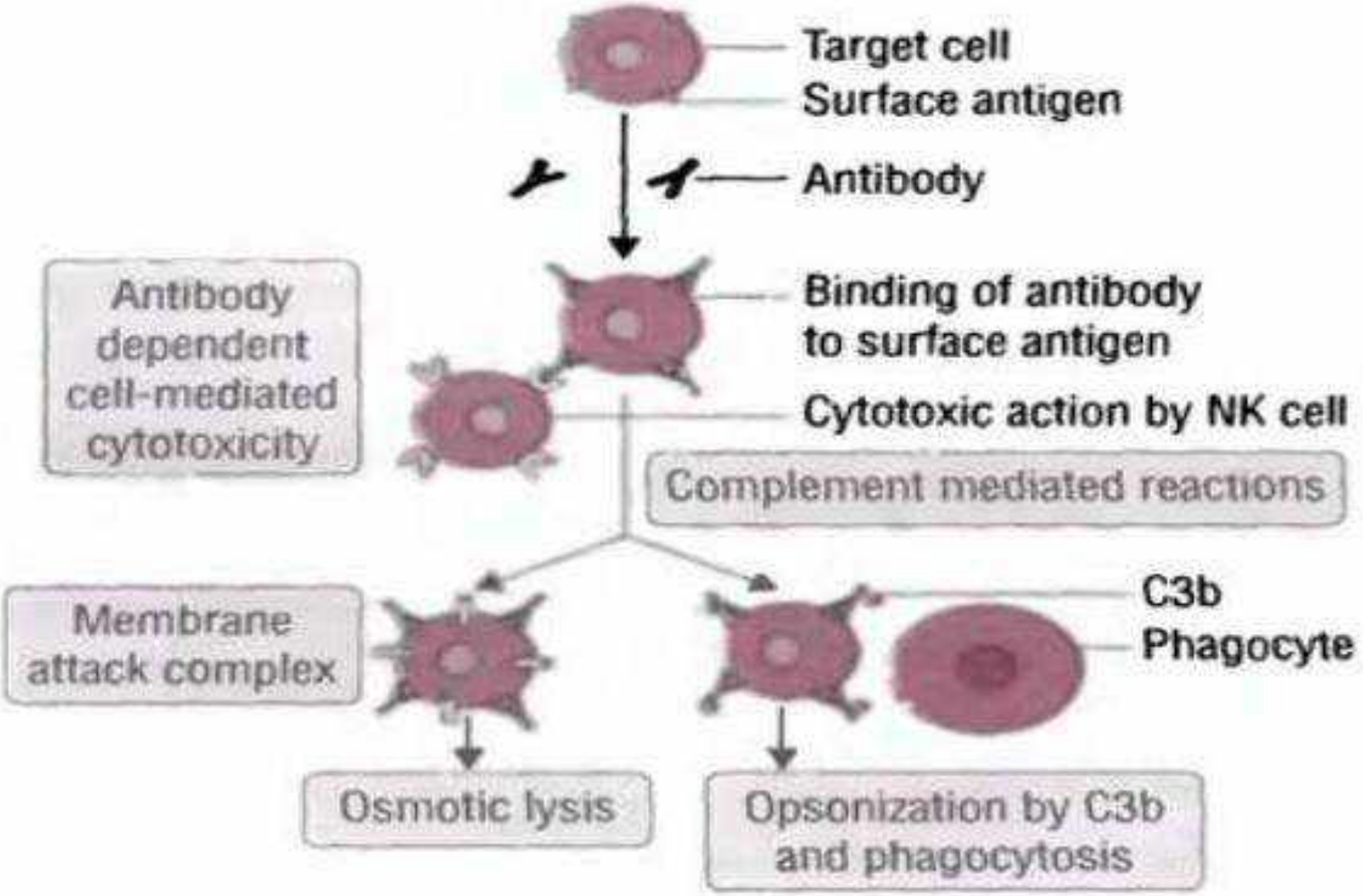
	Group A	Group B	Group AB	Group O
Red blood cell type				
Antibodies in Plasma	 Anti-B	 Anti-A	None	 Anti-A and Anti-B
Antigens in Red Blood Cell	 A antigen	 B antigen	 A and B antigens	None

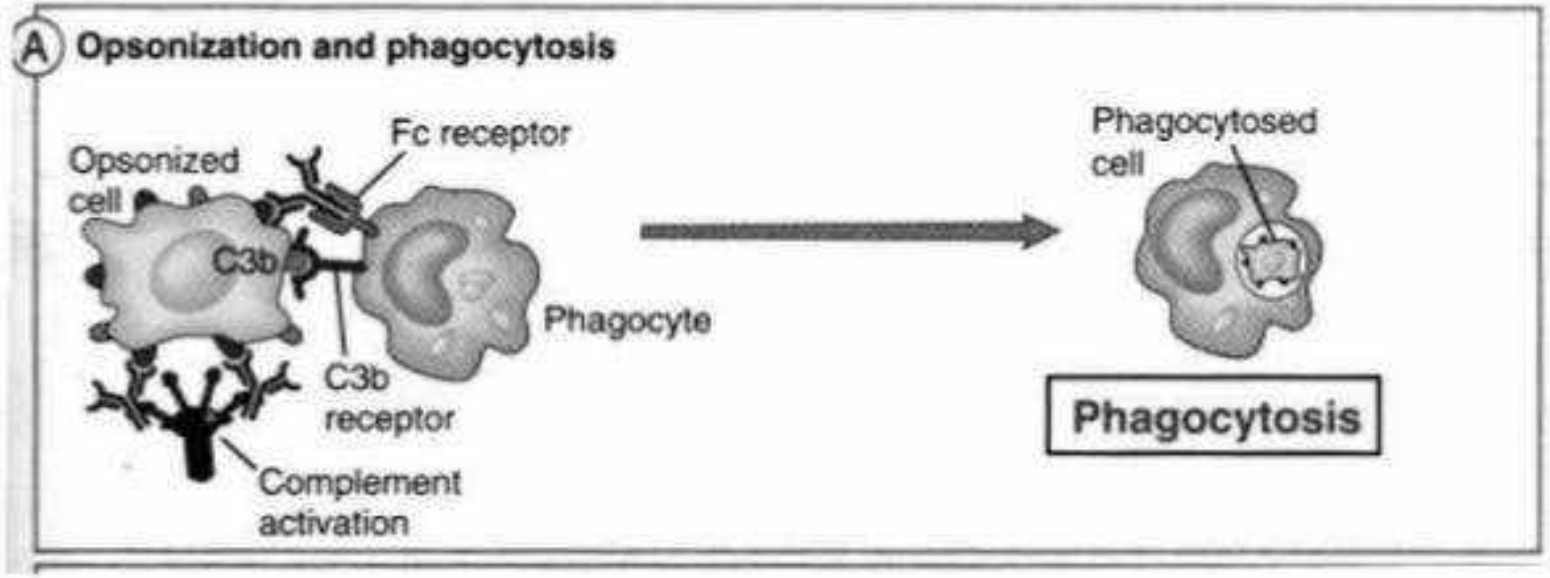
TYPE II (CYTOTOXIC) HYPERSENSITIVITY

- Type II cytotoxic reaction is mediated by antibodies directed against antigens on the cell membrane that activates complement thereby causing antibody-mediated destruction of cells.
- The cell membrane is damaged by a membrane attack complex during activation of the complement.
- The reactions involve combination of IgG or IgM antibodies with the cell-fixed antigens or alternately circulating antigens absorbed onto cells.
- Antigen-antibody reaction leads to complement activation, resulting in the formation of membrane attack complex.


Antigen
Red blood cell
IgM
IgM antibody attaches to cellular antigen
Complement
Opsonization
Complement is activated
Phagocytosis
Lysis of cell

TYPE II (CYTOTOXIC) HYPERSENSITIVITY




A Opsonization and phagocytosis
Opsonized cell
Fc receptor
C3b
Phagocyte
C3b receptor
Complement activation
Phagocytosed cell
Phagocytosis

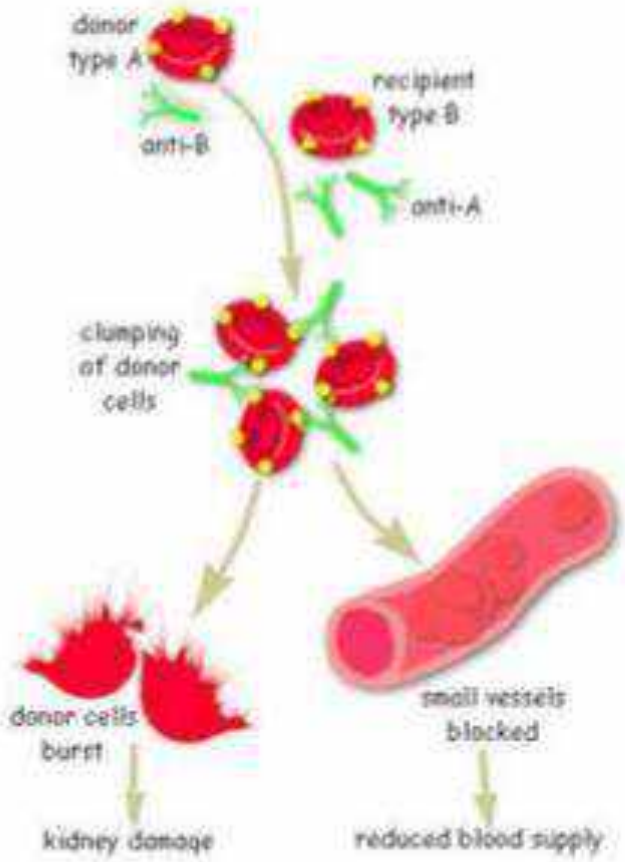
TYPE II (CYTOTOXIC) HYPERSENSITIVITY

- This complex then acts on the cells, causing damage of the cells, as seen in complement-mediated lysis in Rh hemolytic disease, transfusion reaction, or hemolytic anemia.
- Antibody-dependent cell-mediated cytotoxicity (ADCC) is another mechanism which involves the binding of cytotoxic cells with Fc receptors in the Fc binding part of the antibodies coating the target cells.
- The antibody coating the target cell can also cause its destruction by acting as an opsonin. This mechanism is important in immunity against large sized pathogens such as the helminthes.

TRANSFUSION REACTIONS

- A large number of proteins and glycoproteins are present on the surface of RBCs, of which A, B, and O antigens are of particular importance.
- Antibodies to these antigens are called isohemagglutinins and are of IgM class.
- When transfusion with mismatched blood occurs, a transfusion reaction takes place due to the destruction of the donor RBCs through the isohemagglutinins against the foreign antigen.
- The clinical manifestations result from the massive intravascular hemolysis of the donor cells by antibody and complement.

Type II Hypersensitivity:



Produced by mismatched blood types

- Destroys foreign RBC by complement-mediated lysis triggered by IgG
  - Produces fever, intravascular clots, lower back pain, Hgb in urine

- Free Hgb produced has 2 fates:
  - passes to the kidneys – hemoglobinuria
  - Breaks down to bilirubin. Can be toxic

Mechanism of ABO compatible blood group transfusion reaction

- Delayed heamolytic transfusion reactions generally occurs in individuals who have received repeated transfusion of ABO compatible blood that is incompatible forother blood group antigens . The reaction develop between 2 and 6 days after transfusion.
- The most commmon blood group antigens that induce delayed transfusion reaction are Rh, Kidd,Kell and Duffy.
- The antibodies against these antigens are Ig G type, which are less effective than Ig M in activating complement
- These transfusion cells are destroyed at extra vascular sites by agglutination , opsonosation and subsequent phagosytosis by macrophages.
- Symptoms includes fever, low hemoglobin increased bilirubin, mild jaundice and anemia.
- Free haemoglobin is usually detected in the plasma or urine in these reactions because RBC destruction occurs in extra vascular sites.

Treatment

❖ For ABO incompatibility

Prompt termination of transfusion and maintenance of urine flow with diuretic

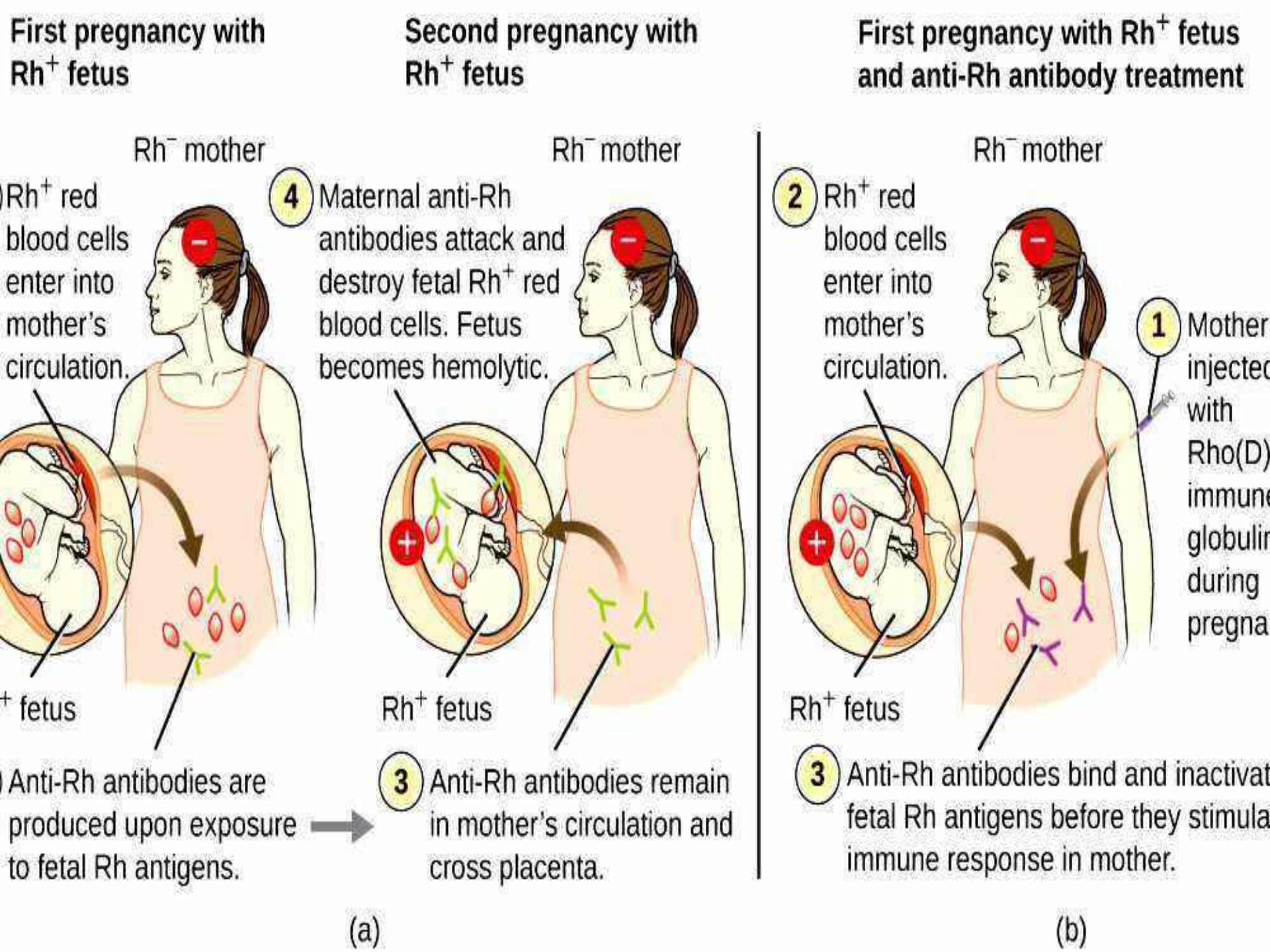
.

❖ For ABO compatibility

Transfusion reaction can be prevented by proper cross matching between the donor and recipient blood

ERYTHROBLASTOSIS FETALIS

- This condition develops when maternal antibodies specific for fetal blood group antigens cross the placenta and destroy fetal RBCs.
- This condition is seen in cases where a pre-sensitized Rh-negative mother mounts an immune response against Rh-positive RBCs of the fetus.
- This results in severe hemolysis, leading to anemia and hyperbilirubinemia which can even be fatal.


First pregnancy with Rh+ fetus
Second pregnancy with Rh+ fetus
First pregnancy with Rh+ fetus and anti-Rh antibody treatment
Rh− mother
Rh− mother
Rh− mother
Rh+ red blood cells enter into mother's circulation.
4 Maternal anti-Rh antibodies attack and destroy fetal Rh+ red blood cells. Fetus becomes hemolytic.
2 Rh+ red blood cells enter into mother's circulation.
1 Mother injected with Rho(D) immune globulin during pregna
+ fetus
Rh+ fetus
Rh+ fetus
Anti-Rh antibodies are produced upon exposure to fetal Rh antigens.
3 Anti-Rh antibodies remain in mother's circulation and cross placenta.
3 Anti-Rh antibodies bind and inactivat fetal Rh antigens before they stimula immune response in mother.
(a)
(b)

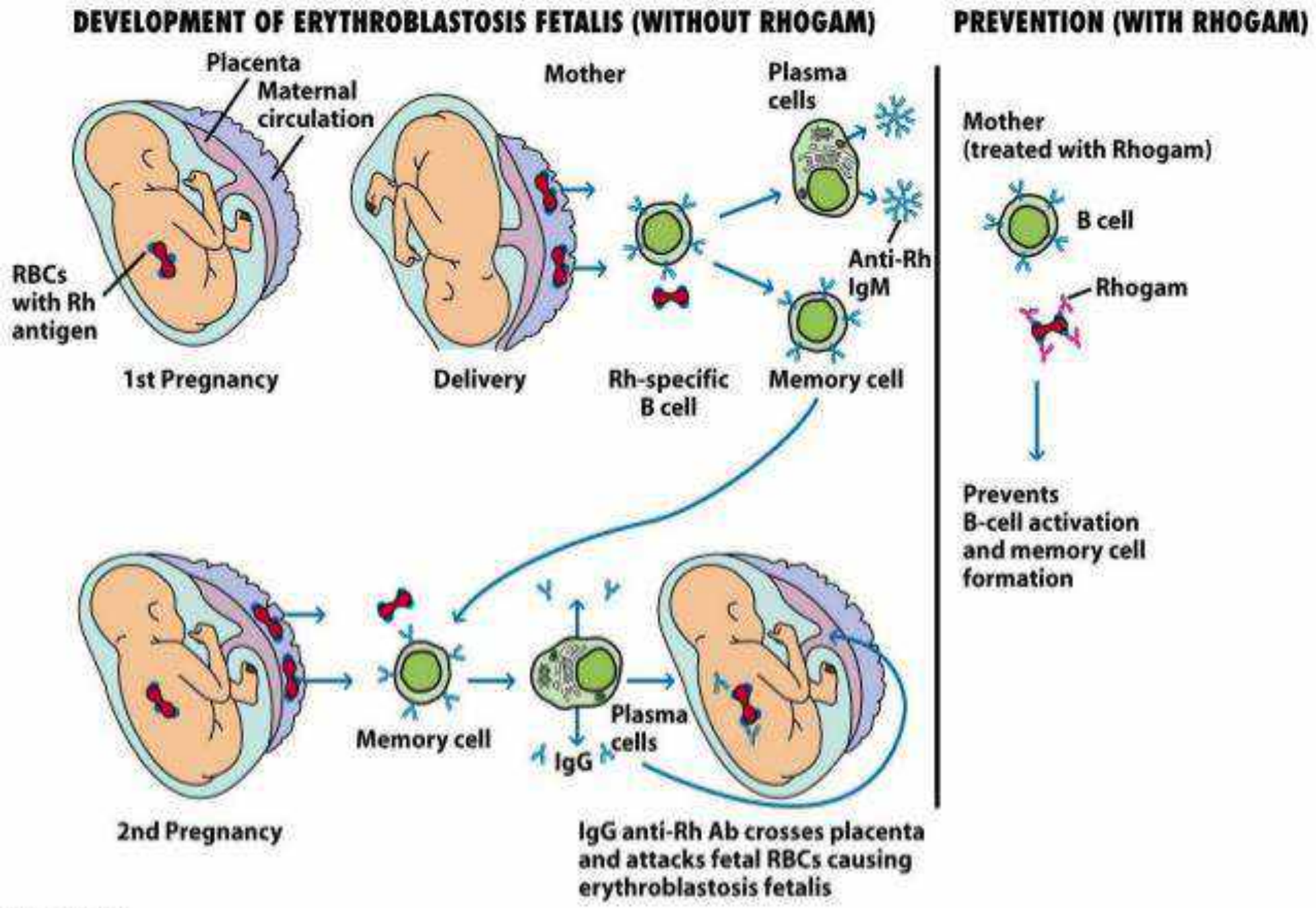


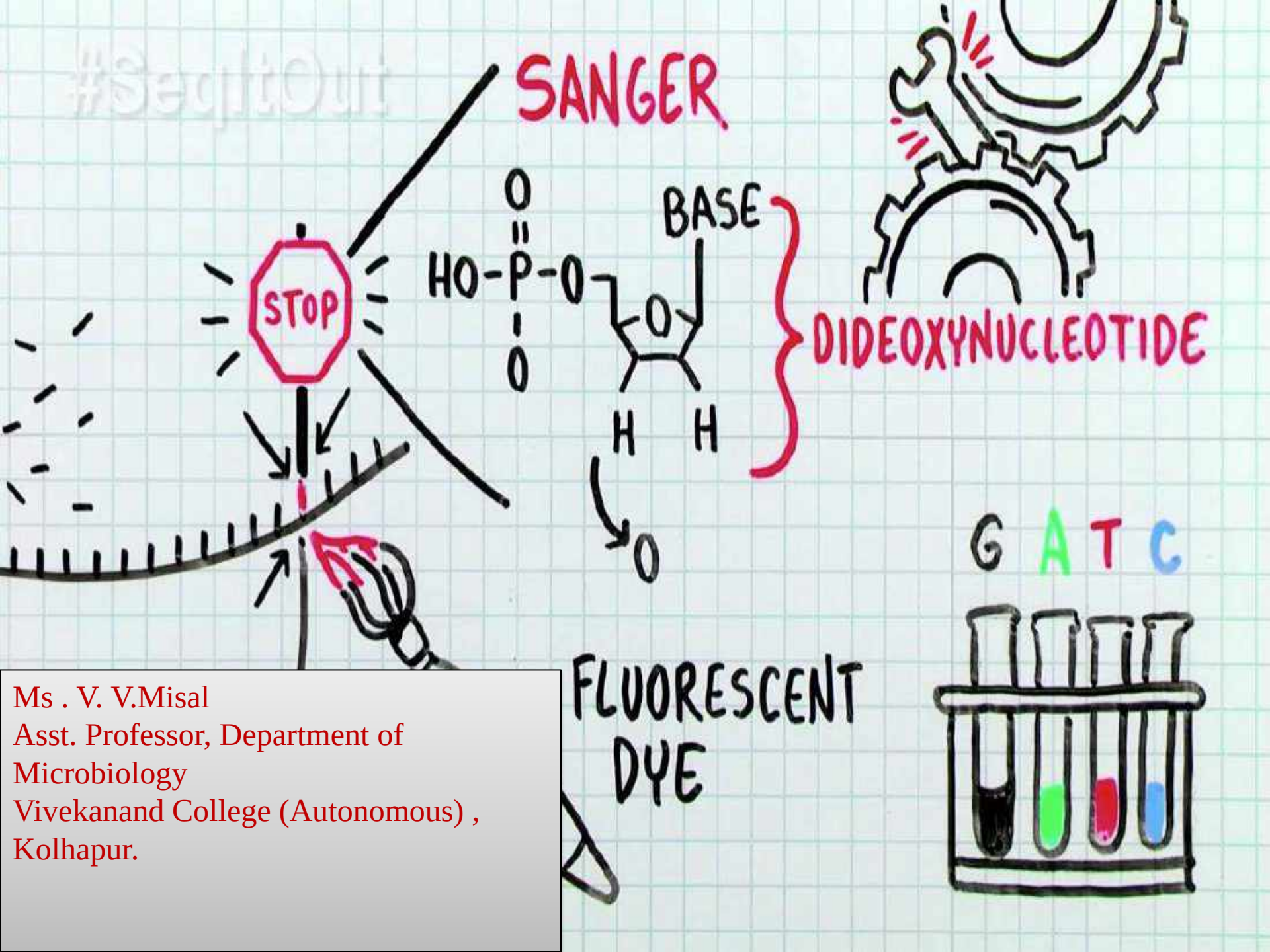
Figure 15-14
Kuby IMMUNOLOGY, Sixth Edition

DRUG-INDUCED HEMOLYSIS

- Certain drugs such as penicillin, quinidine, phenacetin, etc., may induce hemolysis of red blood cells.
- Drugs are attach to the surface of red blood cells and induce formation of IgG antibodies.
- These autoantibodies then react with red blood cell surface, causing hemolysis.
- Similarly, quinacrines attach to surface of platelets and induce autoantibodies that lyse the platelets, causing thrombocytopenia.

RHEUMATIC FEVER

- In this condition, antibodies are produced against group A streptococci that cross-react with cardiac tissues and activate complement and release of components of complement, which in turn causes damage of cardiac tissues.



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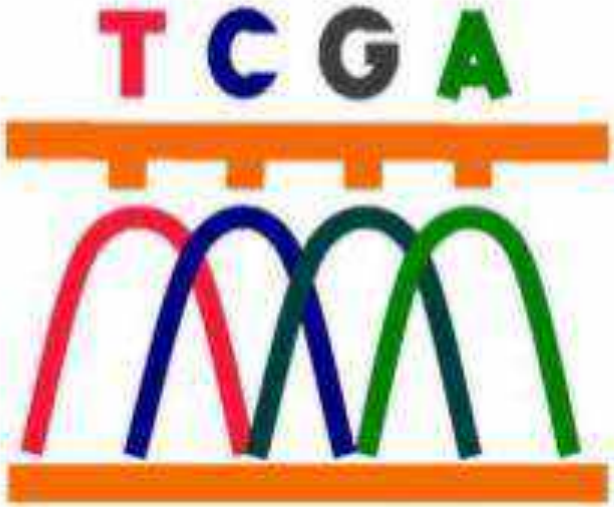
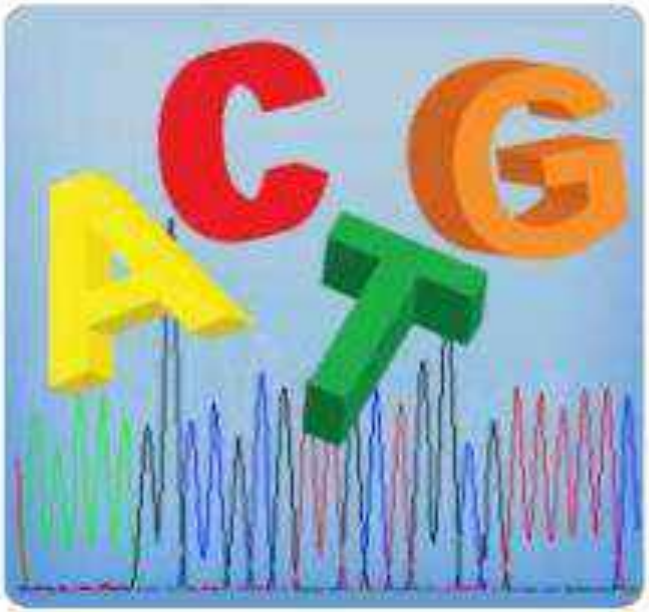
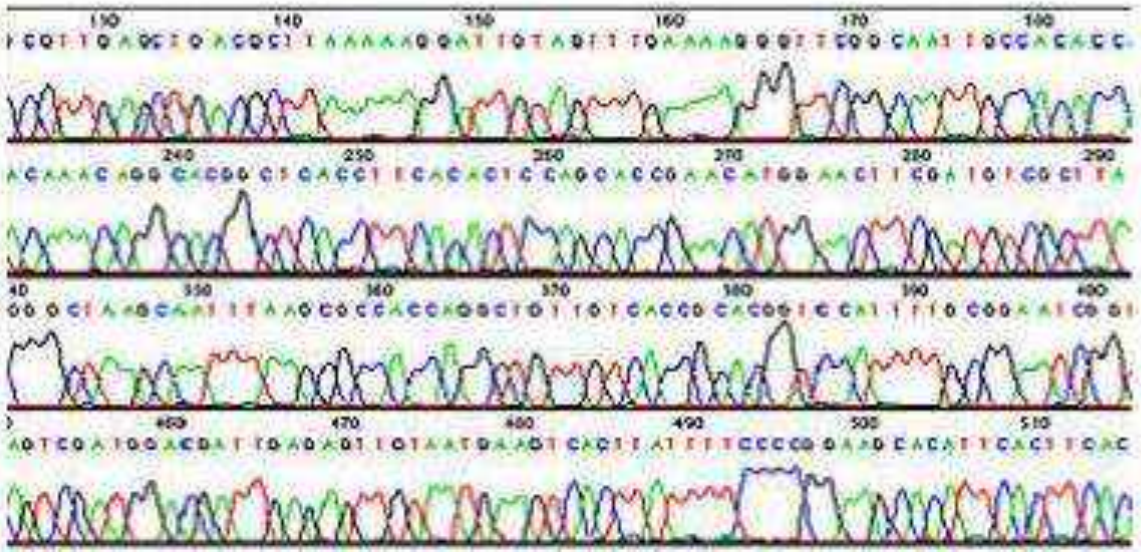
What is DNA Sequencing ?







- The process of determining the order of nucleotides adenine (A), thymine (T), cytosine (C), and guanine (G) along a DNA strand.

- We need to know the order of nucleotide bases in a strand of DNA for sequencing.

- All the information required for the growth and development of an organism is encoded in the DNA of its genome.

- So, DNA sequencing is fundamental to genome analysis and understanding the biological processes in general.

Historical Timeline

1870 – Friedrich Miescher discovers DNA

1940 - Avery: Proposes DNA as 'Genetic Material'

1953 – Watson & Crick "double helical structure"

1970 - Wu: Sequences λ Cohesive End DNA

1977 – Sanger: Dideoxy Chain Termination

1977 – Gilbert: Chemical Degradation

1986 – Partial Automation

1990 – Cycle Sequencing, Improved Sequencing Enzymes,

Improved fluorescent detection schemes

2002 – NGS: 454 Pyrosequencing

SEQUENCING METHODS

To determine the order of the nucleotide bases adenine, guanine, cytosine, and thymine in a molecule of DNA two methods were used

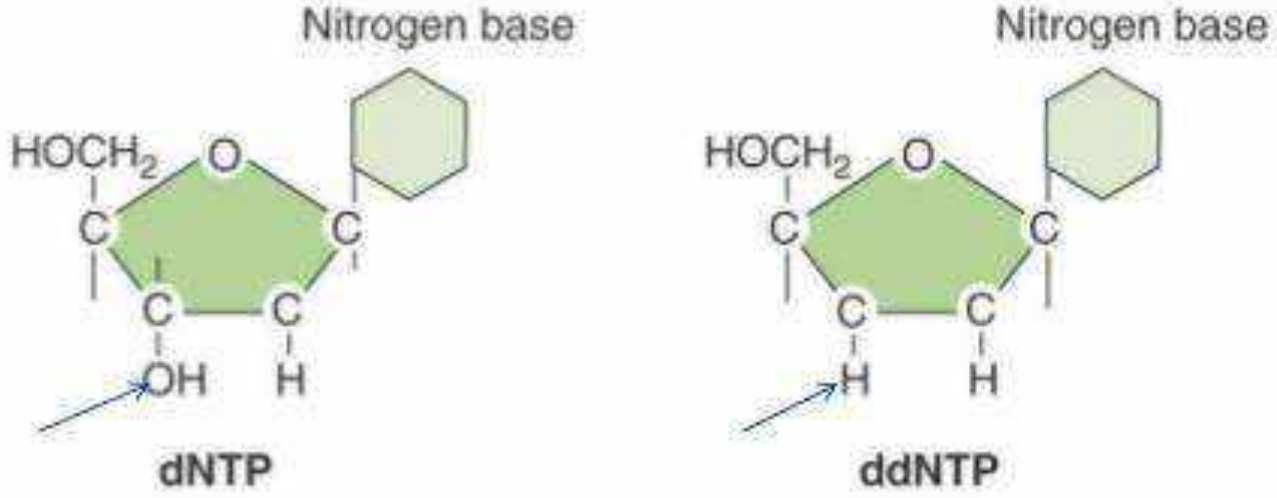
1. Sanger; Chain Termination Sequencing method
2. Maxam and Gilbert; Chemical Sequencing method

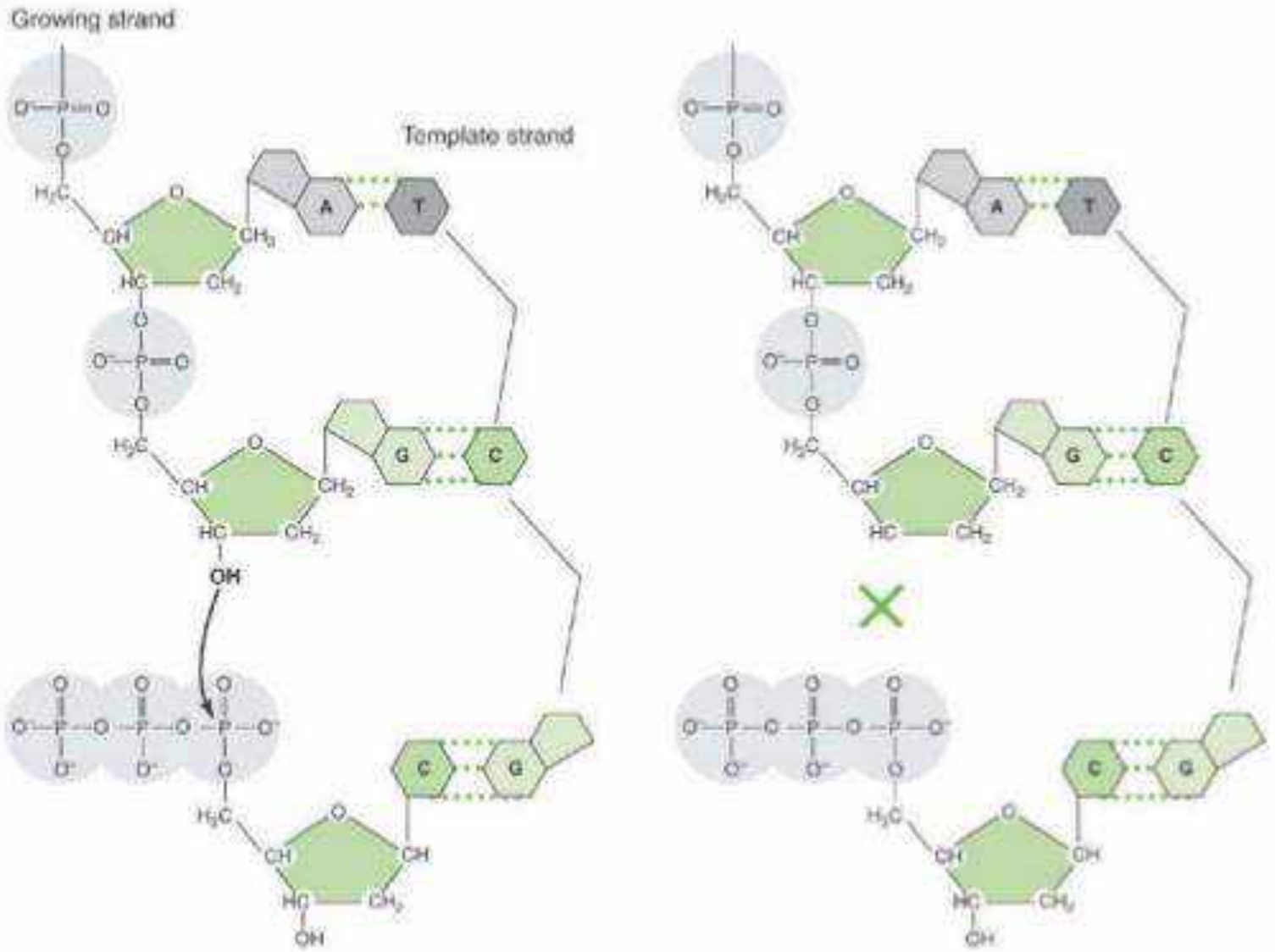
These two methods are most popular conventional methods

- Robotics and automated sequencing are based on these methods

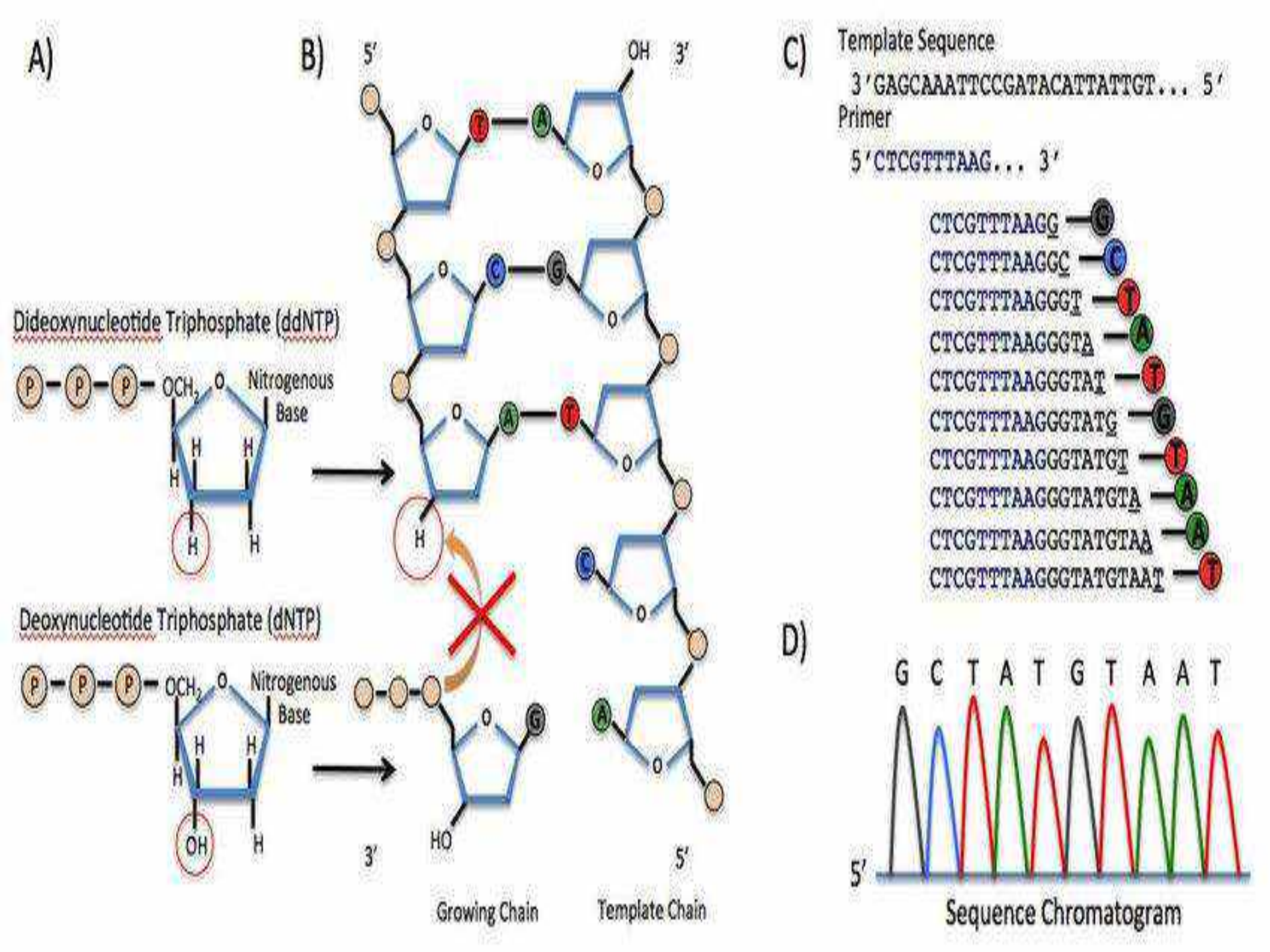
Sanger's- Chain Termination Sequencing

- It is PCR based method
- A modified DNA replication reaction
- Growing chains are terminated by dideoxynucleotides





The 3′-OH group necessary for formation of the phosphodiester bond is missing in ddNTPs.


A)
B)
C)
D)
Dideoxynucleotide Triphosphate (ddNTP)
Deoxynucleotide Triphosphate (dNTP)
Nitrogenous Base
Nitrogenous Base
5′
OH
3′
3′
HO
5′
Growing Chain
Template Chain
Template Sequence
3′GAGCAAATTCCGATACATTATTGT... 5′
Primer
5′CTCGTTTAAG... 3′
CTCGTTTAAGG
CTCGTTTAAGGC
CTCGTTTAAGGGT
CTCGTTTAAGGGTA
CTCGTTTAAGGGTAT
CTCGTTTAAGGGTATG
CTCGTTTAAGGGTATGT
CTCGTTTAAGGGTATGTA
CTCGTTTAAGGGTATGTAA
CTCGTTTAAGGGTATGTAAT
G C T A T G T A A T
5′
Sequence Chromatogram

Sanger Sequencing: Process

1. Get enough quantity of DNA (Run PCR)
2. Aliqot DNA into four different tubes
3. Prepare PCR reaction mix as below:

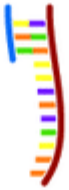
- Primer, Taq polymerase, template(ssDNA), dNTPS (All) and ddNTPs(ddATP, ddGTP,ddCTP & ddTTP respectively)

1. Run PCR
2. Perform Gel Electrophoresis
3. Interpret results

1. denature dsDNA using heat

2. make multiple copies of a segment

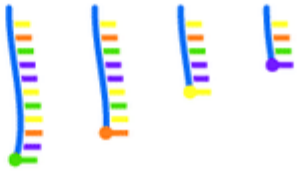
3. attach a primer



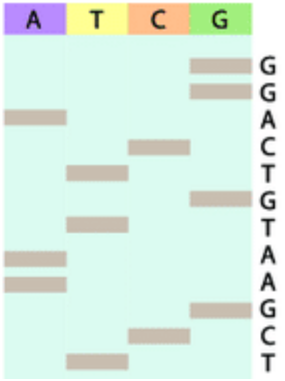
4. add to four polymerase solutions

5. grow complementary chains until termination dye

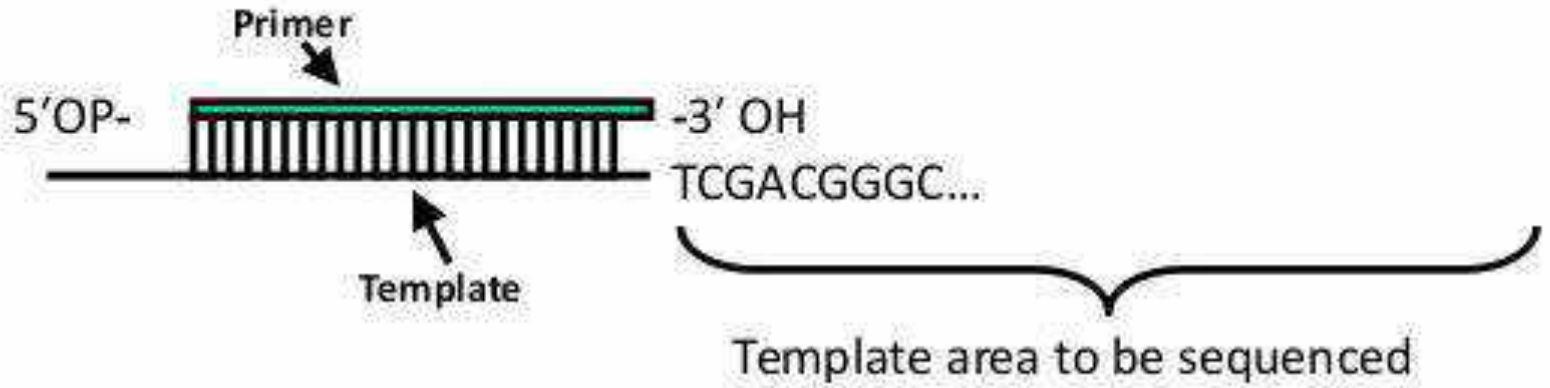
6. denaturate the grown chains



7. electrophorese the four solutions



- A sequencing reaction mix includes labeled primer and template.



- Dideoxynucleotides are added separately to each of the four tubes.

- With addition of enzyme (DNA polymerase), the primer is extended until a ddNTP is encountered.
- The chain will end with the incorporation of the ddNTP.
- With the proper dNTP:ddNTP ratio, the chain will terminate throughout the length of the template.
- All terminated chains will end in the ddNTP added to that reaction.

- The collection of fragments is a sequencing ladder.
- The resulting terminated chains are resolved by electrophoresis.
- Fragments from each of the four tubes are placed in four separate gel lanes.

Sanger Sequencing: An Example

5’-TACACGATCGA-3’

3’-ATGTGCTAGCT-5’

Denature the sequence

Use only forward primer i.e. use 3’-5’ strand of DNA

Amplification in ddTTP

3’-ATGTGCTAGCT-5’

5’-T-3’

5’-TACACGAT-3’

Amplification in ddATP

3’-ATGTGCTAGCT-5’

5’-TA-3’

5’-TACA-3’

5’-TACACGA-3’

5’-TACACGATCGA-3’

Amplification in ddGTP

3’-ATGTGCTAGCT-5’

5’-TACACG-3’

5’-TACACGATCG-3’

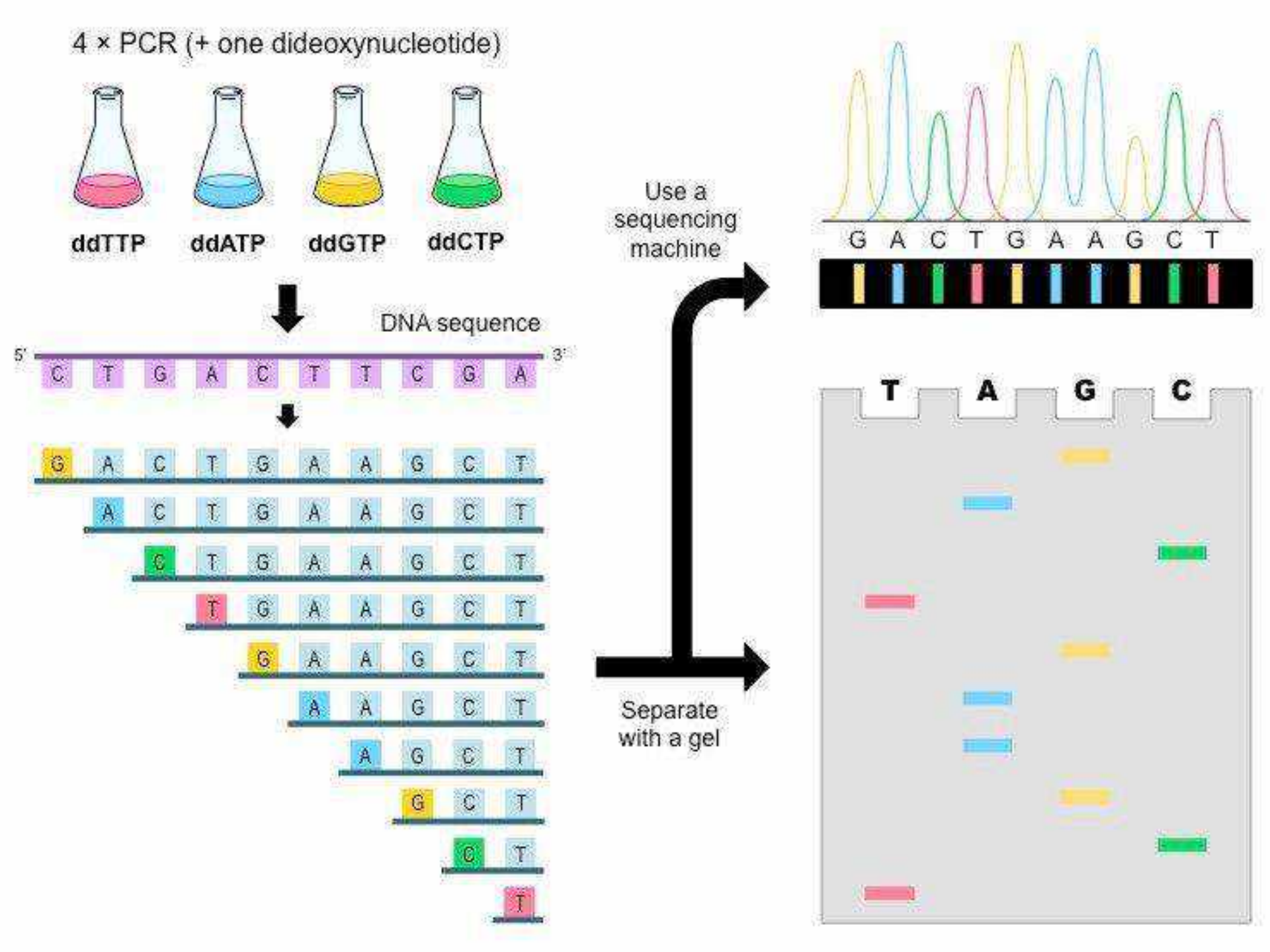
Amplification in ddCTP

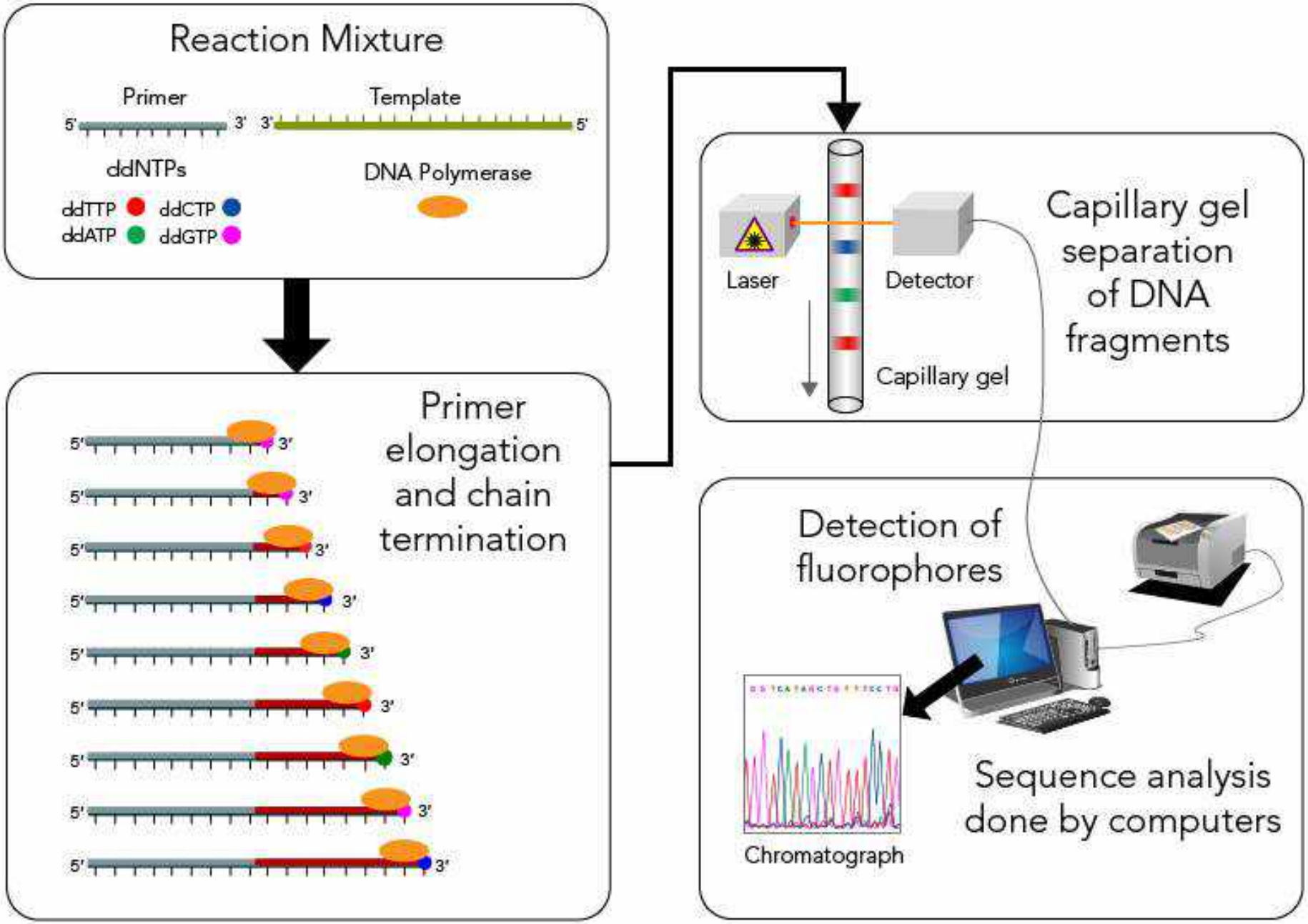
3’-ATGTGCTAGCT-5’

5’-TAC-3’

5’-TACAC-3’

5’-TACACGATC-3’


4 × PCR (+ one dideoxynucleotide)
ddTTP
ddATP
ddGTP
ddCTP
DNA sequence
5'
3'
C T G A C T T C G A
G A C T G A A G C T
A C T G A A G C T
C T G A A G C T
T G A A G C T
G A A G C T
A A G C T
A G C T
G C T
C T
T
Use a sequencing machine
Separate with a gel
G A C T G A A G C T
T A G C

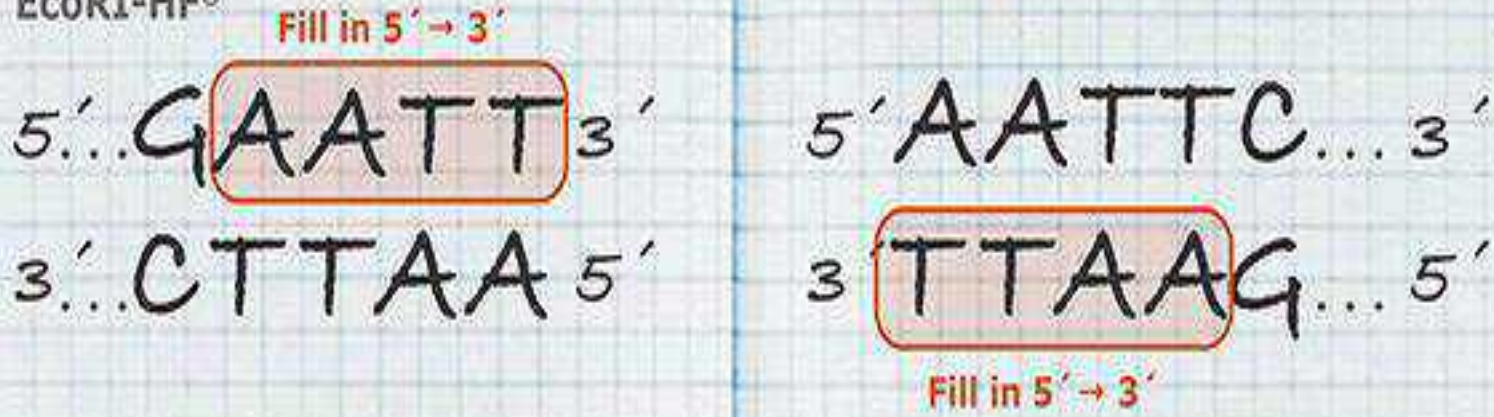

Reaction Mixture
Primer
Template
5'
3'
3'
5'
ddNTPs
DNA Polymerase
ddTTP
ddCTP
ddATP
ddGTP
Primer elongation and chain termination
Laser
Detector
Capillary gel
Capillary gel separation of DNA fragments
Detection of fluorophores
Chromatograph
Sequence analysis done by computers

BLUNTING WITH DNA POLYMERASES

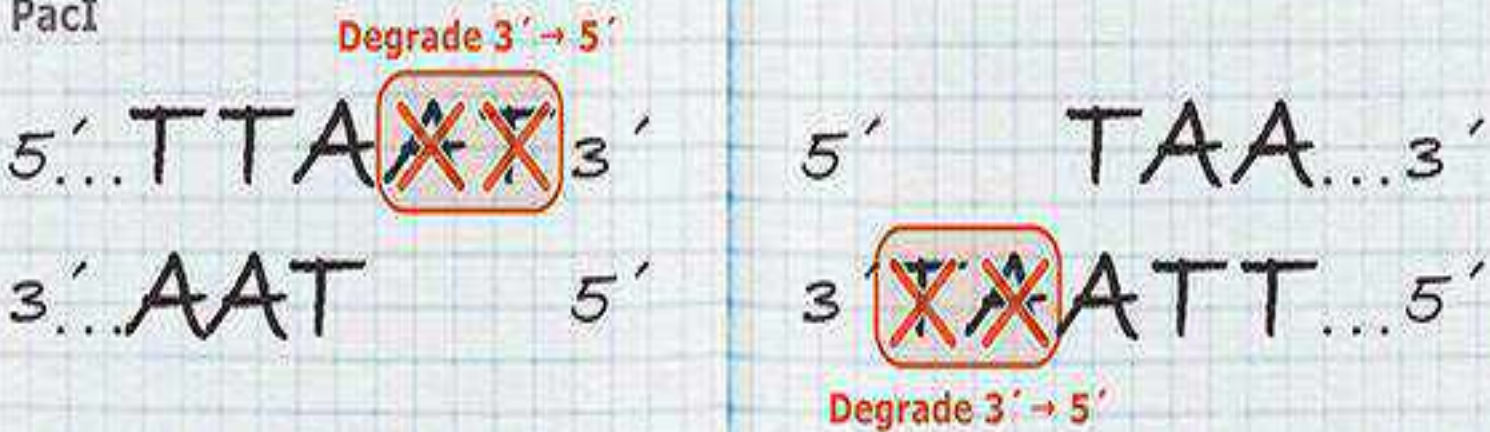
Examples:

- **T4 DNA Polymerase**
- **DNA Polymerase I, Large (Klenow) Fragment**

EcoRI-HF®



PacI



Fundamental reasons for knowing the sequence of DNA molecule:

- To charecterise the newly cloned DNA.
- For predictions about its fuctions.
- To facilitate manipulation of the molecule.
- To confirm the identity of a clone or a mutation.
- To check the fidility of newly created mutation and ligation junction.
- Screening tool to identify polymorphisms and mutation in genes of particular interest.

ONE CISTRON ONE POLYPEPTIDE HYPOTHESIS

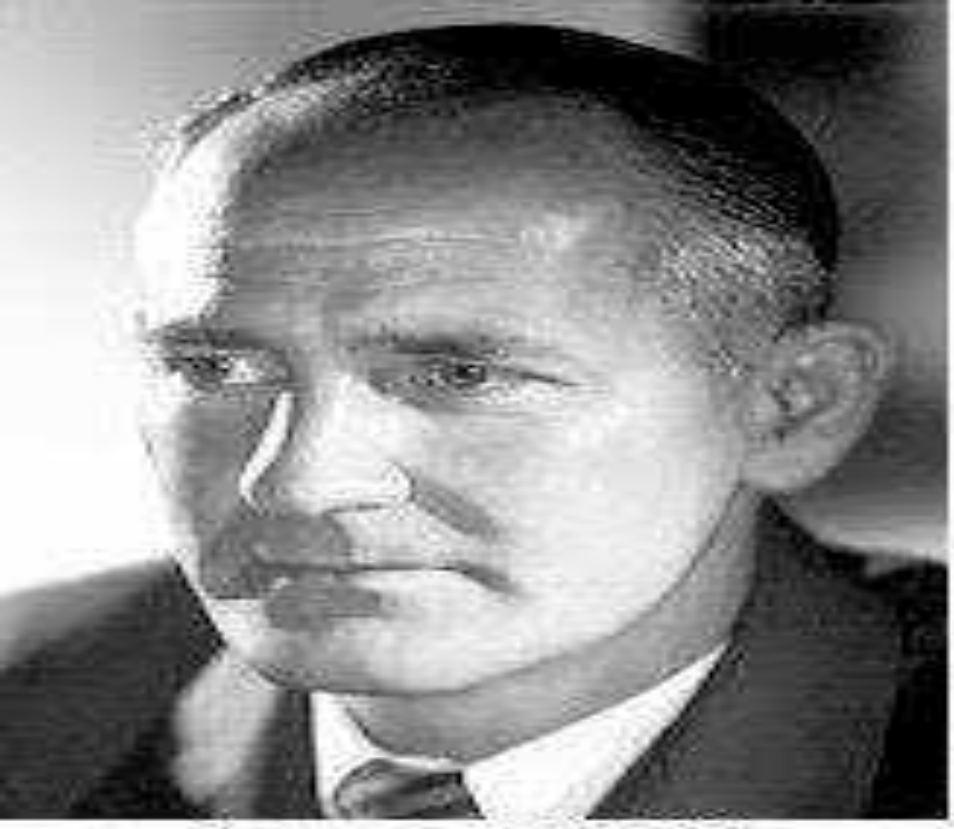
Ms . V. V.Misal
Asst. Professor, Department of Microbiology
Vivekanand College (Autonomous) , Kolhapur.

The existence of genes was first suggested by Gregor Mendel (1822–1884), who, in the 1860s, studied inheritance in pea plants (*Pisum sativum*) and hypothesized a factor that conveys traits from parent to offspring.

He spent over 10 years of his life on one experiment. Although he did not use the term *gene*, he explained his results in terms of inherited characteristics.

Modern concept of gene

- **i) Genes as unit of transmission or cistron :**
- The part of DNA specifying a single polypeptide chain is termed as cistron. A cistron can have 100 nucleotide pairs in length to 30,000 nucleotide pairs. It transmits characters from one generation to other as unit of transmission.
- **ii) Genes as unit of recombination or recon :**
- The smallest segment of DNA capable of being separated and exchange with other chromosome is called recon. A recon consists of not more than two pairs of nucleotides.
- **iii) Gene as unit of mutation or muton :**
- Muton is the smallest unit of genetic material which when changed or mutated produce a phenotypic trait. Thus muton is delimited to a single nucleotide.

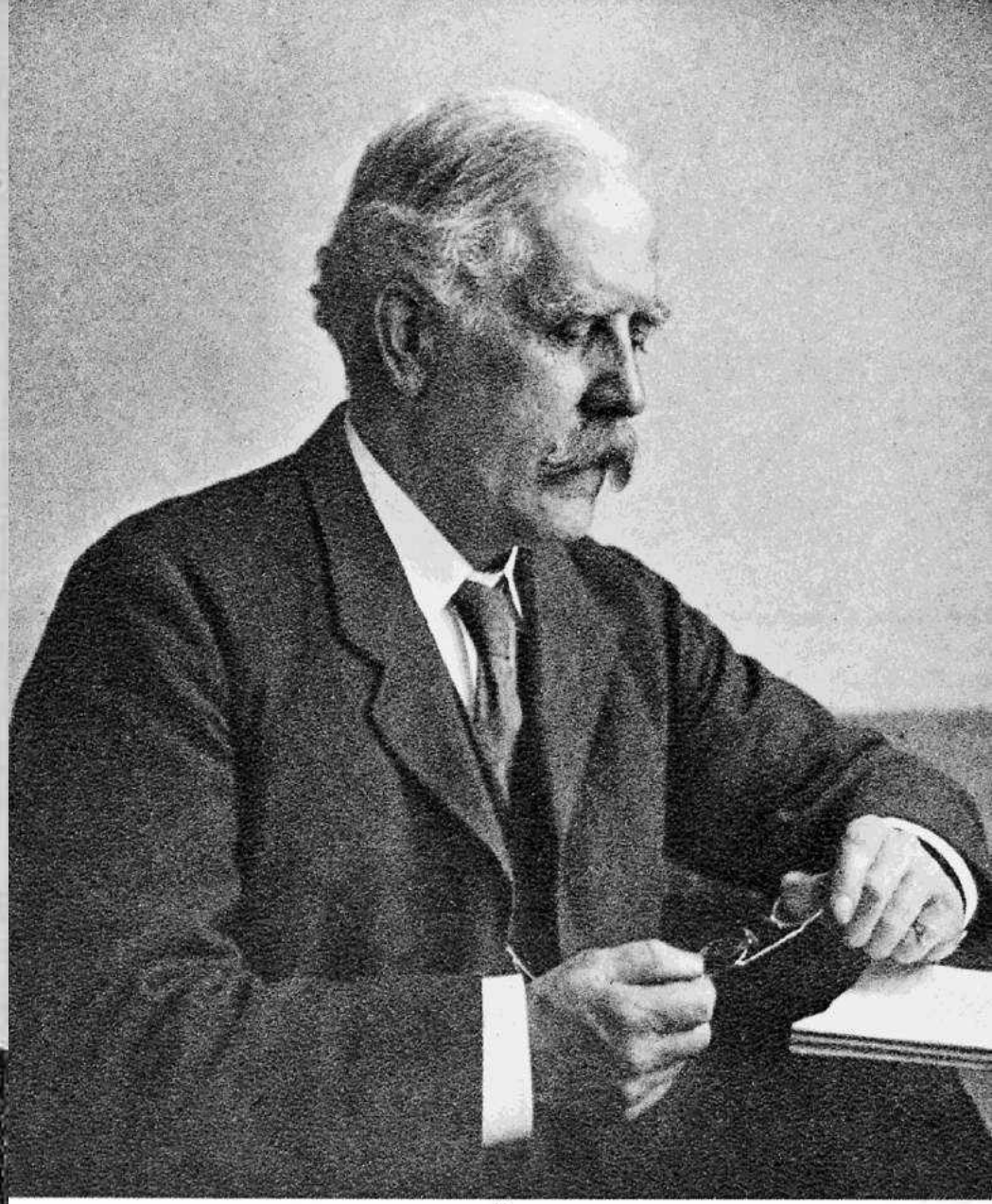


George Wells
Beadle
(1903 - 1989)

Edward Lawrie
Tatum
(1909 - 1975)

1941- "One gene one enzyme hypothesis"
1959 – "One gene one protein hypothesis"
1962- Vernon Ingram modification – "One gene one polypeptide hypothesis"
(Cistron is equivalent to gene)

VERNON INGRAM



A. E. Garrod.

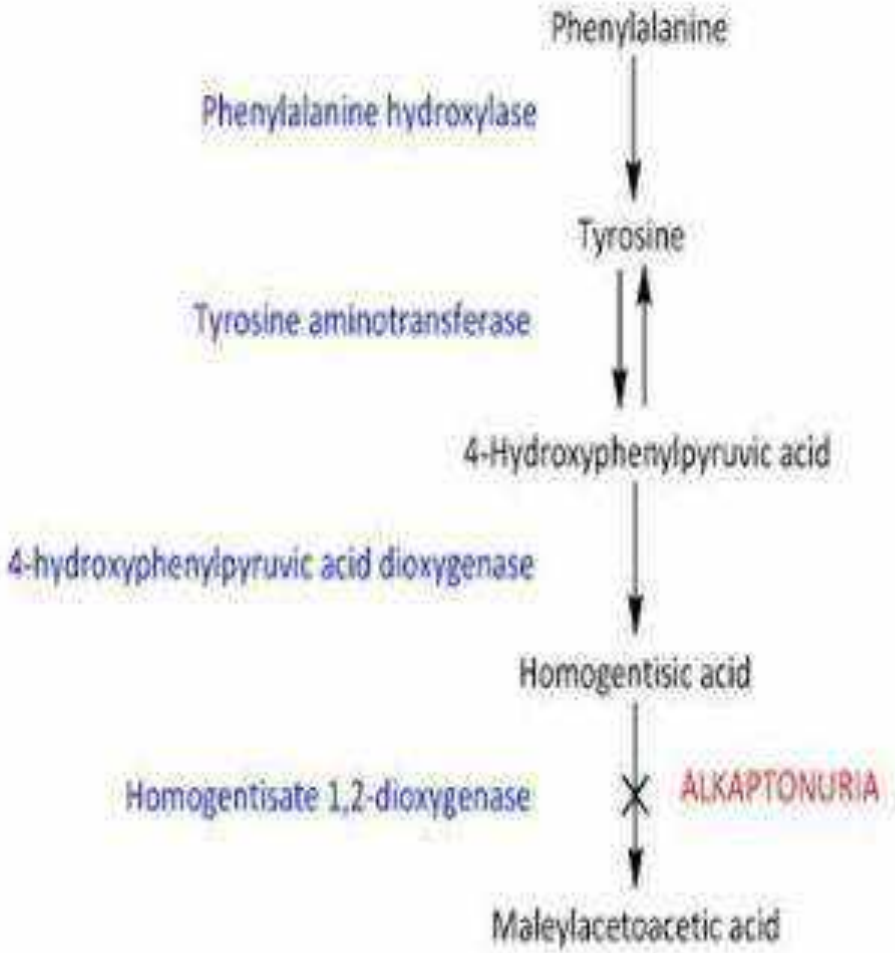
SNAPDRAGON

INTRODUCTION

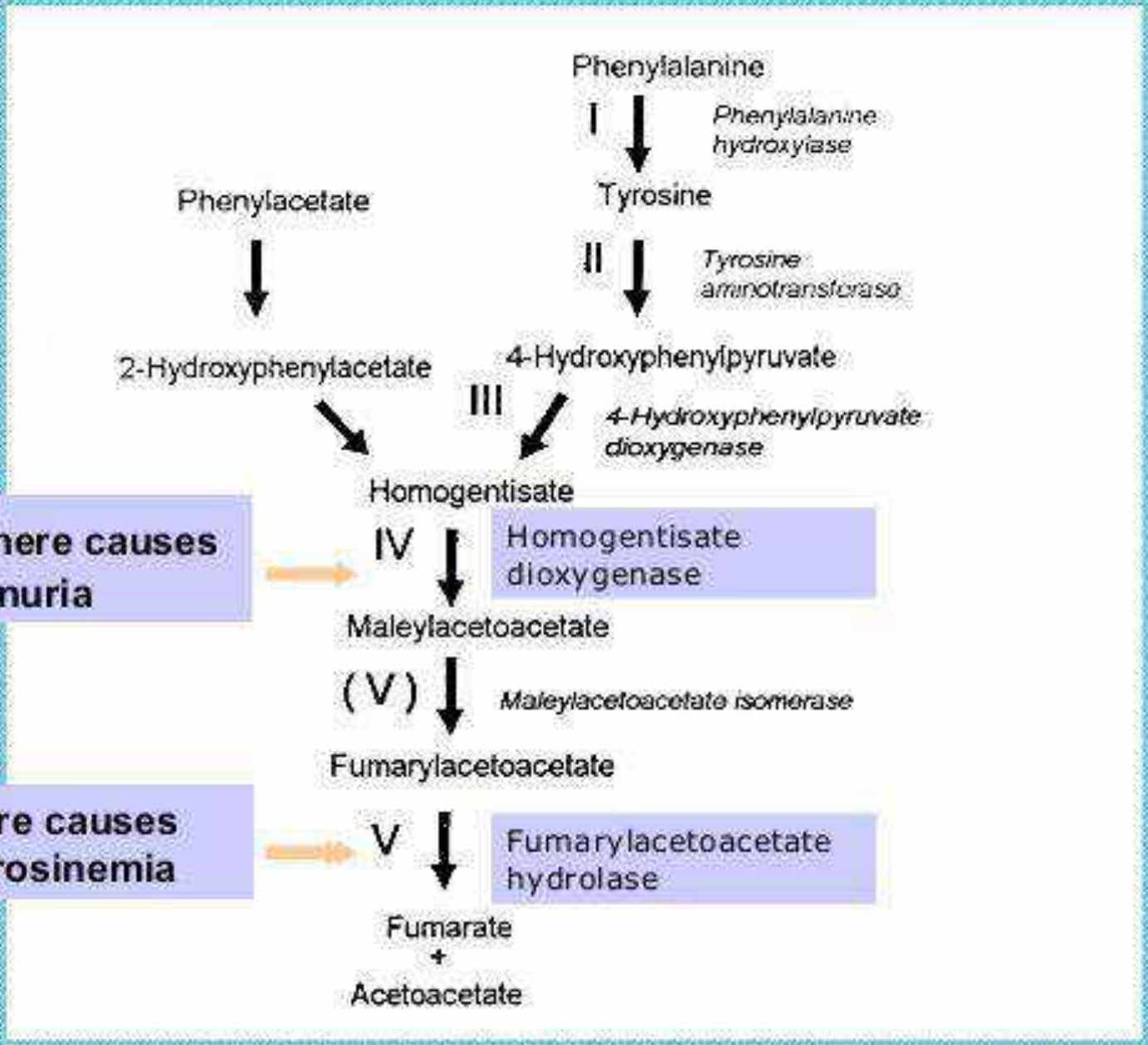
- Black urine disease or black bone disease is an inborn error of amino acid metabolism.
- It is a rare inherited genetic disorder of phenylalanine (Phe) and tyrosine (Tyr) metabolism.
- Autosomal recessive condition (both the parents must have the gene in order to pass to their progeny).

CAUSE

- Mutation or defect in HGD gene which causes lack of the enzyme homogentisate dioxygenase (HGD).
- This causes a build up of homogentisic acid (HGA) in the bones, cartilage and urine.
- HGA is an intermediate in the degradation pathway of the amino acids (Phe & Tyr) to the Krebs cycle.



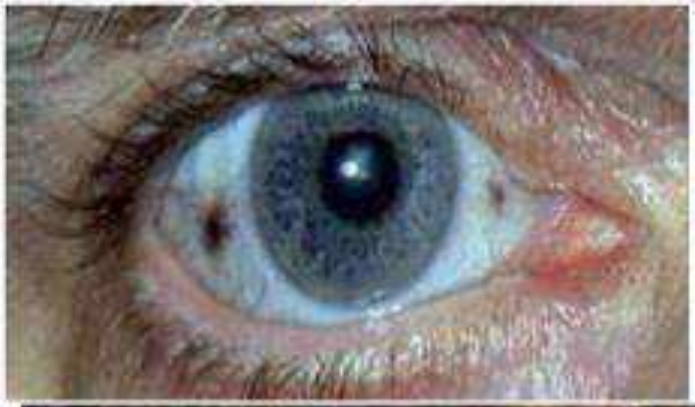
Catabolic pathway for phenylalanine and tyrosine

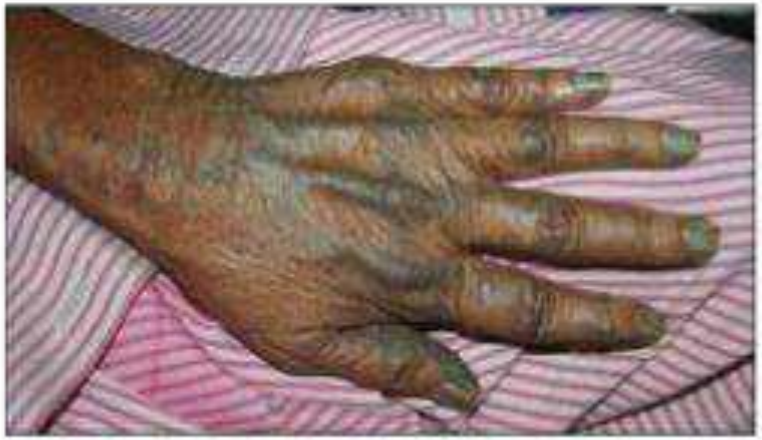


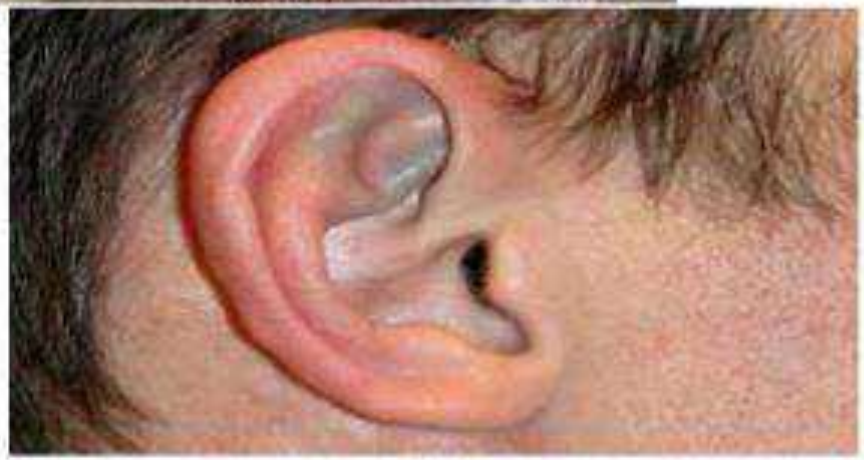
SYMPTOMS

- Urine becomes black when exposed to air.
- Osteoarthritis (mainly spine, hips, shoulders and knees).
- Black spots in the sclera of the eye (Ochronosis).
- Discolored ear and dark earwax.
- Heart valves are affected by the accumulation of HGA.
- Blue-black speckled discoloration of the skin.
- Kidney, prostate and bladder stones due to the build-up of HGA in the genito-urinary tract, during urine production.

Fig. 3: Comparison of Colour of Freshly Voided Urine and Urine after 24 Hours

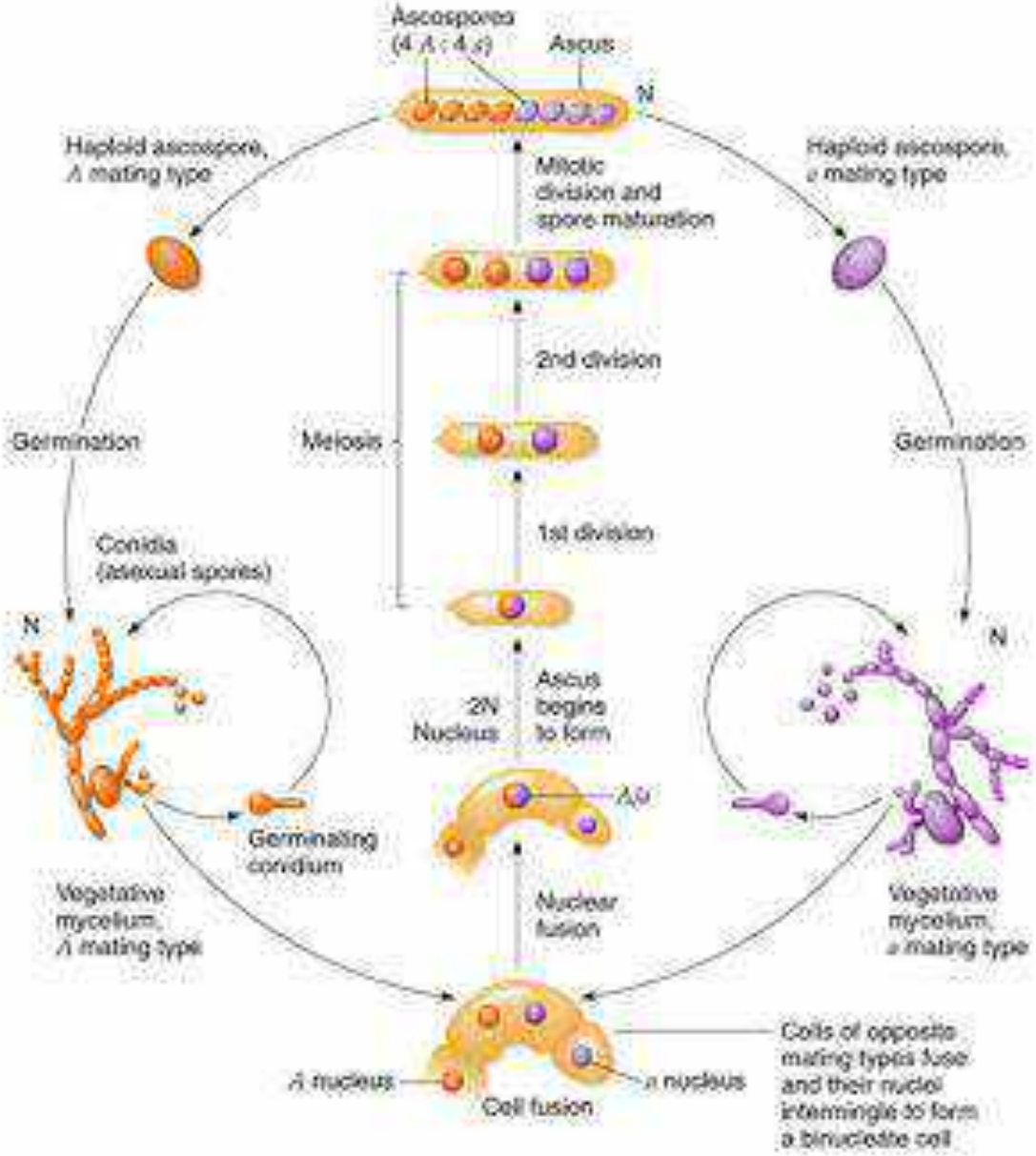




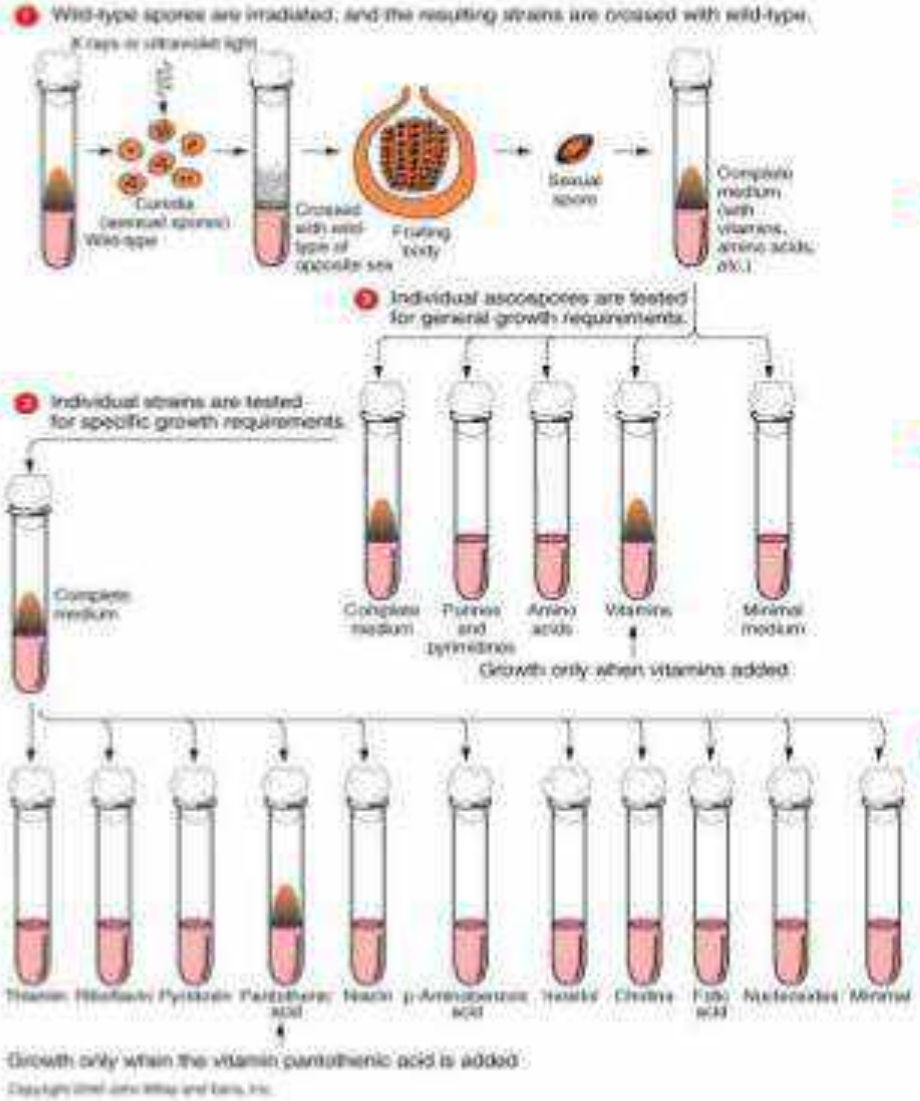



WiseGEEK

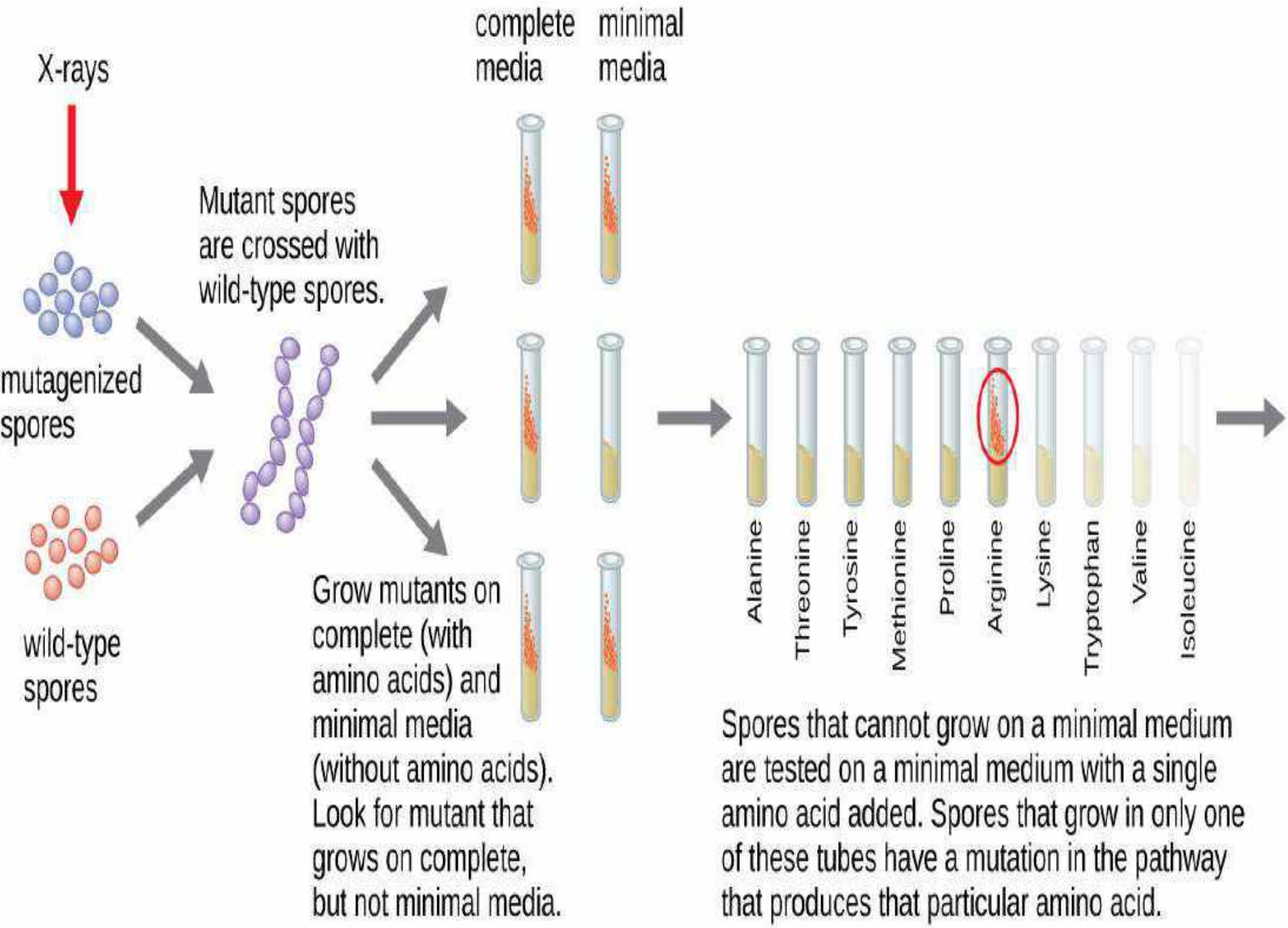
Neurospora Life Cycle

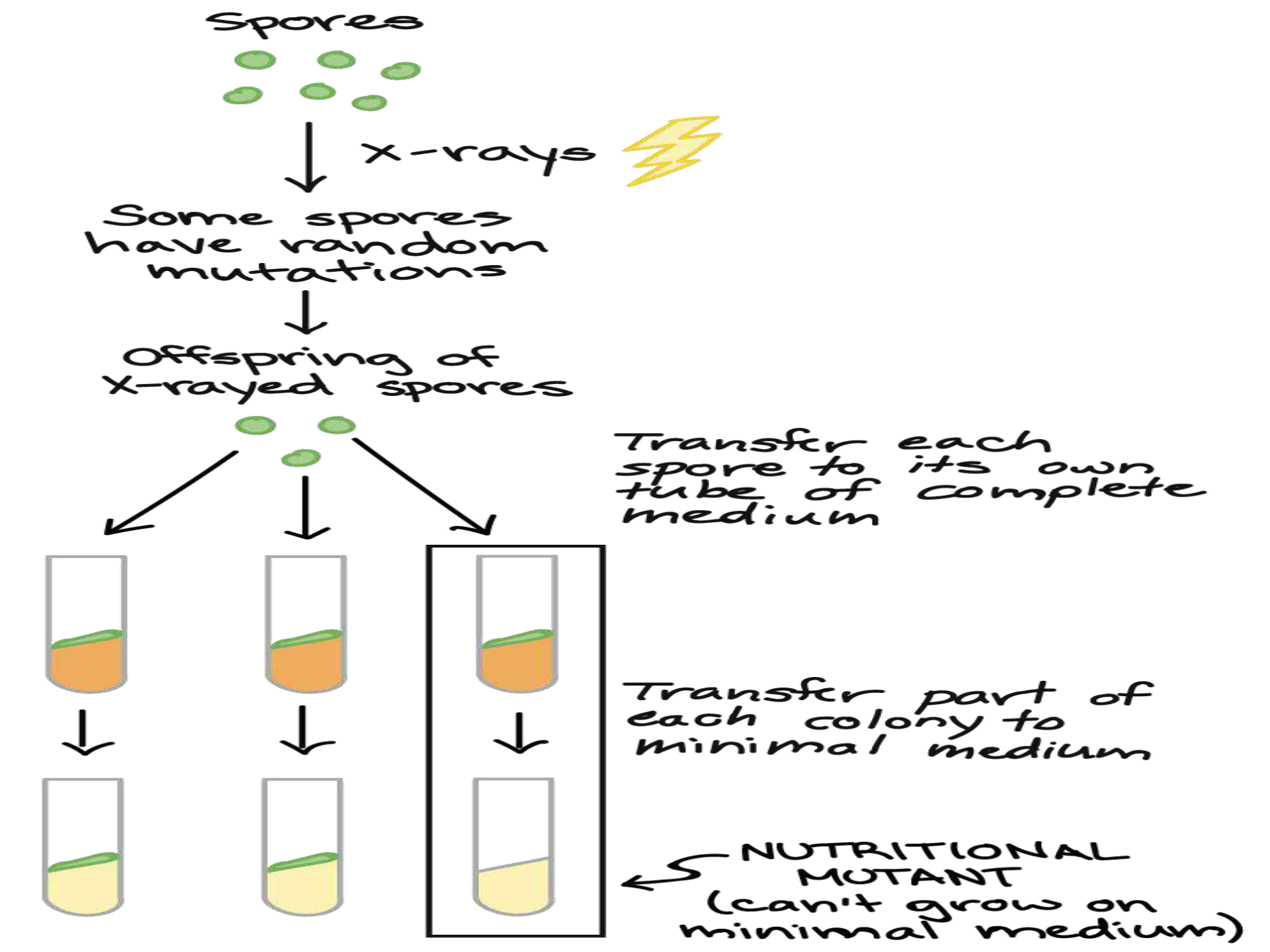


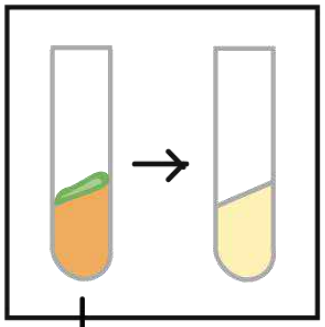
Beadle and Tatum (1942)--One Gene, One Enzyme



- Bread mold *Neurospora* can normally grow on minimal media, because it can synthesize most essential metabolites.
- If this biosynthesis is under genetic control, then mutants in those genes would require additional metabolites in their media.
- This was tested by irradiating *Neurospora* spores and screening the cells they produced for additional nutritional requirements (**auxotrophs**).


X-rays
mutagenized spores
wild-type spores
Mutant spores are crossed with wild-type spores.
complete media
minimal media
Grow mutants on complete (with amino acids) and minimal media (without amino acids). Look for mutant that grows on complete, but not minimal media.
Alanine
Threonine
Tyrosine
Methionine
Proline
Arginine
Lysine
Tryptophan
Valine
Isoleucine
Spores that cannot grow on a minimal medium are tested on a minimal medium with a single amino acid added. Spores that grow in only one of these tubes have a mutation in the pathway that produces that particular amino acid.


Spores
X-rays
Some spores have random mutations
Offspring of X-rayed spores
Transfer each spore to its own tube of complete medium
Transfer part of each colony to minimal medium
NUTRITIONAL MUTANT
(can't grow on minimal medium)



NUTRITIONAL MUTANT (can't grow on minimal medium)

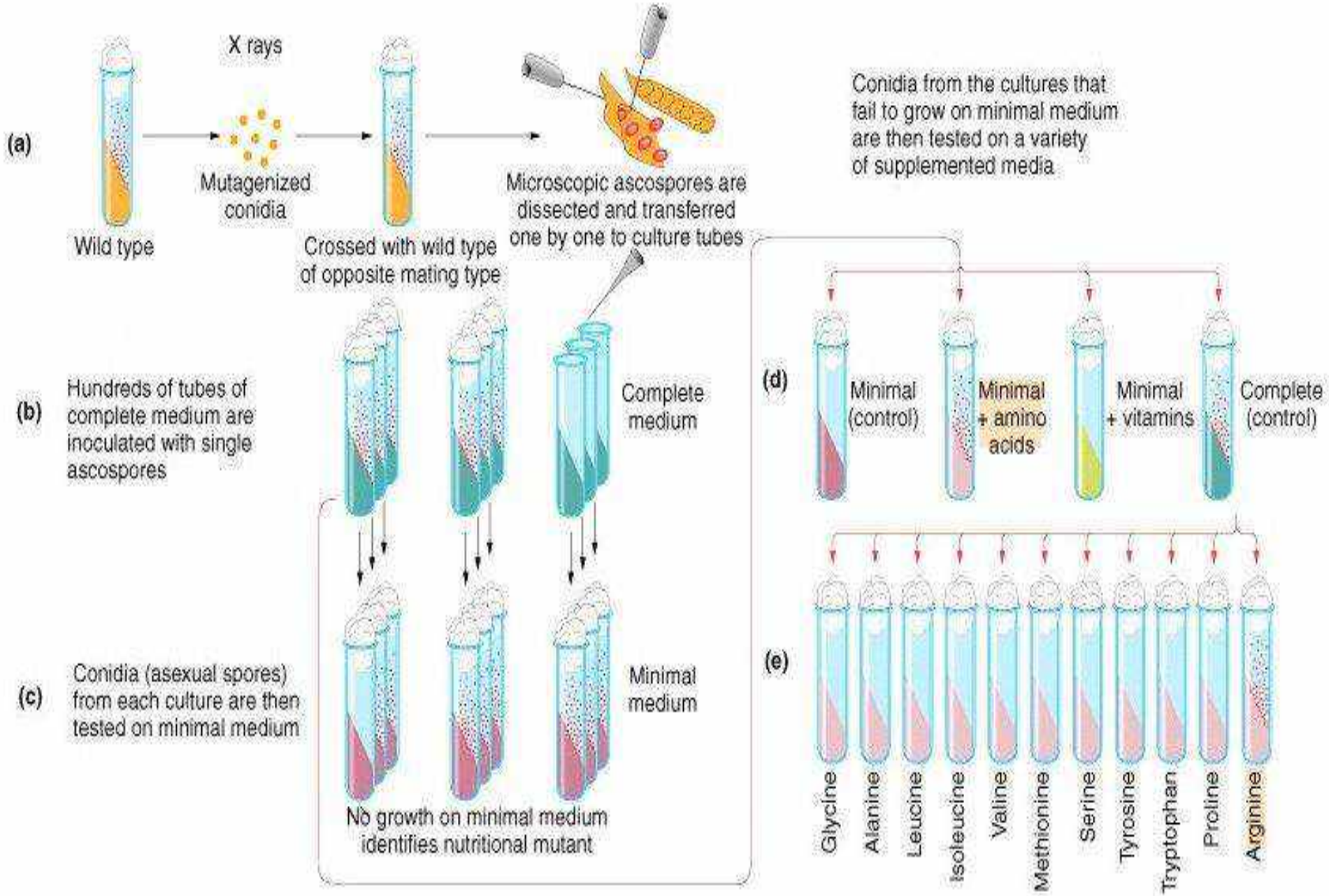
Minimal medium + full set of vitamins

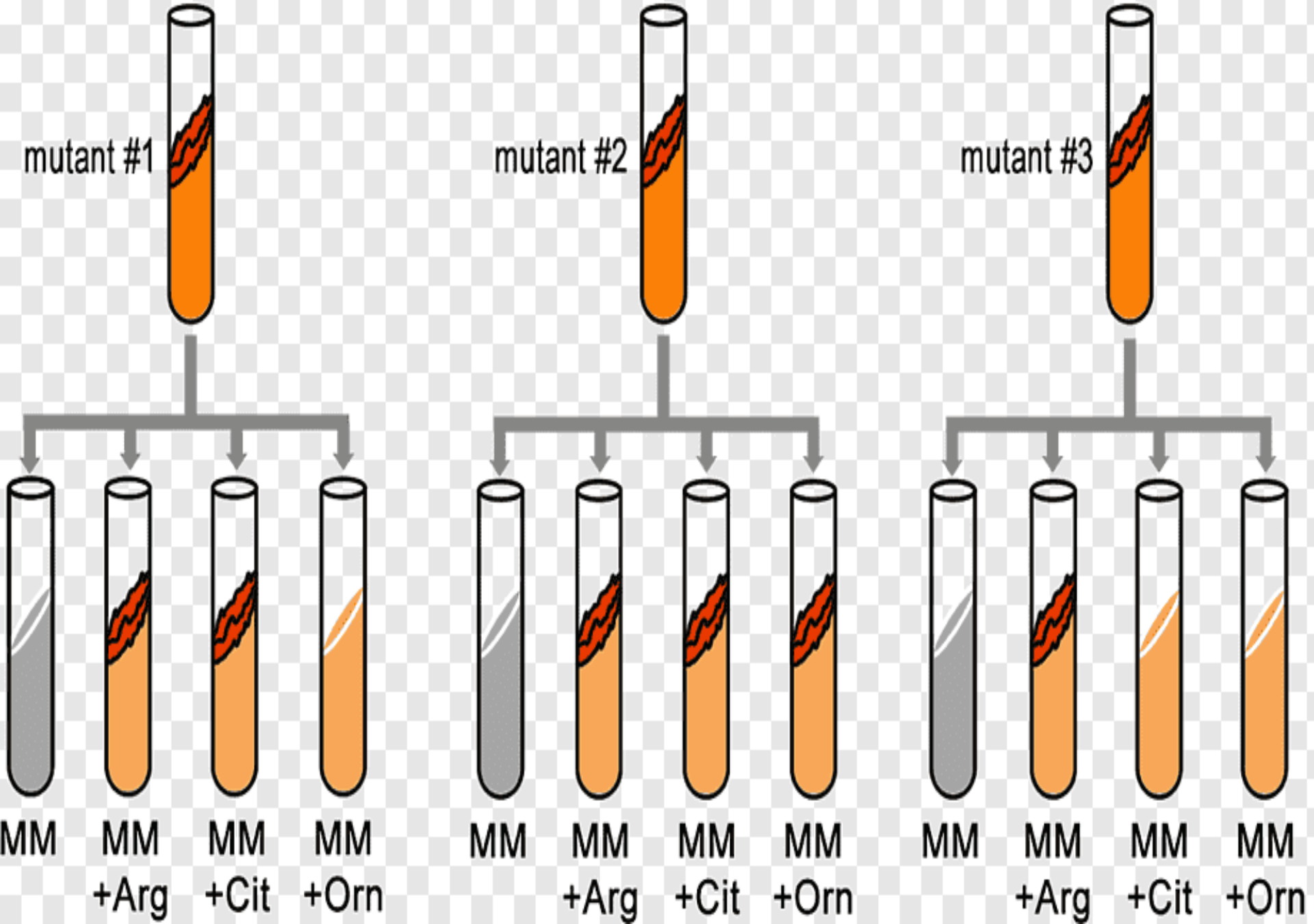
Minimal medium + 20 amino acids

Mutant is rescued by amino acid mix → mutation must block synthesis of one or more amino acids

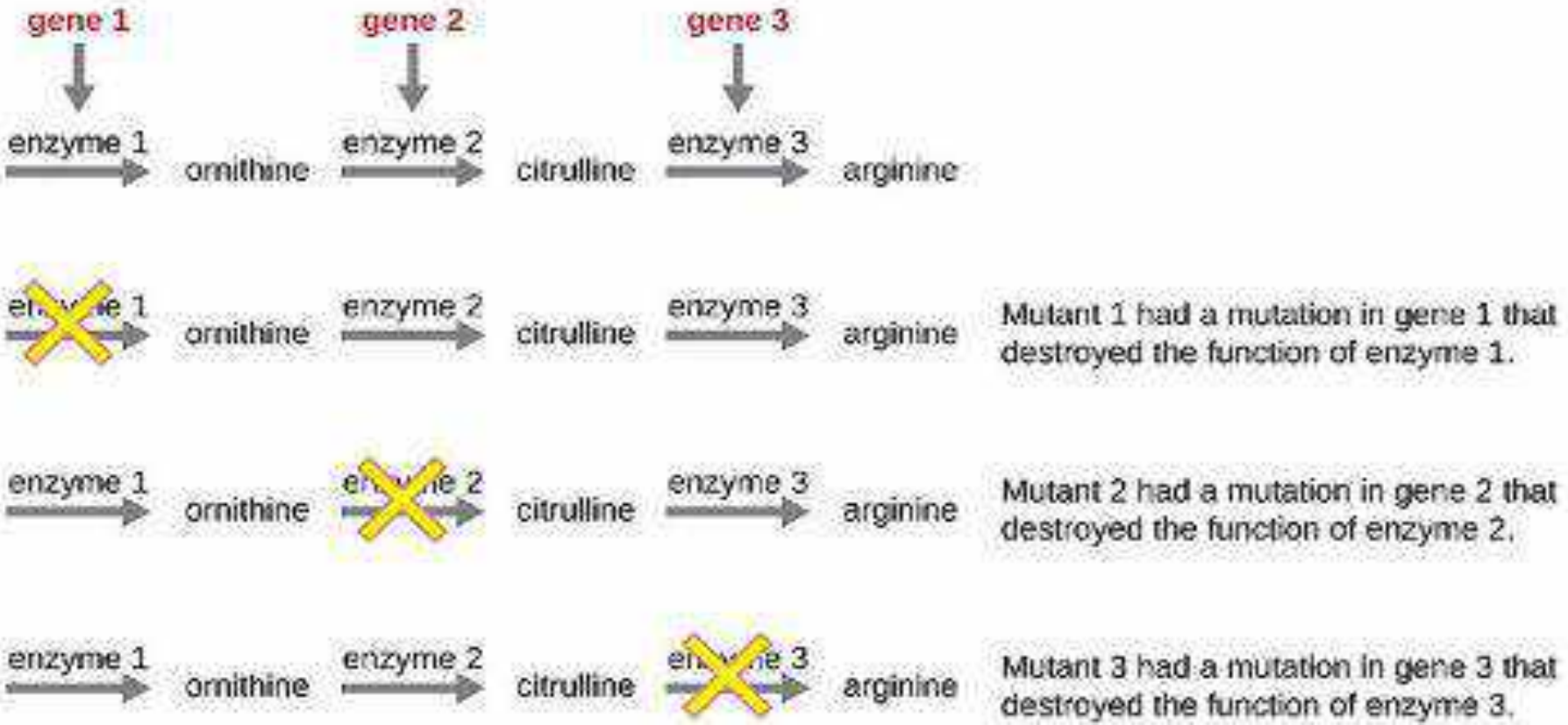
Gly Phe Ala His Trp Ser Met Arg Thr Leu Tyr Asp Glu Gln Asn Cys Val Ile Pro Lys

Mutant is rescued by arginine → mutation must disrupt arginine biosynthesis

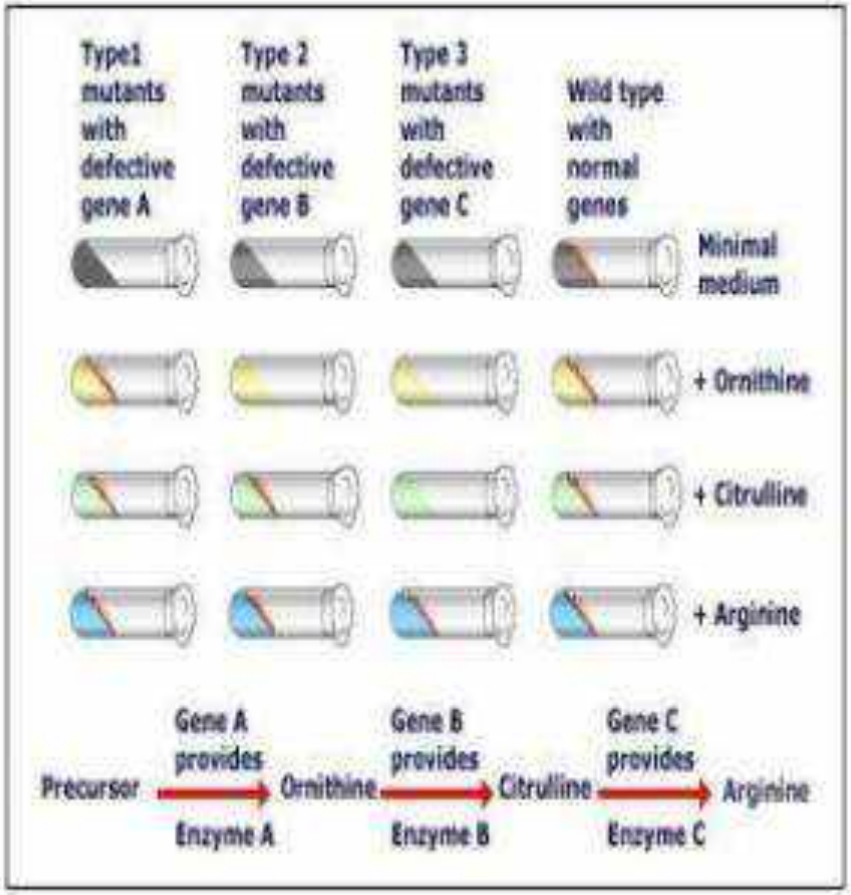

(a)
X rays
Wild type
Mutagenized conidia
Crossed with wild type of opposite mating type
Microscopic ascospores are dissected and transferred one by one to culture tubes
(b)
Hundreds of tubes of complete medium are inoculated with single ascospores
Complete medium
(c)
Conidia (asexual spores) from each culture are then tested on minimal medium
Minimal medium
No growth on minimal medium identifies nutritional mutant
Conidia from the cultures that fail to grow on minimal medium are then tested on a variety of supplemented media
(d)
Minimal (control)
Minimal + amino acids
Minimal + vitamins
Complete (control)
(e)
Glycine
Alanine
Leucine
Isoleucine
Valine
Methionine
Serine
Tyrosine
Tryptophan
Proline
Arginine
Addition of arginine to minimal medium restores growth


mutant #1
mutant #2
mutant #3
MM
MM +Arg
MM +Cit
MM +Orn
MM
MM +Arg
MM +Cit
MM +Orn
MM
MM +Arg
MM +Cit
MM +Orn

Beadle and Tatum Experiments				
Bread Mold	Minimal Medium (MM)	MM + Ornithine	MM + Citrulline	MM + Arginine
Wild type	grew	grew	grew	grew
Mutant 1	did not grow	grew	grew	grew
Mutant 2	did not grow	did not grow	grew	grew
Mutant 3	did not grow	did not grow	did not grow	grew



ONE-GENE-ONE POLYPEPTIDE HYPOTHESIS



- This hypothesis proposed by Ingram accounts for monomeric, dimeric enzymes & for non enzyme proteins.
- According to this hypothesis, a gene specifies a single polypeptide chain.