Lecture 3: Prokaryotic and Eukaryotic Cells

Introduction- Higher eukaryotes have multiple organs to perform specific functions such as liver, kidney and heart. Each Organ has specific tissue and each tissue is composed of cells. **"Cell is the structural and functional unit of life"** and it contains all necessary infrastructure to peform all functions. Based on cellular structure, cells are classified as prokaryotic and eukaryotic cells. In most of the cases, prokaryotes are single cells where as eukaryotes are either single cells or part of multicellular tissues system. Besides this, both types of cells have several structural and metabolic differences as given in Table 3.1 and are discussed later in the lecture.

TABLE 3.1 DIFFERENCE BETWEEN PROKARYOTIC AND EUKARYOTIC CELLS

Feature	Prokaryote	Eukaryote
Size	Small, in µm range	Variable size, upto 40µm in diameter.
Genetic material	Circular DNA present in cytosol as free material	DNA in the form of linear chromosome present in well defined double membrane nucleus, no direct connection with cytosol
Replication	Single origin of replication	Multiple origin of replication.
Genes	No Intron	Presence of Intron
Organelles	No membrane bound organelles	Membrane bound orgelles with well defined function.
Cell walls	Very complex cell wall	Except Fungi and plant, eukaryotic cells are devoid of a thick cell wall.
Ribosome	70S	80S
Trancription and translation	Occurs together	Transcription in nucleus and translation in cytosol

Structure of Prokaryotic cells- A prokaryotic cell is much simpler and smaller than eukarotic cells. It lacks membrane bound organelles including nucleus. A typical prokaryotic cells is shown in Figure 3.1, A. The description of different structural feature of prokaryotic cells is as follows-

1. Outer Flagella: A flagellum attached to the bacterial capsule is a central feature of most of the prokaryotic cell especially of the motile bacteria. It provides motion or locomotion to the bacteria and be responsible for chemotaxsis of bacteria. Movement of bacteria towards a chemical gradient (such as glucose) is known as chemotaxsis. Flagellum is a part of cell wall and its motion is regulated by motor proteins present inside the cell. Flagellar motion is an energy consuming process and it is governed by an ATPase present at the bottom of the shaft. It is made up of protein flagellin and reduction or suppression of flagellar protein reduces bacterial infectivity (pathogenicity) and ability to grow.

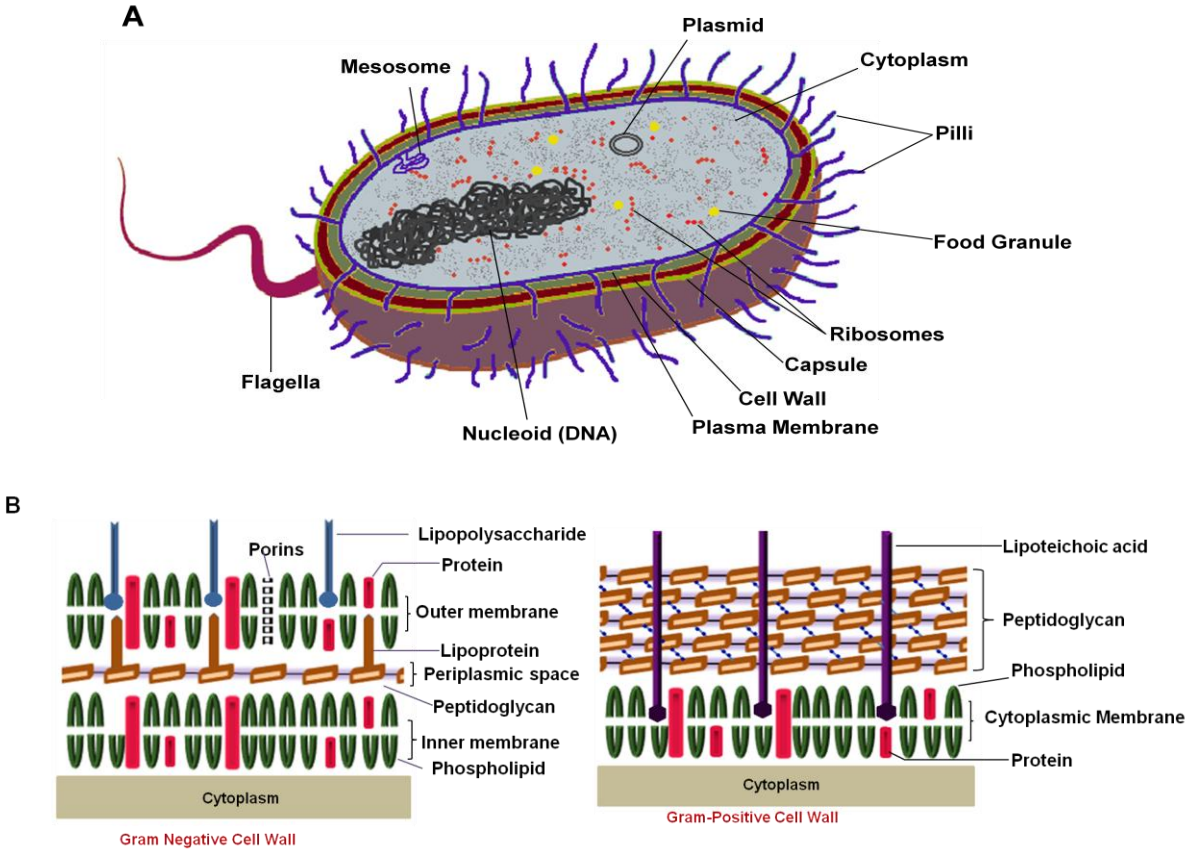


Figure 3.1: Structural details of a typical prokaryotic cell. (A) Whole cell and (B) composition of cell wall of gram negative and positive bacteria.

2. Bacterial surface layers: Bacteria posses 3 anatomical barriers to protect the cells from external damage. Bacterial capsule is the outer most layer and made up of high molecular weight polysaccharides. It is impermeable to the water or other aqueous solvent and it is responsible for antigenicity of bacterial cells. Cell wall in bacteria and its response to gram staining is the basis of classification of bacterial species.

WHAT IS GRAM STAINING? Gram staining is developed by a Danish scientist Hans Christian Gram. This technique differentiates bacterial strains based on their cell wall composition, especially thickness of peptidoglycan layer. A detail staining procedure is given in following paper (**Use of the gram stain in microbiology. Beveridge, TJ (2001) *Biotech Histochem* 76 (3): 111–8. Pubmed ID: 11475313**). During the staining procedure bacterial sample is stained with two dyes, **crystal violet** and **safarin**. During a washing step with non-polar solvents such as alcohol or acetone (decolorization), gram –ve bacteria leave the **blue** stain due to a thin peptidoglycan layer in cell wall whereas gram +ve bacteria retains both stains and appear as **Pink**.

Cell wall composition in gram-ve and gram +ve bacteria is different. Bacterial cell wall has different constituents and be responsible for their reactivity towards gram stain.

A. Peptidoglycan layer: peptidoglycan layer is thick in gram +ve bacteria and thin in gram –ve bacteria. Peptidoglycan is a polymer of NAG (N-acetyl-glucosamine) and NAM (N-acetyl-muramic acid) linked by a β-(1,4) linkage. Sugar polymer are attached to peptide chain composed of amino acids, L-alanine, D-glutamic acid, L-lysine and D-alanine. Peptide chain present in one layer cross linked to the next layer to form a mesh work and be responsible for physical strength of the cell wall. Peptidoglycan synthesis is targeted by antibiotics such as pencillin where as lysozyme (present in human saliva or tears) degrades the peptidoglycan layer by cleaving glycosidic bond connecting NAG-NAM to form polymer.

B. Lipoteichoic acids: Lipoteichoic acid (LTA) are only found in gram +ve bacteria cell wall and it is an important antigenic determinant.

C. Lipopolysaccharides (LPS)- Lipopolysaccharides (LPS) are found only in gram –ve bacterial cell wall and it is an important antigenic determinant.

3. Cytosol and other organelles-Prokaryotic cells do not contain any membrane bound organelle. The organelles are present in cytosol such as ribosome (70S), genetic material where as electron transport chain complexes are embedded within the plasma membrane.

4. Chromosome and extra chromosomal DNA-Prokaryote cell contains genetic material in the form of circular DNA, known as "**bacterial chromosome**". It contains genetic elements for replication, transcription and translation. Bacterial chromosome follows a rolling circle mode of DNA replication. The genes present on chromosome does not contains non coding region (introns) and it is co-translated to protein. Besides main circle DNA, bacteria also contains extra chromosomal circular DNA known as "plasmid". Presence of plasmid containing resistance gene confers resistance towards known antibiotics. Exchange of extra-chromosomal DNA between different bacterial strains is one of the mechanisms responsible for spread of antibiotic resistance across the bacterial population. Details of plasmid and its structural features will be discussed in a later lecture.

Struture of Eukaryotic cell- The eukaryotic cell is much more complex and it contains many membrane bound organelles to perform specific functions. It contains a nucleus isolated from cytosol and enclosed in a well defined double membrane. A typical eukaryotic animal and plant cell is shown in Figure 3.2 and the difference between these types of cells is given in Table 3.2.

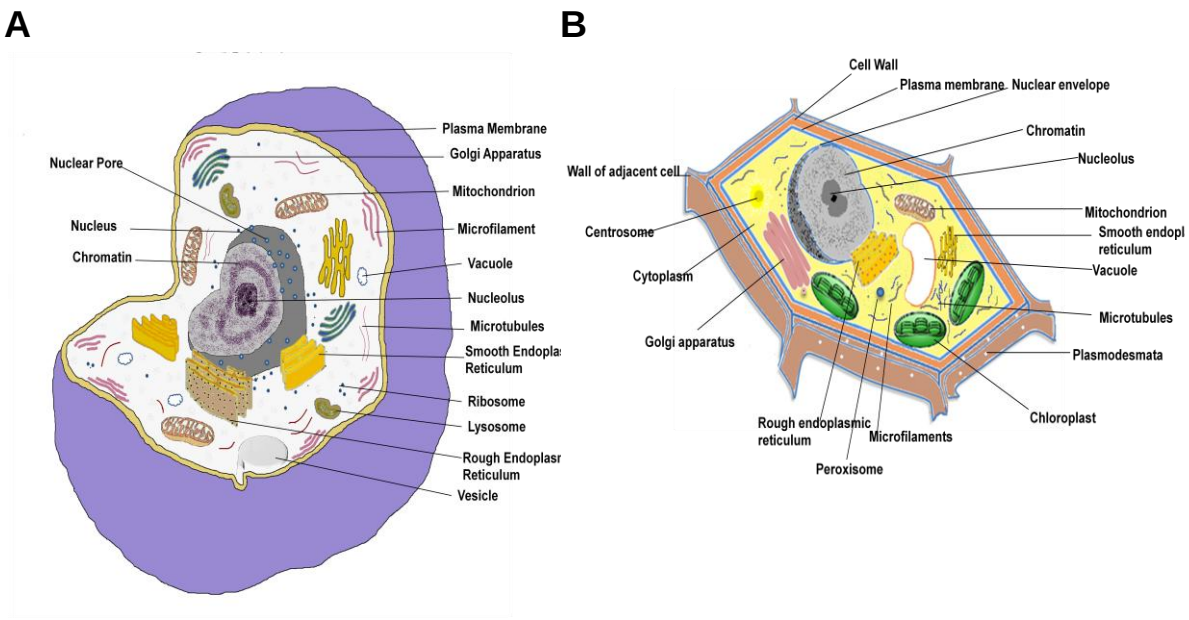


Figure 3.2 : Structure of Eukaryotic cell. (A) Animal Cell (B) Plant Cell

TABLE 3.2 DIFFERENCE BETWEEN ANIMAL AND PLANT CELLS

FEATURE	PLANT CELL	ANIMAL CELL
Cell wall	Present	Mostly absent
Size	Large	Comparatively small
Chlorophyll	Present	Absent
Vacuole	Large Central	Small and many in number
Mitochondria	Few	More
Lysosome	Almost absent	Present
Glyoxysomes	Present	Absent
Cytokinesis	By Plate method	By constriction

The description of different structural feature of eukaryotic cell is as follows-

Different organelles of Eukryotic cells (Animal)

1. Cytosol-Cytosol is the liquid part filled inside the cell and it contains water, salt, macromolecules (protein, lipid, RNA). It has an array of microtubule fiber running through out the cytosol to give vesicular structure to its destination. Besides this, cytosol exhibits "Sol" to "Gel" transition and such transition regulates multiple biochemical and cellular processes.

2. Nucleus-Nucleus is the central processing unit of cell and homologous to the processor in a typical computer (Figure 3.3, A). The liquid filled inside nucleus is called as **nucleoplasm**. It is a viscous liquid containing nucleotides and enzymes to perform replication, transcription, DNA damage repair etc. It contains genetic material (DNA) in a complex fashion involving several proteins (histones) to pack into nuclear bodies or chromosomes. The chromatin in eukarotic nucleus is divided into **euchromatin** or **heterochromatin**. Euchromatin is a part of chromatin where DNA is loosely packed and it is transcriptionally active to form mRNA where as Heterochromatin is more densily packed and it is transcriptionally inactive. Nuclei in eukarytotic cells are present in a double layer of membrane known as **nuclear envelope** (Figure 3.3, B). Outer membrane of nuclear envelope is continuous with the rough endoplasmic reticulum and has ribosome attached to it. The space between these two membranes is called as **perinuclear space**. Nuclear envelope often has **nuclear pore** and as per calculation an average nucleus has 3000-4000 pores per nuclear envelope.

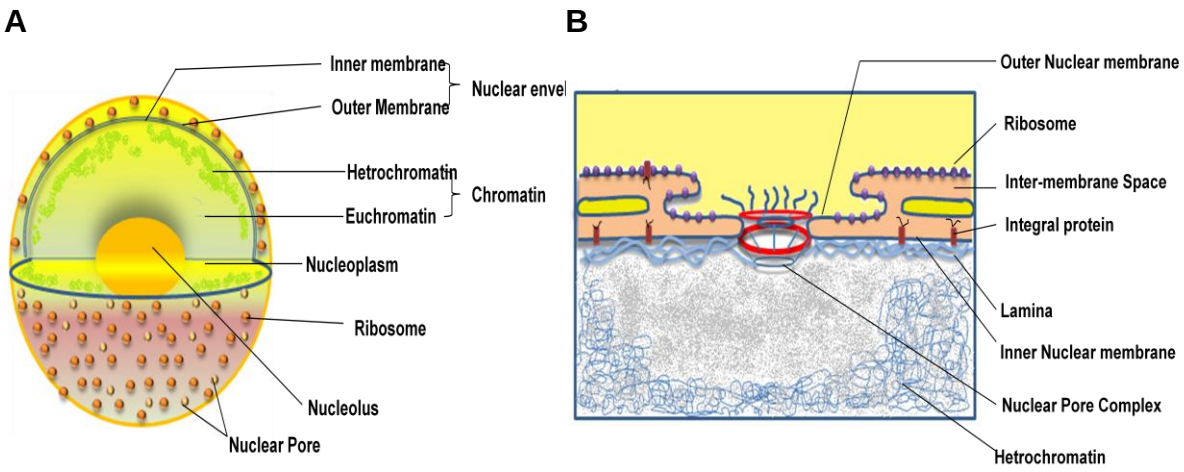


Figure 3.3: Structural details of nucleus. (A) whole and (B) enlarged view of nuclear pore.

Nuclear pore is 100nm is diameter and consists of several proteins. It is a gateway for transfer of material between nucleus and cytosol. RNA formed after transcription from DNA within the nucleus and move out of the nucleus into the cytosol through nuclear pore. Similarly protein from cytosol crosses nuclear pore to initiate replication, transcription and other processes.

Lecture 4: Prokaryotic and Eukaryotic Cells (Part II)

Summary of Previous Lecture: In the previous lecture we discussed the structure of prokaryotic cells, differences between prokaryotic and eukaryotic cells and lastly we started the discussion about the structure of eukaryotic cells. In continuation to previous lecture, in the current lecture we will discuss remaining cellular organelles of eukaryotic cells.

1. Mitochondria- It is popularly known as "**power house of the cell**" as the organelle is actively involved in the generation of ATP to run the cellular activities. Mitochondria is a double layered membrane-bound organelle with different structural properties (Figure 4.1, A). Outer membrane is smooth and cover the complete organelle with large number of integral proteins, known as **porins**.

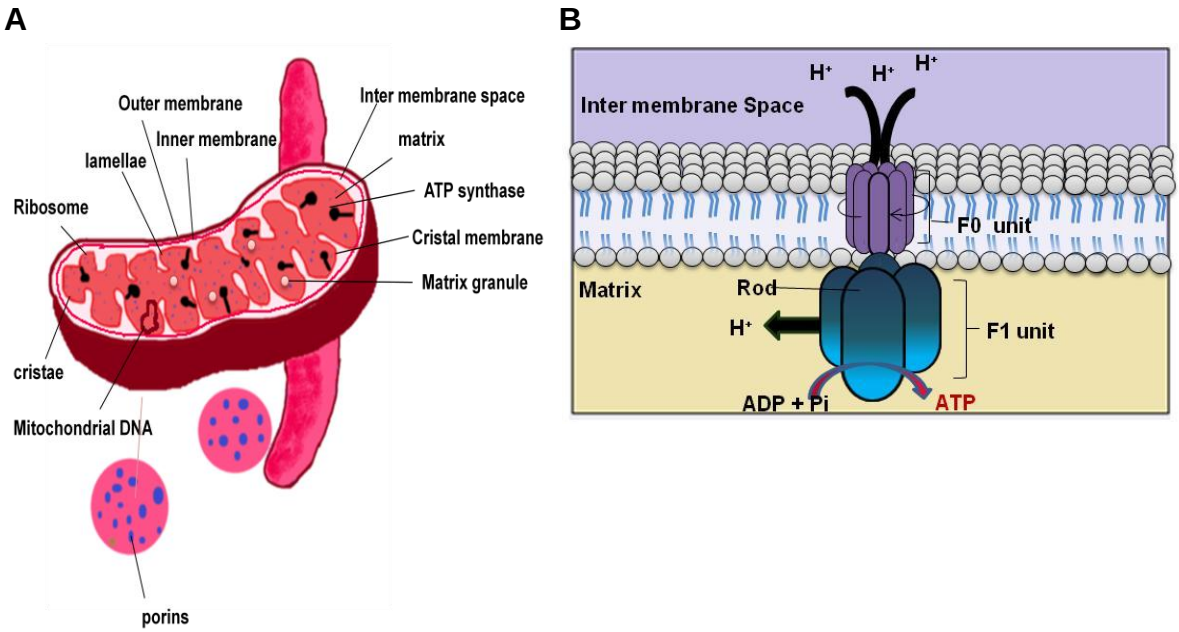


Figure 4.1: Mitochondria. (A) Struture of mitochondria and (B) enlarged view of ATP Synthase.

Porin allows free movement of molecules less than 5000da within and outside mitochondria. Where as larger molecules or proteins moves into the mitochondria through transporters involving signal peptides known as "**mitochondrial targeting sequence**". Inner membrane is folded into membrane projections to form **cristae**. Cristae occupies major area of membrane surface and house machinery for anaerobic oxdidation and electron transport chain to produce ATP. Due to presence of inner and outer membrane, mitochondria can be divided into 2 compartments: first in between the inner and outer membrane, known as **intermembrane space** and second inside the inner membrane known as **matrix**. The proteins present in intermembrane space have a role in executing "**programmed cell death**" or "**apoptosis**". Matrix is the liquid part present in the inner most compartment of the mitochondria and it contains ribosome, DNA, RNA, enzymes to run Kreb's cycle and other proteins. Mitochondrial DNA is circular and it has full machinery to synthesize its own RNA (mRNA, rRNA and t-RNA) and proteins. Marked differences exist between mitochondrial DNA and DNA present in nucleus and these differences are not discussed here due to space constrain. Electron transport chain components (complex I to complex V) are integral proteins, present in the inner membrane of mitochondria. During metabolic reactions such as glycolysis, Kreb's cycle [metabolic reaction are discussed later] produces large amount of reducing equivalent in the form of $NADH_2$ and $FADH_2$. Electron transport chain process reducing equivalent and flow of the electron through different complexes (Complex I to Complex IV) causes generation of proton gradient across the membrane. Proton expelled in the intermembrane space returned back to the matrix through complex V (ATP synthase) to generates ATP. ATP synthase (Figure 4.1, B) is a mushroom shaped multimeric protein complex, mainly composed of two proteins Fo and F1. Fo is a membrane bound portion where as F1 is the complex present into the lumen towards matrix. F_oF_1 complex of mitochondria harvest the proton motive force to catalyze phosphorylation reaction involving ADP and phosphate to generate ATP.

Functions of mitochondria-

1. Production of ATP

2. Generation of **Reactive Oxygen Species** (ROS) in immune cells to kill infectious agents.

3. Used to track tree of a family.

3. Role in programmed cell death or **"apoptosis"**

Apoptosis: Apoptosis is the programmed cell death involving a series of events involving cellular metalloprotease known as caspases. In an adverse event of exposure of cell to the cyto-toxic agent or environmental condition, it activates cell surface signaling to activate cytosolic caspases. In addition, it disturbs mitochondrial membrane potential to cause the release of CytC. Ultimately, these cellular events activates DNase activity within nucleus and degrade genominc DNA to cause cell death.

2. Chloroplast-Chloroplasts are found in plant, algae and other lower invertebrates such as euglena. Contrasting to mitochondria, chloroplast has outer membrane, an inner membrane and then light pigment containing inner most thylakoid membrane (Figure 4.2, A). Outer membrane is porous to the small molecules but protein or large molecules are transported by **TOC** (translocon on the outer chloroplast membrane) complex. Movement of material passed through outer membrane gets into the inner membrane through **TIC** (translocon on the inner chloroplast membrane) complex. In between outer and inner membrane is intermembrane space filled with aqueous liquid.

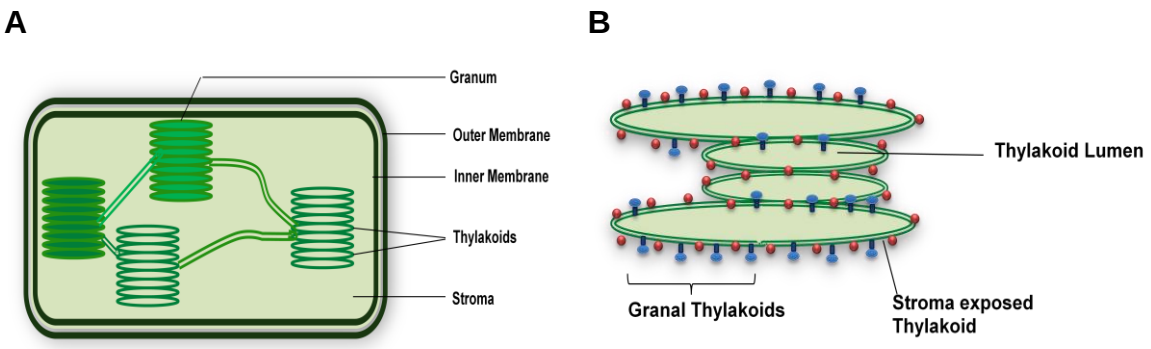


Figure 4.2: (A) Struture of Chloroplast, (B) Arrangement of thylakoid membrane in chloroplast.

The inner membrane of the chloroplast further folds to a flattend membrane system known as **thylakoids**. The photosynthsis machinery such as light absorbing pigments, electron carriers and ATP synthesizing machinery is present on inner membrane as intergral protein complex. Thylakoid membranes are arranged like stack of coin to form **granum** (Figure 4.2, B). The granum throughout the chloroplast are connected by tubule to share the material. Over-all structure of chloroplast is similar to mitochondria but it has few significant structural and biochemical differences. Thylakoid membrane contains photosynthetic green colored pigment chlorophyll.

$$6CO_2+6H_2O+\text{Solar Energy}\rightarrow C_6H_{12}O_6+6O_2 \quad \text{.....(4.1)}$$

Photosynthesis is an assimilation reaction involving CO_2 and water to produce sugar in the presence of solar energy (photons) that catalyzes fusion reaction as given Eq. 4.1. The photo system present on thylakoid membrane consists of two photo system, **photo system-I (PS-I)** and **photo system complex II (PS-II)**. PS-II absorbs the photon from solar energy to excite the electron to the higher energy state, and catalyze water break down into the proton and oxygen. The electron pass through multiple electron carrier and during this proton are exported out of the thylakoid membrane into the lumen. The proton passes through **ATP synthase** and returns back into the stroma to generate ATP. The electron from PS-II is eventually been received by PS-I and been excited after absorbing photon from sun light to high energy state. The energy associated with these electrons are used to generate NADPH in the stroma. Hence as a result of photosynthesis, solar energy is trapped by photo synthesis apparatus to generate **ATP** and **NADPH** into the lumen. Both of them are used to run **Calvin cycle** to assimilate environmental CO_2 to form sugar.

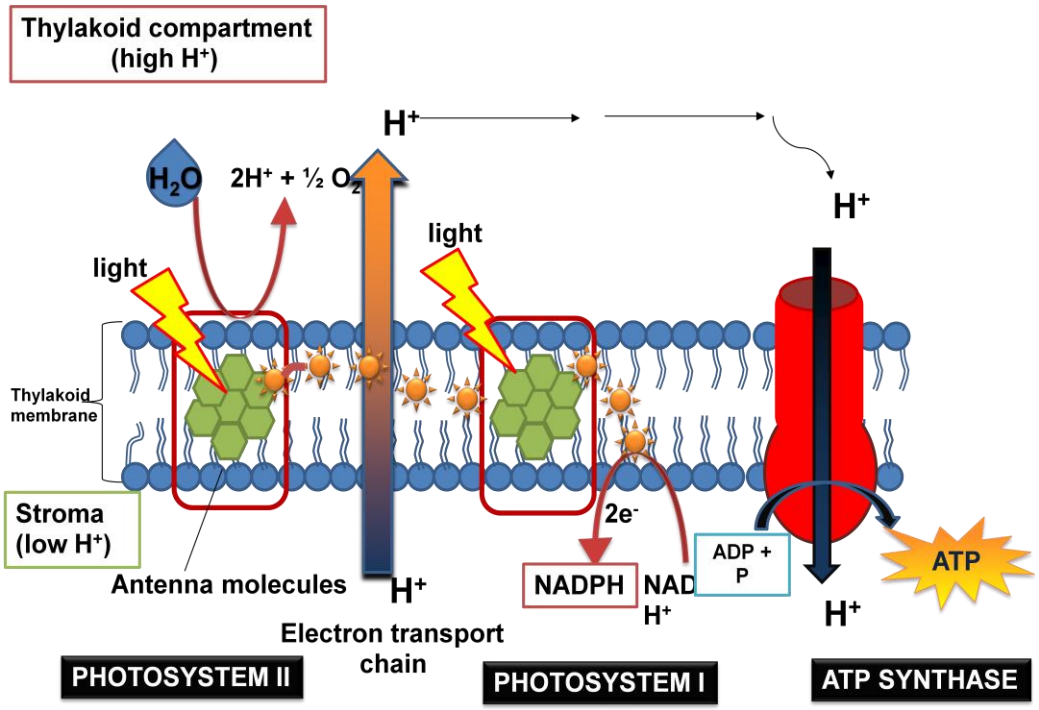


Figure 4.3: Different Steps of Photosynthesis.

5. Organelles of Vesicular Trafficking System: The main function of these organelles is to manage the distribution of material (food particles or proteins) throughout the cell. 3 different organelles such as endoplasmic reticulum, Golgi apparatus and lysosome, co-ordinately work together to maintains vesicular transport of material across the cell (Figure 4.3). Eukaryotic cell takes up the solid material from outside through a process called "**endocytosis**" whereas uptake of liquid is through a process called as "**pinocytosis**". Similarly material is secreted out of the cells through "**exocytosis**". In addition, intravesicular system delivers protein synthesized in endoplasmic reticulum to different organelles.

During endocytosis, material present outside the cells binds to the cells surface through cell surface receptors and trap it in a membraneous structure called as **endosome**. Endosomal vesicles are fused with the lysosomes to form late endosome. In late endosome, with the help of lysosomal enzymes material is digested and then endosome is fused with the Golgi bodies and deliver the content for further distribution. In the similar manner, during secretion, vesicles originate from Golgi bodies and fuse with the plasma membrane to release the content outside of the cell.

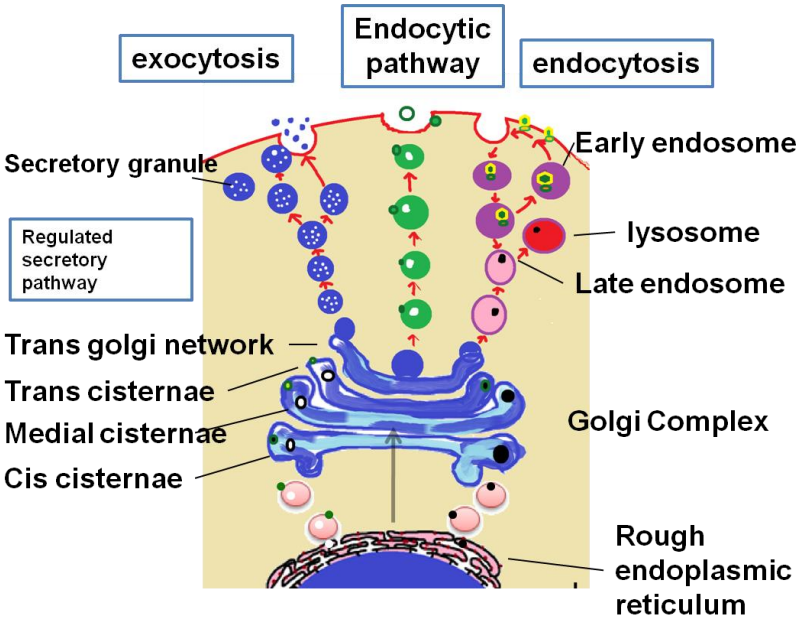


Figure 4.3: Intra cellular vesicular trafficking system of cell.

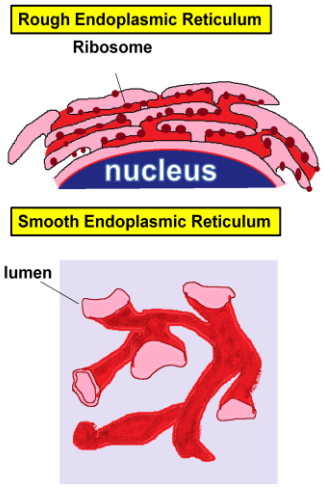


Figure 4.4: Endoplasmic reticulum.

Endoplasmic Reticulum- The vesicular network starts from nuclear membrane and spread throughout the cytosol constitutes endoplasmic reticulum (Figure 4.4). There are two different types of endoplasmic reticuli present in the cell, 1) Rough endoplasmic reticulum (RER), and 2) smooth endoplasmic reticulum (SER). RER has ribosome attached to it to give a rough appearance whereas smooth endoplasmic reticulum is devoid of ribosomes. Protein synthesis on ribosome attached to RER are sorted into 3 different catagories, such as integral membrane proteins, proteins for secretion and protein destined for different organelles. Proteins are synthesized with a n-signal peptide and these signal peptides are recognized by signal recognition particle on their the target organelles. For example, if a protein is synthesized with a signal peptide for mitochondria, it will attach to signal recognition particle and receptor onto the outer mitochondrial membrane to deliver the protein. The proteins without any signal peptide tags are supposed to remain in the cytsol.

Functions of endoplasmic reticulum:

1. Synthesis of steroid hormone in gonad cells.
2. Detoxification
3. Ca^{2+} sequestration
4. Synthesis of protein, phospholipid and carbohydrate.
5. Protein sorting to different organelles.
6. Protein modifications such as glycosylation etc.

Golgi Bodies- Golgi bodies were first visualized by a metallic stain invented by **Camillo golgi** and it is made of flattend, disk like cisternae arranged in a stacked manner to give 3 distinct zones (Figure 4.5). **Cis-face** receives material or vesicles from endoplasmic reticulum, **medial Golgi** is the actual place where protein are covalently modified with the sugar. **Trans Golgi** is the face of Golgi towards plasma membrane and this site sorts vesicle for their destined organelles or plasma membrane.

Functions of golgi bodies

1. Protein sorting

2. Protein modifications (Glycosylation)

3. Proteolysis

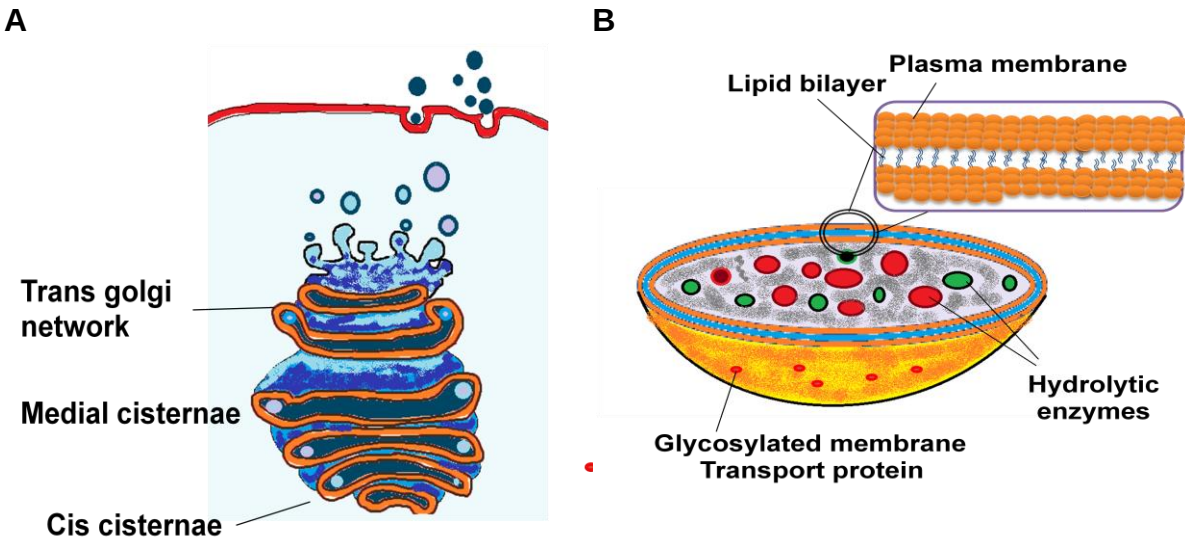


Figure 4.5: Scematic structure of (A) Golgi bodies and (B) Lysosome.

Lysosomes-Lysosomes are discovered by De Duve. They are membrane bound organelles and an important component of intracellular vesicular system (Figure 4.5). They are popularly known as suicidal bags due to their role in autophagy, a cellular process probably operates in cells during starvation to meet their energy requirements. [for more details of molecular mechanism of autophagy and underlying signaling mechanism could be find here: **Annu Rev Genet. 2009;43:67-93. Regulation mechanisms and signaling pathways of autophagy**]. Lysosome lumen is extremely acidic and contains protease, cytolytic enzymes to degrade the ingested material.

Functions of lysosomes

1. Degradation of ingested food material for delivery through vesicular system.

2. Degradation of pathogenic bacteria

3. Degradation of old protein.

Quiz

Q1: Which organelle is the destination for destruction of cellular proteins ?

Answer: Lysosome. The proteins that are either aged or misfolded are sent to lysosome for degradation. Lysosome with the help of acidic environment and proteases degrade protein into the smaller peptide. In addition, bacteria or other pathogenic organism also follows the same path to degrade into smaller pieces for antigen presentation in immune cells. Besides lysosome mediated protein degradation, proteins targeted for degradation are also sent to the proteasome complex. Proteasome complex is a non-membranous multimeric protein complex present in cytosol as free particles and they identify and degrade aged and misfolded proteins. **[Student can refer to following article for further details of proteasome: Structural biology of the proteasome. Annu Rev Biophys. 2013;42:29-49. doi: 10.1146].**

Q2: Which organelle can be visualzed by basic dye Hematoxylin ?

Answer: Nucleus. Nucleus contains genomic DNA (deoxy-ribonucleic acid) and then internal pH is acidic. As a result hematoxylin concentrates into the nucleus and visualize genomic DNA. It can also stain circular DNA in mitochondria but sensitivity of the dye is not optimal for visualization of mitochondrial DNA.

Q3: Treatment of mitochondria with molecule X destroys the proton gradient. These molecular are called as …….

Ans: Uncoupler. The electron transport chain consists of 4 complexes involved in relaying electron and complex V for harvesting proton gradient. Any molecule which can destroy the membrane permeability via making pores will eventually destroy the protein gradient and ultimately affects the ATP production.

Q4. Decribe the structural details of the molecular complex responsible for harvesting proton gradient in the mitochondria?

Ans: ATP Synthase. Please go through the structural details of ATP synthase from web and attempt to collect the information to describe the structure of ATP synthase.

Lecture 5: Metabolism-I: Glycolysis

Introduction: In the previous lecture we discussed the structure of prokaryotic and eukaryotic cells. Cellular integrity is maintained at the expense of energy produced by a set of chemical reactions, collectively known as metabolism. It is a summation of two different types of chemical processes:

Anabolism, the reactions which are responsible for formation of new compounds. It is alternatively known as biosynthetic pathway.

Catabolism, the reactions which are responsible for utilization of organic nutrients to produce energy in the form of ATP, NADH, FADH. ATP is the readily available form of energy whereas NADH and FADH needs to go mitochondria for ATP generation. Although carbohydrate, protein and fat undergo catabolism to produce energy but carbohydrate is most preferred choice for this purpose and henceforth topic of choice to discuss in the current course.

Carbohydrate Metabolism- Post digestion, food material is digested into the amino acid, fatty acid and glucose. All these final digestion products are absorbed by intestine and enter into the blood stream. Glucose enters into blood and distribute to the different organs for storage purpose but liver is the prime site for storage. Glucose is converted into the glycogen with the help of an enzyme **glycogen synthase**. Glucose is oxidized into the glycolysis and Kreb's cycle to produce ATP and other reducing equivalent to produce energy.

Glycolysis- Glycolysis is central to carbohydrate metabolism and it is the universal pathway found in prokaryotic or eukaryotic cells. It is a breakdown of 6 membered glucose into two 3 membered carbon suger to feed Kreb's cycle (in the presence of oxygen) or to send for anaerobic oxidation (in the absence of oxygen). Hence, it plays a crucial role for adopation of a living organism under differet types of stress conditions. The glycolysis is a 10 step chemical reaction to enable glucose for its optimal oxidation. All these reactions are given in Figure 5.1.

STEP-1: Phosphorylation of glucose-Glucose produced after glycogen breakdown is phosphorylated by **glucokinase** (in liver) or **hexokinase** in all other tissues especially in muscles. In the phosphorylation reaction, phosphate (**γ-phosphate**) group of **ATP** is transferred to glucose to form glucose-6-phosphate. The phosphorylation reaction of glucose to produce glucose-6-phosphate marks the molecule for glycolysis. One molecule of ATP is utilized in this step.

STEP 2: Conversion of glucose-6-phosphate to fructose-6-phosphate-Phosphorylated sugar produced in step-1 is converted into the fructose-6-phosphate by the action of **phospho-hexose isomerase**.

STEP 3: Phosphorylation of fructose-6-phosphate- In this step, sugar is further phsophorylated at carbon 1 to produce fructose-1,6 bis phosphate by the action of **Phosphofructokinase**. In the phosphorylation reaction, phosphate (**γ-phosphate**) group of **ATP** is transferred to phosphorylated sugar to form fructose-1,6 bis phosphate. One molecule of ATP is utilized in this step.

STEP 4: Clevage of fructose 1,6-bis phosphate-This step is catalyzed by enzyme **aldolase** or **fructose 1,6 bis aldolase** to generate **glyceraldehyde-3 phosphate** (aldose) and **dihydroxy acetone phosphate** (ketose).

STEP 1-4: First 4 reactions of enzymatic conversion of glucose (6 carbon sugar) to **glyceraldehydes-3 phosphate** (aldose) and **dihydroxy acetone phosphate** (ketose) are considered as preparative phase of glycolysis and during this phase, two major events happen:

1. Commitment of Sugar for glycolysis- Phosphorylated products are negatively charged and impermeable to the cell membrane through passive diffusion. Glycolysis operates in cytosol and as a result first step of phosphorylation inhibits the passive movement of the particular glucose moiety and drive it to participate in further steps of glycolysis.

2. Activation of sugar- In the 1^{st} and 3^{rd} step of glycolysis, two phosphorylation reactions add potential energy into the molecule and hence activate the sugar to participate into the cleavage reaction to form two 3 carbon sugar moiety.

STEP 5: Interconversion of the triose phosphates-Three carbon sugar formed in step 4 undergoes internal conversion and as **glyceraldehyde-3 phosphate** can readily be able to enter into the next step, the ketose generated in step 4 is reversibly convereted into the **glyceraldehydes-3 phosphate** by **triose-3-phosphate isomerase.**

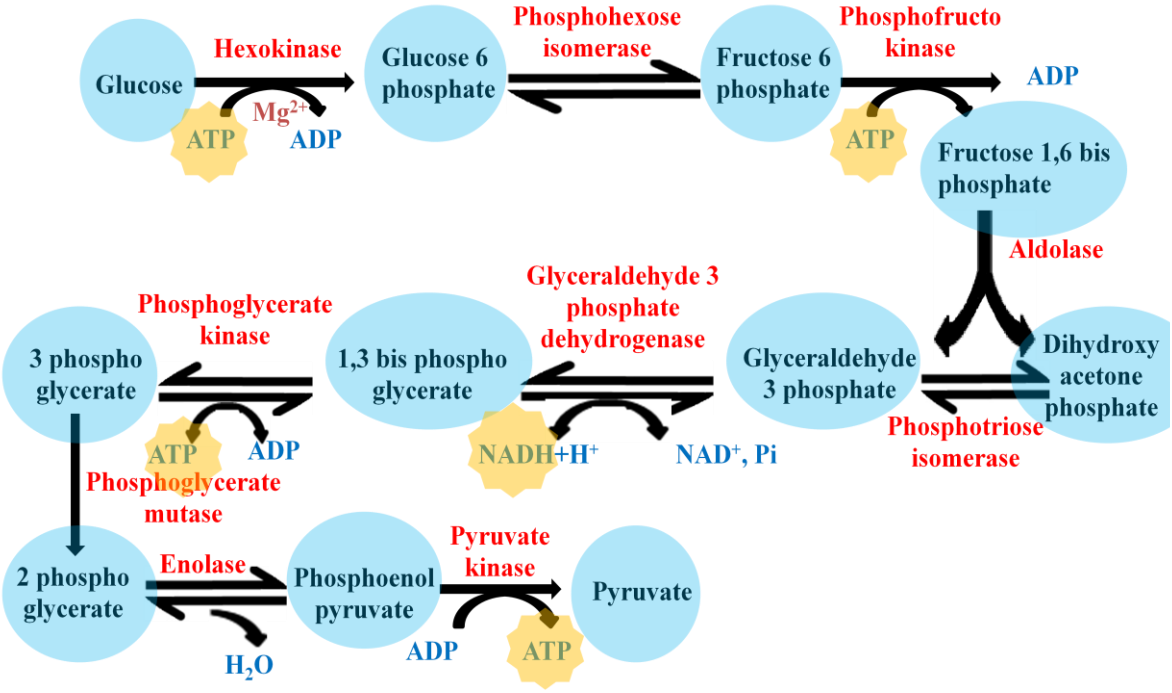


Figure 5.1: Different Reactions of Glycolysis.

STEP 6: Glyceraldehyde-3-phosphate to 1,3 bis-phospho-glycerate-In this step, one molecule of NADH is produced after oxidation of aldehyde group of glyceraldehyde-3-phosphate with the help of enzyme **glyceraldehyde-3-phosphate dehydrogenase**.

STEP 7: In this step, phosphate group from 1,3 bis-phosphoglycerate is removed by **phosphoglycerate kinase** with an acyl phosphate group transfer to ADP to generate ATP molecule.

STEP 8: Conversion of 3-phosphoglycerate to 2-phosphoglycerate- In a two step mechanism, phosphoglycerate mutase catalyzes a reversible shift of phosphoryl group to form 2-phosphoglycerate.

STEP 9: Dehydration of 2-phosphoglycerate to phosphoenol pyruvate- The enzyme **enolase** catalyzes the dehydration reaction to produce **phosphoenol pyruvate**, a compound with high phosphoryl group transfer potential.

STEP 10: In the last step of glycolysis, phosphate group from phosphoenol pyruvate is transferred by **pyruvate kinase** with an acyl phosphate group transfer to ADP to generate ATP molecule.

BOX 5.1 CALCULATION OF ATP PRODUCTION DURING GLYCOLYSIS.

The balance sheet of ATP generation from one molecule of glucose is as follow-

STEPS OF GLYCOLYSIS	**Number of ATP Generation (+) or Investment (-)**
1. Step 1-4	**- 2**
2. Generation of 2 molecules of glyceraldehyde-3 phosphate.	**2x3=6**
3. Step 6, generation of NADH, Each NADH in ETS gives 3 ATP	**2x1=2**
4. Step 7, Generation of ATP	**2x1=2**
5. Step 10, Generation of ATP	
NET BALANCE for oxidation of one glucose molecule.	**6+2+2-2= 8 ATP molecules**

Regulation of Glycolysis-

1. Uptake of glucose from blood-The level of glucose present in a cell determines the availability of sugar for oxidation via glycolysis. Glucose transport in cell is regulated by several cell surface receptor which are under the control of insulin (Figure 5.2). Insulin upregulates the level of glucose transporters Glut-3 or Glut-4 and increases the uptake of glucose from blood stream. In addition, insulin also regulates breakdown of glycogen to increase the amount of available glucose.

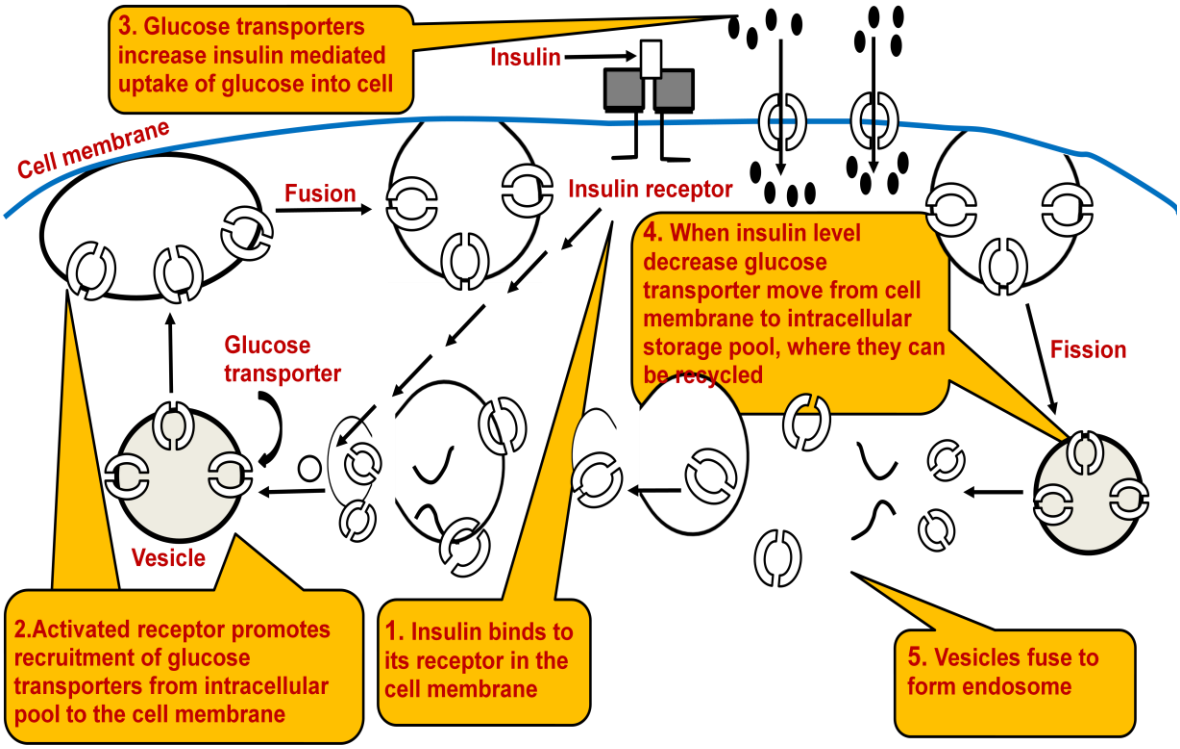


Figure 5.2: Regulation of uptake of glucose in the cell through action of insulin and cell surface receptors.

2. Covalent Modification of Enzyme- Hexokinase, phosphofructokinase and pyruvate kinase are key enzymes responsible for controlling glycolysis. Most of the typical protein kinases are regulated by a reversible phosphorylation and dephosphorylation. In the presence of low glucose in blood, pyruvate kinase is getting phosphorylated by cytosolic enzymes and phosphoryated pyruvate kinase is less active. Similarly in the presence of high blood glucose level, it remains as unphosphorylated and that relive the inhibition caused by phosphorylation (Figure 5.3, A).

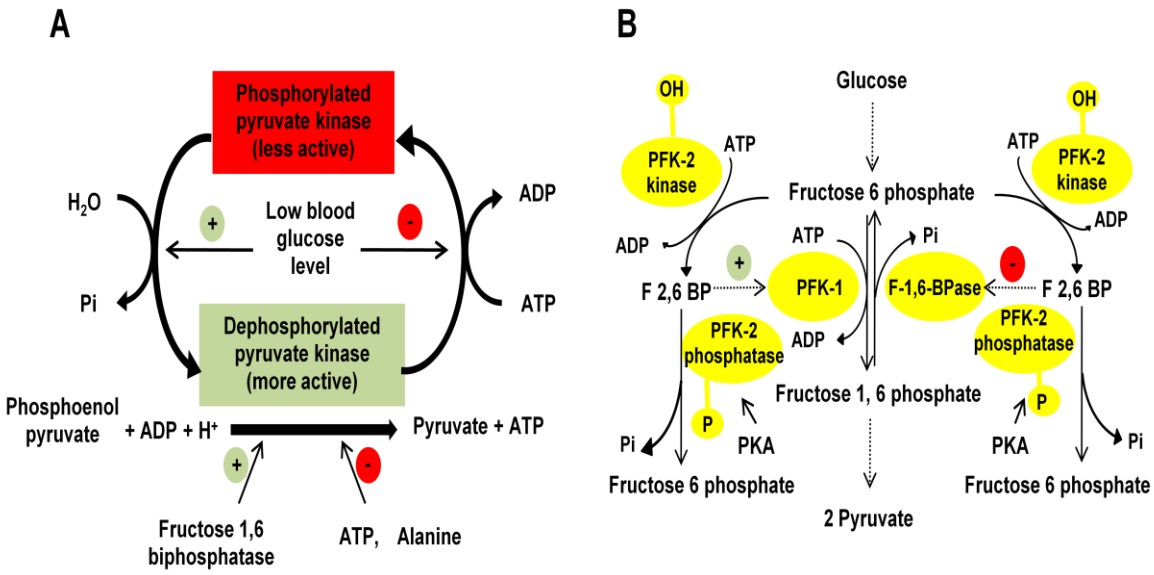


Figure 5.3: Regulation of glycolysis: (A) Covalent Modification (B) Alloteric regulation of enzymes of glycolysis.

3. Allosteric regulation- All the three crucial enzymes Hexokinase, phosphofructokinase and pyruvate kinase of glycolysis are regulated allosterically. In an allosteric regulation, an enzyme binds the allosteric molecules and this modulates the activity of the enzyme either in positive or negative manner. In glycolysis, fructose 2,6 bis phosphate is produced from fructose-6, phosphate by the enzyme phosphofructo kinase-2. fructose 2,6 bis phosphate is allosterically activating the enzymatic activity of phospho fructokinase (PFK-1) and at the same time it is down regulating the activity of fructose 1,6 bis phosphatase. In addition, ATP and citrate is inhibiting the activity of phospho fructokinase where as ADP and AMP is allosterically enhancing the enzymatic activitiy.

HOME ASSIGNMENT

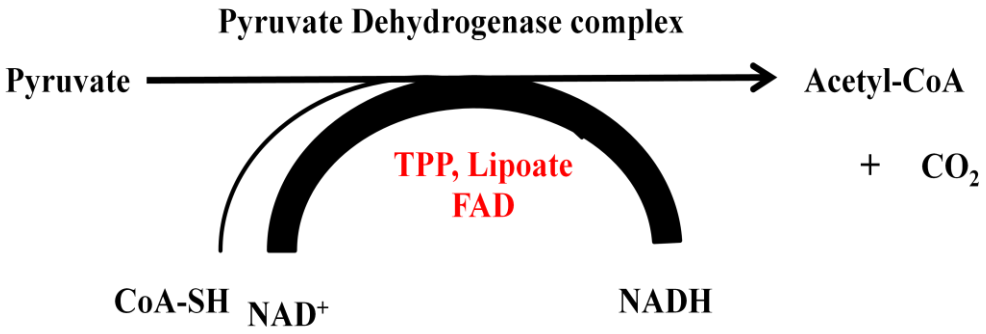
1. Calculate the production of number of ATP molecules from oxidation of one molecule of 1,6 bis-fructose ?

2. Calculate the production of number of ATP molecules from incomplete oxidation (in the absence of oxygen) of one molecule of glucose ?

Lecture 6: Metabolism-II (Kreb Cycle)

Kreb's Cycle: Kreb's Cycle is discovered by professor Hans Kreb and as it has all sugar intermediates with three carbon. It is also known as tricarboxylic acid or citric acid cycle. In higher eukaryotes, Kreb's cycle operates inside the mitochondrial stroma with the different enzymes. In the presence of oxygen, pyruvate formed during glycolysis enters into the Kreb's cycle for further oxidation to produce energy. But pyruvate can not enter directly into the Kreb's cycle, instead it needs further activation to form acetyl Co-A.

Production of Acetyl-CoA: It is a oxidative decarboxylation from pyruvate to release CO_2 and generation of **acetyl CoA** and reducing equivalent **NADH**. It is an irreversible reaction catalyzed by **pyruvate dehydrogenase complex**. Similar to glycolysis, irreversible decarboxylation commits the pyruvate for Kreb's cycle. In addition, acetyl-CoA is the reaction intermediate in fat metabolism and works as feeder point for Kreb's cycle (discussed more later).



Acetyl-CoA enters into the Kreb's cycle and undergoes a chain of 8 different reactions to produce energy. These steps are given in Figure 6.1.

STEP 1: Formation of citric acid- This reaction is catalyzed by citrate synthase where acetyl CoA condense with oxaloacetate to form citric acid. During the reaction, citronyl-CoA is produced due to joining of acetyl CoA and oxaloacetate. This high energy intermediate undergoes hydrolysis to form citrate.

STEP 2: Formation of isocitrate- The reversible transformation of citrate to **isocitrate** with **cis-aconitate** as an intermediate.This reaction is catalyzed by **aconitase**.

STEP 3: Oxidation of Isocitrate to α-keto glutarate- This is the first step of Kreb's cycle where CO_2 is produced with an additional oxidative decarboxylation of iso-citrate to form **α-keto glutarate** catalyzed by **isocitrate dehydrogenase.** One molecule of NADH is generated which will give 3 ATP molecule after oxidative phosphorylation.

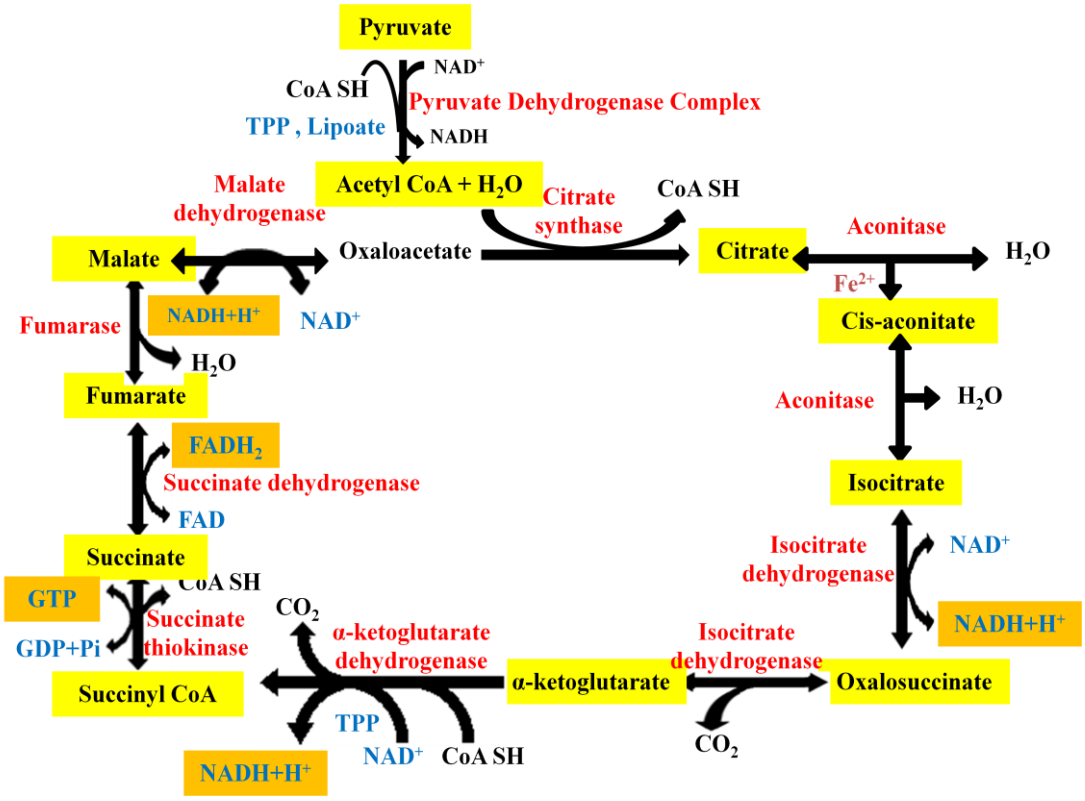
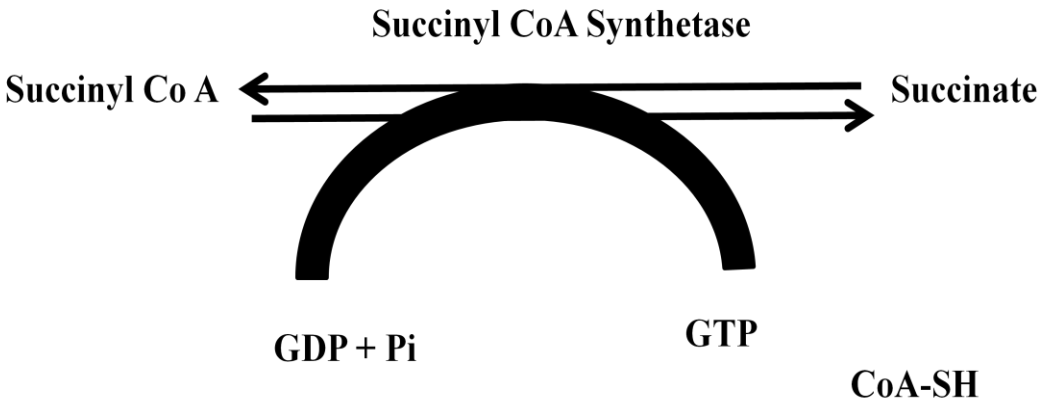


Figure 6.1: Different Reactions of Kreb Cycle.

STEP 4: Oxidation of α-keto glutarate to succinyl CoA-This is the second oxidative decarboxylation to produce **succinyl CoA** and CO_2 in the presence of **α-ketoglutarate dehydrogenase complex**. One molecule of NADH is generated which will give 3 ATP molecule after oxidative phosphorylation. α-ketoglutarate dehydrogenase is a multimeric enzyme complex comprised of 3 enzymes, E1, E2 and E3.

STEP 5: Conversion of Succinyl CoA to Succinate- This is the first step where thio ester linkage containing high energy compound is converted into a low energy product with the help of **succinyl CoA synthetase**. The energy of thio ester bond is utilized by the enzyme to produce **GTP** from condensation of **GDP+Pi**.



STEP 6: Oxidation of Succinate to fumarate-Succinate dehydrogenase, a flavo protein catalyzes conversion of succinate to **fumarate** with the production of FADH. One molecule of FADH is generated which will give 2 ATP molecule after oxidative phosphorylation.

STEP 7: Conversion of fumarate to malate-The dehydration of fumarate causes release of water molecule and generation of malate. This reaction is catalyzed by fumarase, a streospecific enzyme which is capable of making distinction between trans and cis isomer of the molecule.

STEP 8: Oxidation of malate to oxaloacetate- This is the last step of Kreb's cycle where malate is oxidized to oxaloacetate by **malate dehydrogenase**. One molecule of NADH is generated which will give 3 ATP molecule after oxidative phosphorylation. Oxaloacetate again recombines with new molecule of acetyl CoA to start another round Kreb's cycle.

Regulation of Kreb's Cycle- There are 4 rate limiting steps in kreb cycle and the points where it can be regulated. These different steps are shown in Figure 6.2.

1. Conversion of pyruvate into the acetyl CoA is the first step which allow the entry of sugar moiety into the kreb cycle. Pyruvate dehydrogenase complex is allosterically inhibited by high ratio of ATP/ADP, NADH/NAD^+ and acetyl CoA/CoA.

BOX 6.1 CALCULATION OF ATP PRODUCTION DURING KREB CYCLE.

The balance sheet of ATP generation from one molecule of glucose is as follows-

Steps of Kreb Cycle	**Number of ATP produced (+)**
1. Production of Acetyl CoA	**3x1=3**
2. STEP 3, Generation of α-ketoglutarate	**3x1=3**
3. STEP 4, Generation of Succinyl CoA	**3x1=3**
4. STEP 5, Generation of GTP., GTP=ATP	**1x1=1**
5. STEP 6, Genration of fumarate, Generation of FADH,	**2x1=2**
6. STEP 8, Generation of oxaloacetate,	**3x1=3**
NET BALANCE for oxidation of one pyruvate molecule.	**3+3+3+1+2+3=15 ATP molecules**
In glycolysis, two molecules of pyruvate is generated, hence total	**2x15=30 molecules of ATP will**
be generated.	

2. First reaction of Kreb's cycle, catalyzed by citrate synthase is inhibited by high level of NADH, ATP and succinyl-CoA.

3. Isocitrate dehyrogenase is inhibited by high level of ATP, NADH where as Ca^{2+} and ADP stimulate this step.

4. α-ketoglutarate dehydrogenase is inhibited by succinyl CoA and high level of NADH where as Ca^{2+} stimulates this step.

In addition, rate of glycolysis indirectly regulates the Kreb's cycle through availability of pyruvate in the feeding step. To maintain good co-ordination between two metabolic pathways, citrate produced in first step of Kreb's cycle allosterically inhibitphosphofructokinase-1 in the glycolytic pathway.

Significance of Kreb Cycle:

1. As a master regulator of metabolism- Kreb cycle is centrally connected to metabolic intermediates of carbohydrate, protein and lipid metabolism (Figure 6.3). It has several branching points where it can communicate with either protein or lipid metabolism. Lipid metabolism is connected to Kreb's cycle through common intermediated as citrate and acetyl Co-A.

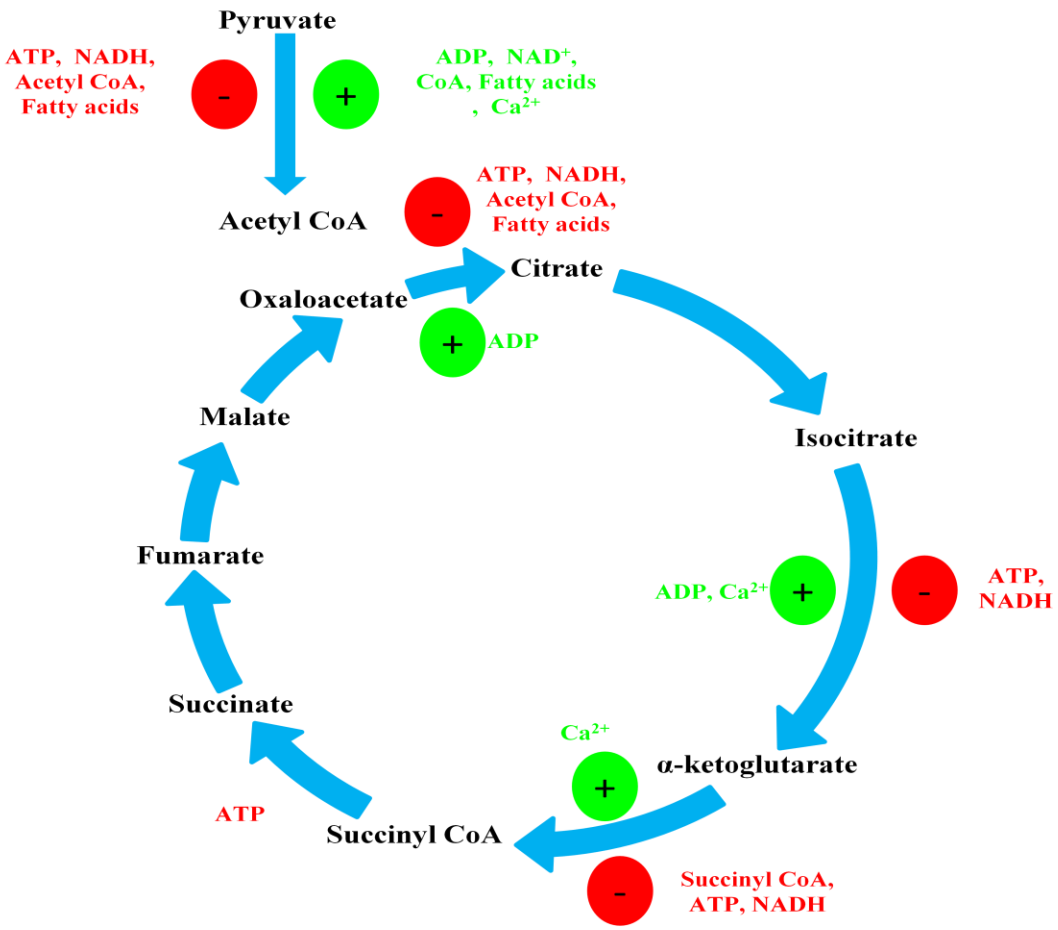


Figure 6.2: Regulation of Kreb Cycle.

Similarly, Protein metabolism shares intermediate at α-ketoglutarate, oxaloacetate. As a result, Kreb's cycle can allosterically or through product inhibition, regulates other metabolic pathways. In addition, it can redistribute intermediates between metabolic pathways and hence help in conversion of sugar to protein, lipid or vice-versa.

2. Role in Evolution- Kreb's Cycle is directly associated with running of electron transport chain and hence depends on availability of oxygen. Development of Kreb's cycle has evolved the organisms to adopt into the high oxygen environment.

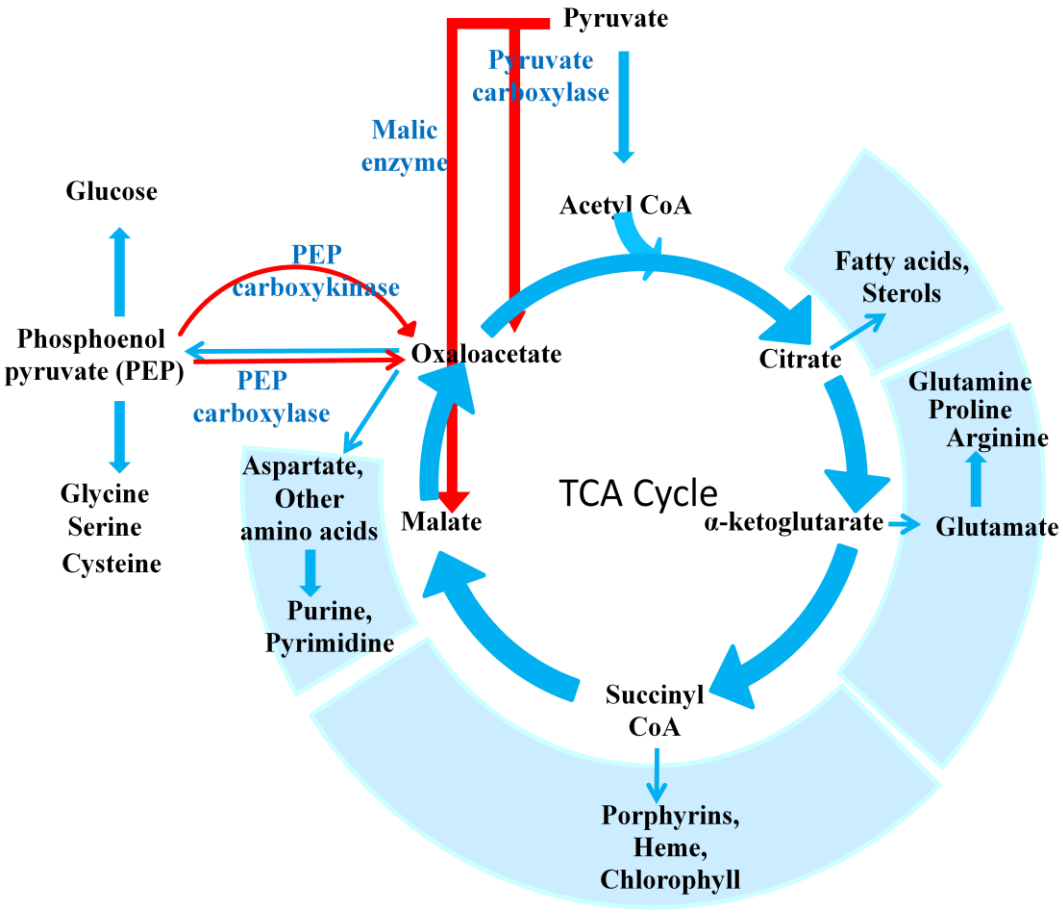


Figure 6.3: Communication of Kreb's cycle with other metabolic pathways.

HOME ASSIGNMENT

1. Do a web search to list and describe crucial experiments performed to determine that citric acid is a cylic pathway?

2. Make a list of glycolyis and Kreb's **cycle inhibitors used as drug ?**

Lecture 7: Anaerobic Oxidation and Fermentation

Anaerobic Oxidation-Glucose enters into the glycolysis produce pyruvate, which in turn enters into the Kreb's cycle for complete oxidation to produce maximum energy. The primary requirement of the oxidative phosphorylation is presence of a well developed electron transport chain to process reducing equivalents to produce ATP. In addition, presence of oxygen is mandatory for this process. Hence, depending upon the environmental conditions, pyruvate produced in glycolysis has multiple routes to follow as given in Figure 7.1. As discussed before, in the presence of oxygen, pyruvate directly enters into the kreb cycle to follow oxidative phosphorylation. In the absence of oxygen, pyruvate accumulates in cytosol and is immediately processed into two routes: (1) direct conversion to lactate with the help of cytosolic enzyme lactate dehyrogenase (LDH). (2) conversion of pyruvate to alchol with acetaldehyde as a intermediate by the concerted action of pyruvate decarboxylase and alchol dehyrogenase.

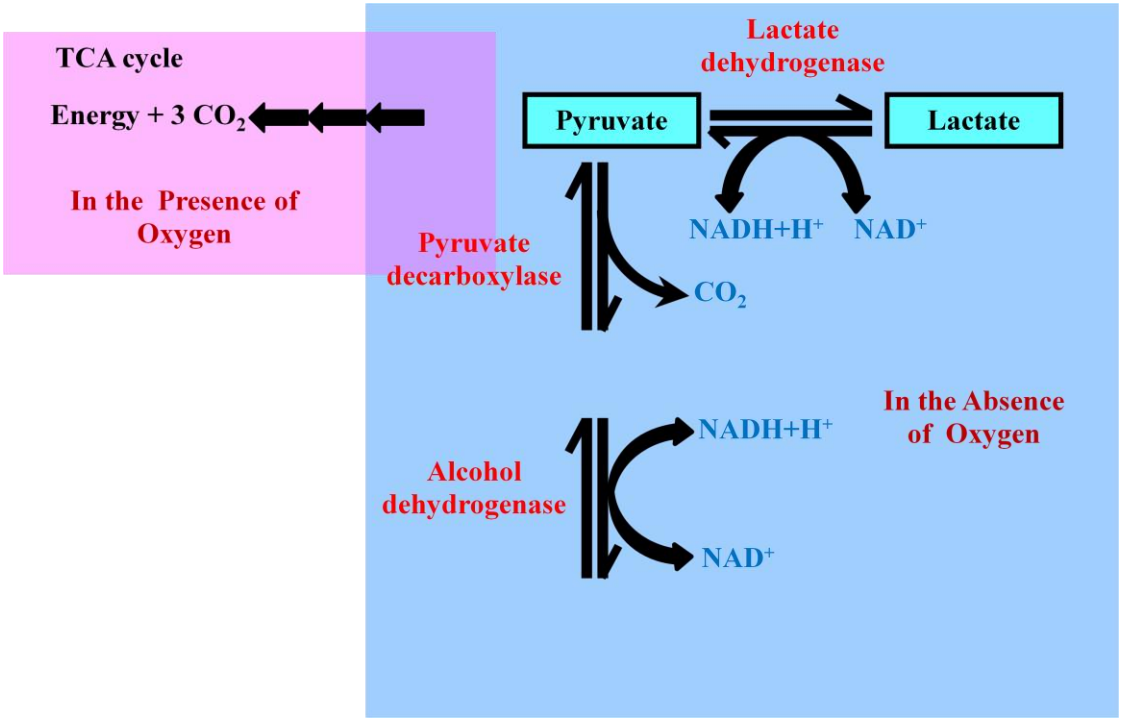


Figure 7.1: The distribution of Pyruvate during carbohydrate metabolism.

These set of reactions operating in the absence of oxygen helps organism in many ways and these possibilities are discussed later in the lecture. Now we will discuss the mechanism of pyruvate conversion to lactic acid or alchol and the significance of these pathways in adopting to the low oxygen environment.

Anaerobic reduction of Pyruvate to Lactate- Pyruvate is reduced to lactate with an enzymatic action of lactate dehyrogenase. In this process, cell spend 1 molecule of NADH and 1 molecule of NAD+ is generated. The NAD+ produced in this process will be used to continue running glycolysis and other metabolic pathways.

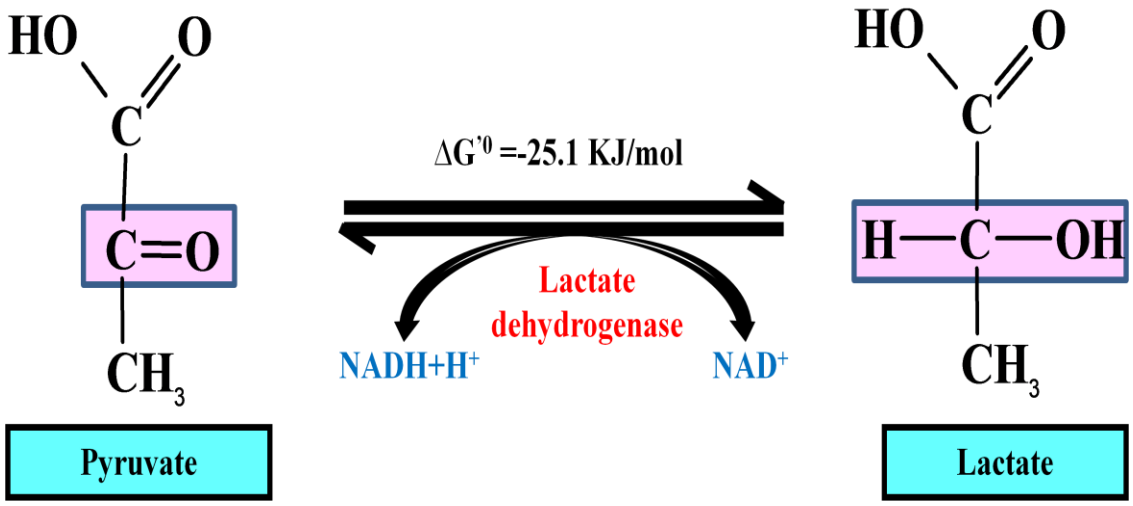


Figure 7.2: Conversion of Pyruvate to Lactate.

The free energy change (-25.1 KJ/mol) of the pyruvate to lactate conversion favors lactate formation inspite of no net gain of NADH, but it allows the glycolysis to keep running in the absence of oxygen.

Pyruvate to Ethanol- It is a two step process, first conversion of pyruvate to acetaldehyde and in the second step conversion of acetaldehyde to alchol. First step is a decarboxylation reaction catalyzed by pyruvate decarboxylase where as second step is reduction reaction catalyzed by alchol dehydrogenase.

The Over all equation of ethanol production from pyruvate is as follows

$$\text{Glucose} + 2\text{ADP} + 2\text{Pi} \rightarrow 2\ \text{ethanol} + 2\text{CO}_2 + 2\text{ATP} + 2\text{H}_2\text{O}$$

Mechanism of pyruvate decarboxylation by pyruvate decarboxylase-Pyruvate decarboxylase requires thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors to catalyze decarboxylation reaction (Figure 7.3). Thiamine pyrophosphate is a co-enzyme present in pyruvate decarboxylase and responsible for stabilizing carbanion intermediate. The sequence of event of reaction catalyzed by pyruvate decarboxylase is as follows-

1. Deprotination of TPP to form TPP carbanion.

2. Carbanion attacks on carbonyl group of pyruvate to form adduct.

3. Release of CO_2.

4. Resonance stabilization of intermediates.

5. Protonation to generate hydroxyl methyl TPP.

6. Release of acetaldehyde and regeneration of TPP from hydroxyl-TPP for next round of enzymatic catalysis.

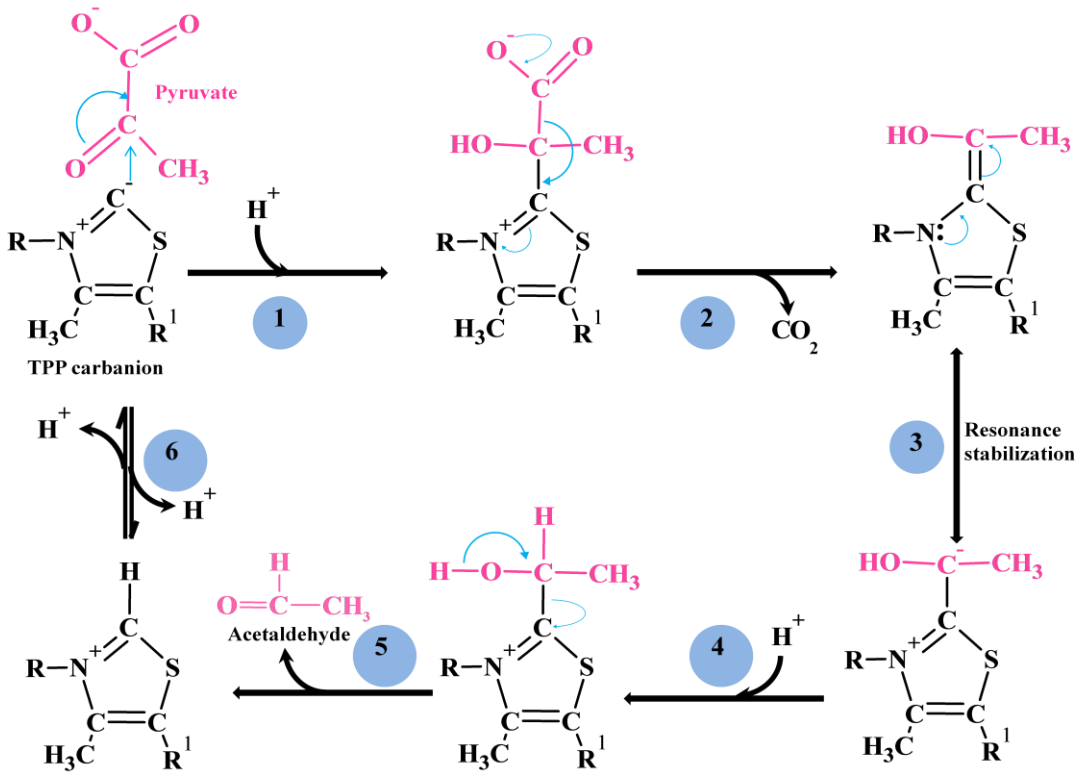


Figure 7.3: Mechanistic details of conversion of pyruvate to acetaldehyde by pyruvate decarboxylase.

Mechanism of Acetaldehyde to alchol-Alchol dehydrogenase is a dimeric metal dependent dehydrogenase present in animal, plant and bacteria. The reaction mechanism discussed below might have some modifications but over-all alchol dehyrogenase follows it. The conversion of acetaldehyde to alchol by alchol dehydrogenase completes in 4 steps:

1. Binding of substrate acetaldehyde to enzyme bound zinc,

2. binding of NADH

3. transfer of hydride ion from NADH to reduce acetaldehyde.

4. Reduced acetaldehyde intermediate acquires a proton from water to form alchol.

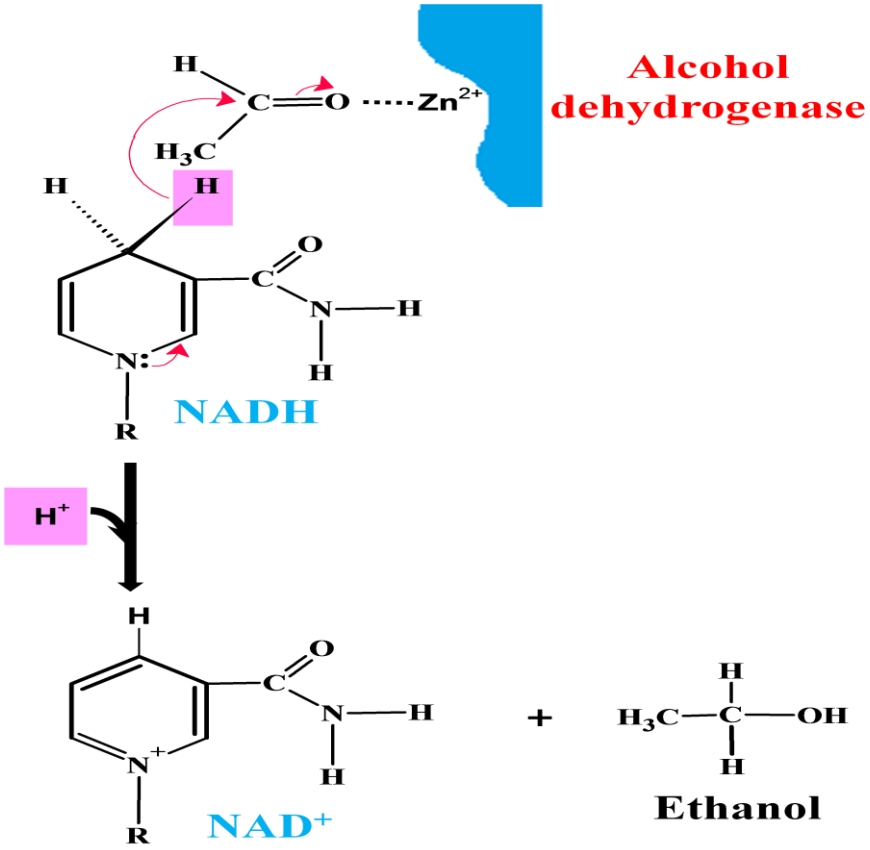


Figure 7.4: Mechanistic details of conversion of acetaldehyde to alchol by alchol dehydrogenase

BOX 7.1 THE BALANCE SHEET OF DURING FERMENTATION OF GLUCOSE TO ALCHOL

The balance sheet is as follow-

STEPS OF GLYCOLYSIS	Number of ATP Generation (+) or Investment (-)
1. Step 1-4	- 2
2. Generation of 2 molecules of glyceraldehyde-3 phosphate.	3x2=6 [If ETS will operate]
3. Step 6, generation of NADH, Each NADH in ETS gives 3 ATP	2x1=2
4. Step 7, Generation of ATP	2x1=2
5. Step 10, Generation of ATP	
6. Oxidation of one glucose molecule.	6+2+2-2= 8
7. Pyruvate to acetaldehyde	0
8. Acetaldehyde to alchol, NADH	-3x2=6
9. NET BALANCE	8-6=2 ATP molecule per glucose

Significance of Anaerobic Oxidation

In the absence of oxygen, cell becomes short of NAD^+ as glycolysis convert all NAD^+ into the NADH. Kreb's cycle is not operating and to continue glycolysis to produce energy, NAD+ is required. To meet the requirement of maintaining NAD^+ pool, metabolism has adopted a futile cycle approach where NADH produced in glycolysis will eventually been utilized in anaerobic oxidation to convert the aldehyde to either lactic acid or alchol. In higher vertebrate, under low oxygen pressure (such as during exercise in muscle) anerobic oxidation produces large amount of lactic acid but once oxygen is available lactic acid produced in muscle is sent to liver to regenerate glucose which will be send back to muscle for oxidative phosphorylation. This cyclic event is known as Cori cycle.

Quiz

Q1: The enzymes of glycolysis are found in ……part of the eukaryotic cell.

Q2: The released energy from kreb cycle is stored in the form of ……..

Q3: One molecule of glucose produces……molecules of pyruvate.

Q4: Explain how presence of oxygen inhibits alchol production from yeast?

Q5: Which enzyme links glycolysis and kreb cycle?

Lecture 8: Growth Media for different Expression Systems

Introduction: Growth and multiplication of host organisms used as expression system, requires a suitable biochemical and biophysical conditions. The biochemical (nutritional) conditions can be provided by the use of various nutrient media. Depending upon the special needs, different types of media have been developed for expression system to achieve growth, multiplication and over-expression of protein.

Growth Media for Bacterial Expression System

Bacterial expression systems are mainly utilized for protein over-expression because of its rapid growth rate, low cost, ease of high-cell-density fermentation, and availability of excellent genetic tools. Growth of bacterial expression system requires different types of media based on the requirement which can be divided into either **complex or defined media**. The complex media comprises of natural substances and rich in nutrients therefore suitable for culturing fastidious organism (Table 8.1). On the other hand, defined media are simple and made up of known components put together in the required amounts (Table 8.2).

TABLE 8.1 COMMON MEDIA CONSTITUENTS FOR BACTERIAL GROWTH

Constituents	Source
Amino-Nitrogen	Peptone,protein hydrolysate, infusions and extracts
Growth Factors	Blood,serum, yeast extract or vitamins, NAD
EnergySources	Sugar, alcohols and carbohydrates
BufferSalts	Phosphates, acetates and citrates
Mineral salts and Metals	Phosphate, sulfate, magnesium, calcium,iron
Selective Agents	Chemicals, antimicrobials and dyes
Indicator Dyes	Phenol red, neutral red
Gelling agents	Agar,gelatin,alginate,silicagel

Preparation of Bacterial Expression media: The composition of the selected bacterial expression media is given in Table 8.2. For preparation of bacterial media dissolve the components in 1 liter of distilled water. Cover the top of the flask with cotton plug or aluminium foil and autoclave the solution at 121°C for 20 minutes. The various antibiotics or nutrient supplement should be added to the media when the temperature is less than 50°C after autoclaving (Figure 8.1).

For making of solid media agar plates, 1.5% agar is added to the media and autoclaved. After autoclaving allow the media to cool up-to 50°C.Transfer the warm media into a petri dishes until it is 1/3 full, loosely close the lid and allow the media to solidify.

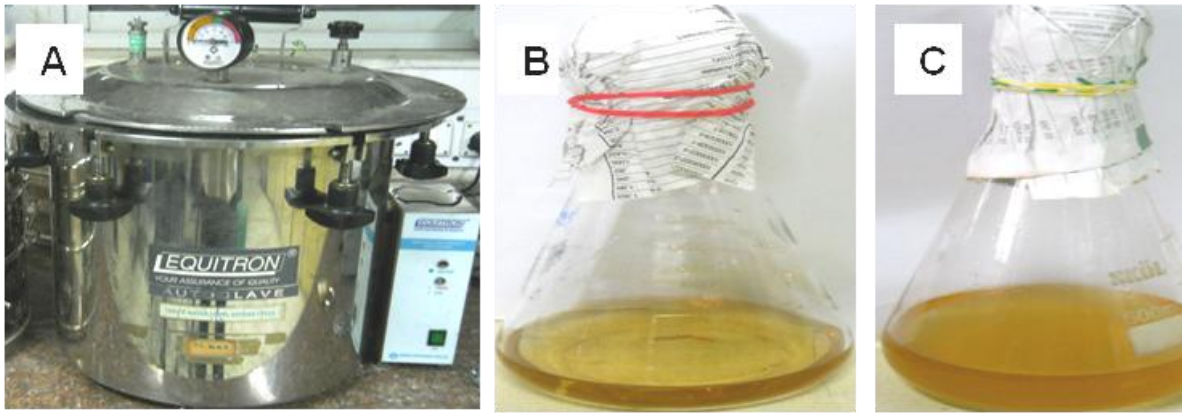


Figure 8.1:Equipments and media required for sterilization and growth of bacterial expression system.(A)autoclave (B)autoclaved LB broth (C) E.coli grown in LB broth.

TABLE 8.2: COMPOSITION OF SELECTED MEDIA FOR BACTERIAL GROWTH

Growth media	Compositions	Applications
M9 minimal media	0.6% disodium hydrogen phosphate 0.3% potassium dihydrogen phosphate, 0.05%, Sodium chloride 0.1% ammonium chloride	For cultivation and maintenance of *Escherichia coli* (*E. coli*) strains.
M63 minimal media	0.2% ammonium sulfate 1.36% potassium dihydrogen phosphate monobasic 0.00005% ferrous sulfate.$7H_2O$	For cultivation and maintenance of *E. coli* strains.
LB (Luria Bertani) Miller broth	1% peptone 0.5% yeast extract 1% NaCl	For *E.coli* growth; plasmid DNA isolation and protein production
LB (Luria Bertani) Lennox Broth	1% peptone 0.5% yeast extract 0.5% NaCl	For *E.coli* growth; plasmid DNA isolation and protein production
SOB medium	2% peptone 0.5% Yeast extract 10mM NaCl 2.5mM KCl, 20mM $MgCl_2$	To make high efficiency competent cells.
SOC medium	SOB + 20mM glucose	growth of competent cells.
2x YT broth (2x Yeast extract and Tryptone)	1.6% peptone 1% yeast extract 0.5% NaCl	Phage DNA production
Terrific Broth) medium	1.2% peptone, 2.4% yeast extract 72 mM K_2HPO_4 17 mM KH_2PO_4 0.4% glycerol	For protein expression and plasmid production.
Super Broth) medium	3.2% peptone, 2% yeast extract 0.5% NaCl	High yield plasmid DNA and protein production
TYGPN media	2% Tryptone, 1% Yeast extract, 1ml 80% Glycerol, 1%Potassium Nitrate, 0.5% Sodium Phosphate dibasic	For rapid growth of *E. coli.*

Growth Media for yeast Expression System

Yeast expression system offers advantages of speedy growth, easy genetic manipulation, low cost media with the characteristics of higher eukaryotic systems such as post translational modifications and secretory expression. Yeast expression system mainly utilizes *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*) strains for cloning and protein overexpression. For the growth, propagation and protein overexpression of these strains specific formulations and ingredients are required (Table 8.3). The common type of yeast expression media are given in Table 8.3.

TABLE 8.3: SELECTED GROWTH MEDIA FOR YEAST EXPRESSION SYSTEM

Growth media	Composition (For 1 litre)	Applications
CSM Media	CSM (without tryptophan)0.74gm, Yeast Nitrogen Base6.66gm, AmmoniumSulfate5gm,Glucose20gm, Agar 20 gm	For making agar plates that enable the growth of Saccharomyces cerevisae MaV203 competent cells.
YPD Broth	10gm Yeast extract 20gm Bacto peptone 20gm Dextrose (glucose)	Commonly used yeast media for maintenance and propagation of *P. pastoris* and *S. cerevisae* .
YPGal	10gm Bacto Yeast Extract 20gm Bacto Peptone 100ml of 20% Galactose 15gm Bacto Agar	Standard medium for *S.cerevisae* omitting glucose repression
Standard Minimal Medium (SD) /Yeast Nitrogen Base (YNB)	6.7gm yeast nitrogen base with ammonium sulfate and without amino acids and 20gm dextrose plus any amino acids or nucleotides required for growth at ~50 ug/ml each).	Base medium for preparation of minimal and synthetic defined yeast media

Method of Preparation. Media preparation of yeast expression system is similar to the microbiology media. As per the media composition, constituents are in 950 ml of water and autoclave. Allow medium to cool to 50°C and then add 50ml of filtered sterile 40% dextrose (glucose) so that the final concentration become 2% . Adjust the final volume to 1 litre, if necessary.

Growth Media for Insect cell culture

In modern biotechnology insect cell culture is gaining a considerable attention for production of recombinant proteins. Insect cell systems provide improved target protein solubility and important post-translational modifications for increased activity. Baculovirus Expression Vector System (BEVS) is the well known system to utilize the insect cell lines for the production of recombinant proteins. The media used for insect cell culture is a complex mixture of Amino acid, Monosaccharide, Vitamin, Inorganic ion, trace elements, fetal bovine serum (FBS) and broad spectrum antibiotics. The popular culture media required for the growth of various insect lines are given in Table 8.4.

TABLE 8.4: SELECTED GROWTH MEDIA FOR INSECT CELL CULTURE

Growth media	Compositions[#]	Applications
Grace's Insect medium supplemented	Unsupplemented media actalbumin hydrolysate yeastolate	Growth of *Spodeptera frugiperda* cells, Sf9 and Sf21 cell lines
Hink's TNM-FH Insect Medium	supplemented Grace's, 4.1 mM L-glutamine, 3.33g/Llactalbuminhydrolysate(LAH)	For the culture of cabbage looper, *Trichoplusia ni* cells
IPL-41 Insect Medium Modified	IPL-41 media Calcium chloride 200mM L-glutamine Sodium bicarbonate	Growth of *Spodeptera frugiperda* cells, Sf9 and Sf21 cell lines
TC-100 Medium	TC-100 Medium 200mM L-glutamine Sodium bicarbonate	For the production of baculovirus in lepidopteran cell lines.
Mitsuhashi/Maramorosh Insect Medium	Mitsuhashi/MaramoroshInsect Medium Sodium bicarbonate	For Mosquito cell culture especially *Aedes aegypticus*
Schneider's Drosophila Medium	Schneider's Drosophila Medium Calcium chloride 200mM L-glutamine Sodium bicarbonate	For the in vitro culture of Drosophila melanogaster cells and tissues

[#]NOTE: FBS and antibiotics solution are added after sterilization to make it complete media

General method of preparation: Dissolve dry powder of medium in cell culture grade water (80% of the final volume). Mix powder until dissolved completely. Add required amount of other component, mix completely and adjust the pH to 6.9-7.3 using 1N NaOH or 1N HCl. Make up the final volume with cell culture grade water. Sterilize the solution using a 0.22µm membrane filter. Finally, aseptically add antibiotics and serum to the sterilized incomplete media.

Growth Media for mammalian cell culture:

The media used for animal cell culture is a complex mixture of Amino acid, Monosaccharide, Vitamin, Inorganic ion , trace elements and broad spectrum antibiotics. The other key ingredients of cell culture is a natural medium which may be animal body fluids or medium of tissue extraction, including plasma, serum, lymph, chicken embryos leaching solution, etc. Serum, usually bovine or calf is the most commonly used natural medium. Serum provide a similar osmotic pressure and pH as of body environment. Serum enhances the cell attachment and provides extra nutrients, various hormones like growth factor that promotes healthy growth of the cell. In order to monitor the status of media, phenol red is added as a pH indicator. This will turn yellow if media becomes acidic otherwise media at pH 7.2-7.4 remains red.

TABLE 8.5 RECIPE OF MAMMALIAN CELL CULTURE COMPLETE MEDIA.

Components	Composition
DMEM	13.4 gm/ltr
Sodium bicarbonate	3.7gm/ltr
Fetal bovine serum (FBS)	10%
100X Antibiotic (Pencillin –Streptomycin)	1%

Prepartion of cell culture medium

To explain the method of media preparation, we are taking the example of DMEM media (Figure 8.2). Measure 80 - 90% of the final volume of cell culture grade water. Add 13.4 gm dry powder medium to the water and mix to dissolve it completely. For each liter of DMEM, add 3.7g/L of sodium bicarbonate, mix completely and adjust the pH to 6.9 -7.1 using 1N NaOH or 1N HCl. Finally add cell culture grade water to the media to bring it to the final volume. Sterilize the solution using a sterilized membrane filter with a pore size of 0.22µm. Supplements, such as antibiotics and serum can be added to the sterilized solution using aseptic technique.

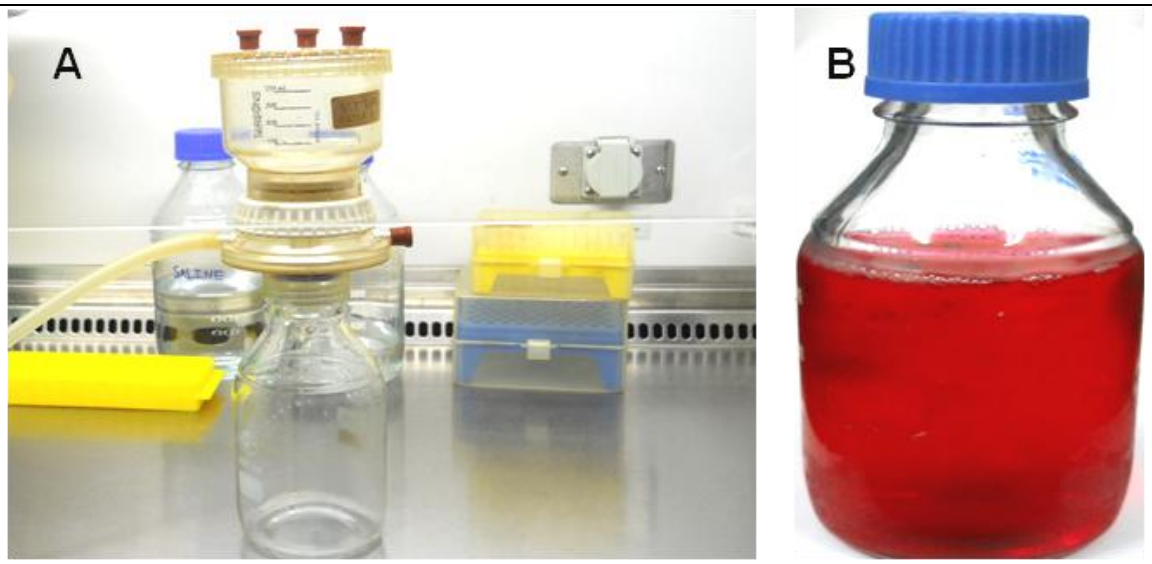


Figure 8.2: Sterilization of cell culture medium (a) filteration unit set up for the filteration of DMEM media through 0.22µm filter. (b) complete media containing 10% serum and 1% pencillin–streptomycin antibiotic.

Lecture 9: Microbial Growth Kinetics

Introduction-Studying growth of a microorganism is the basis of biotechnological exploitation of microflora for production of desired product. Optimization of growth of microorganism in a particular media is desirable due to economical and availability of particular growth constituent in a region. Despite this, some microorganisms have specific requirement and they grow in a particular growth media. Common media for growth of different microorganism, yeast and animal cells is discussed in future lecture. In today's lecture we will discuss bacterial cell division, methods of measuring growth, different phase in bacterial growth and growth kinectics.

Modes of Bacterial Cell Division-

1. Binary division-binary division is the most common mode of cell division in bacteria (Figure 9.1). In this mode of cell division, a single bacteria cell grows transversely with the synthesis of chromosomal DNA. A transverse septum appears in the middle of the cell body that divides the bacterial cell into the two with a distribution of chromosomal DNA, ribosome and other cellular machinery.

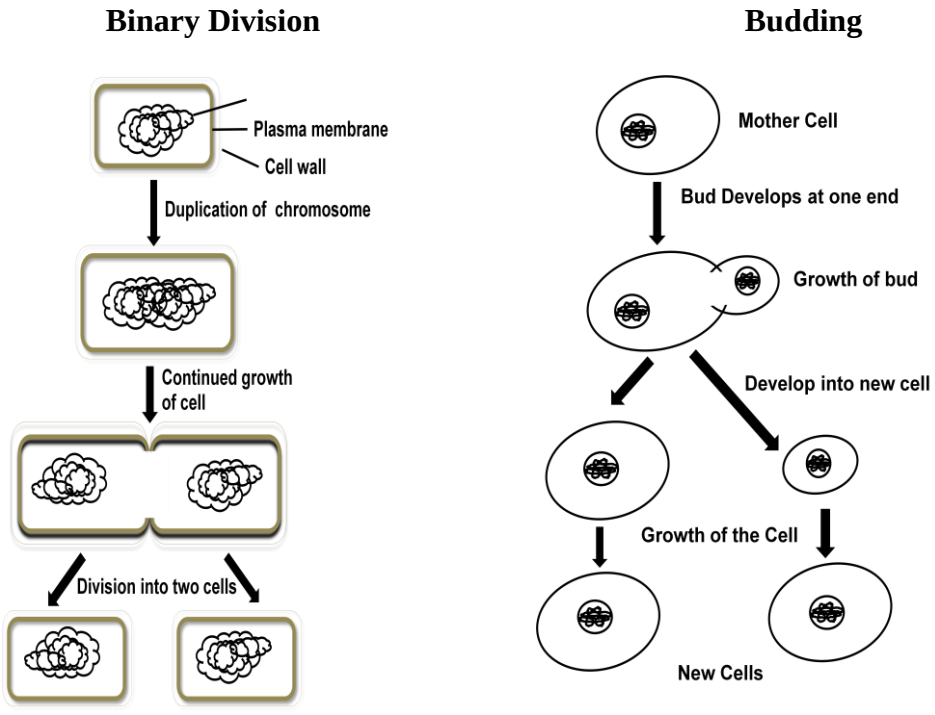


Figure 9.1: Different modes of cell division in bacteria.

2. Budding-In this mode of cell division, chromosomal DNA divides to form two copies. Sister chromosomal DNA moves to the one side of the cell and this portion of the cells protrude from main body to form bud. Eventually bud grows in size and get separated from main cell to develop a new cell.

3. Fragmentation-This mode of asexual division is more common in filamentous bacteria. In this mode, filament of the growing cell gets fragmented into small bacillary or coccoid cells, these cellular fragments eventually develop into new cell.

Measuring Bacterial growth- A number of methds have been developed to measure bacterial growth in liquid media and in solid support media. A few are discussed below:

Microscopic count-bacterial cells can be counted easily on a "petroff-hausser counting chamber" (Figure 9.2). The chamber has a ruling to make square (1/400 mm^2) of equivalent volume. A glass slide is placed (~1/50mm height) to make a chamber filled with bacterial cell suspension. Volume of each chamber is 1/20,000 mm^3. This chamber can be used to observe bacteria with phase contrast microscope. For example, if each chamber has 8 bacteria then there are 8x20,000,000 or $1.6x10^8$ bacteria/ml. A very high or low concentration of bacterial sample can not be counted accurately.

Plate count method-In this method, a defined amount of bacterial culture suspension is introduced onto solid support media to grow and give colonies. If number of colonies on solid media is too high, then serial dilution of original stock can be plated on solid media and number of colony can be counted with a colony counter. A manual colony counter has lamp at the bottom, a grid to divide the bacterial culture plate and a magnifying glass to visualize and count single colony. A plate with colony count of 30-300 can be used to determine the number of bacteria present in original stock.

Number of bacteria per ml= Number of colonies counted on plate X dilution of sample

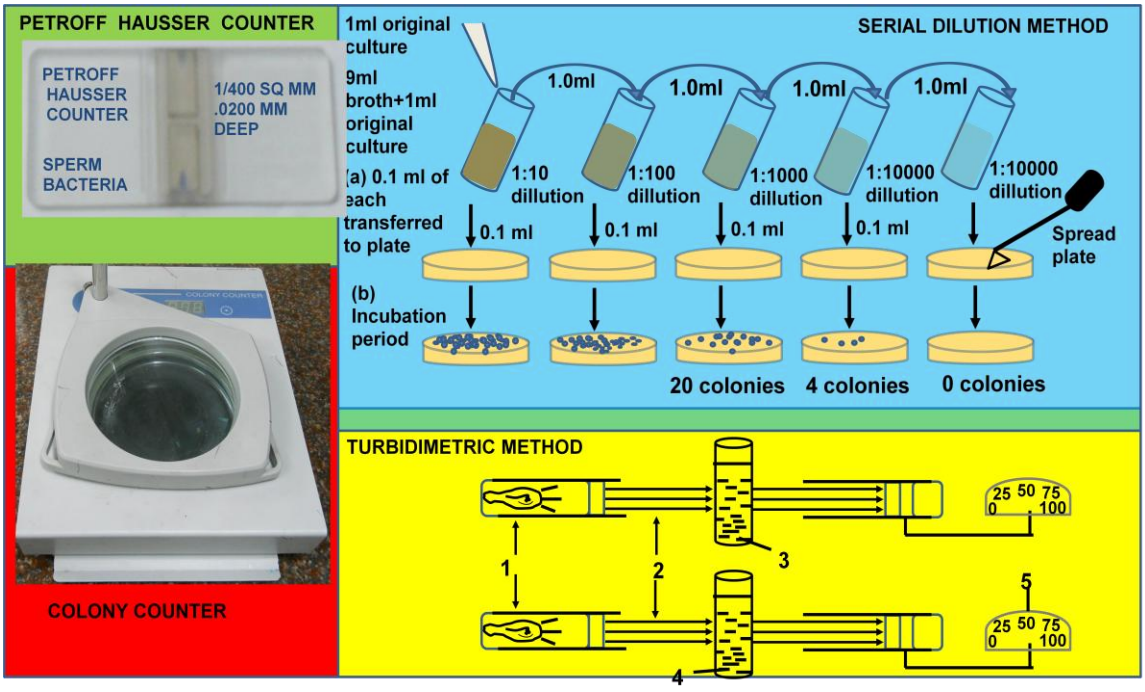


Figure 9.2: Different methods of bacterial counting.

Turbidimetric methods-This method is based on light scattering principles of particulate matter such as bacteria. A bacteria cell suspension is placed in test cuvette and corresponding media in reference cuvette. The optical density or absorbance of the bacterial suspension is used to measure the number of bacteria number. This method can not distinguish between live or dead bacteria as both form contribute to the turbidity.

Nitrogen content and Dry weight- A bacterial cell mass can be measured by direct measuremenof dry weight of culture or nitrogen content.

Growth cycle of bacteria- As discussed earlier, the most common method of bacteria division is binary fission and by this method, one bacteria cell gives two daughter cells. The time a bacteria takes to complete one division is called as generation time and it depends on bacteria species and media properties.

Hence, if we start from one bacteria, it divides after every generation time as follows-

Generation (n)	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**n**
No. of bacteria	**1**	**2**	**4**	**8**	**16**	**32**	**64**	
No. of bacteria	**1**	**2^1**	**2^2**	**2^3**	**2^4**	**2^5**	**2^6**	**2^n**

Hence, After n generations, no of bacteria will be

$N=1 \times 2^n$……. Eq 9.1

But assume if number of bacteria at time 0 is N_o, then

$N=No \times 2^n$……..Eq 9.2

$\text{Log } N=\text{Log } N_o+n \log_{10} 2$……...Eq 9.3

$n= 3.3 (\text{Log}_{10} N-\text{Log}_{10} N_o)$…….Eq 9.4

Eq 9.2 can be used to determine number of bacteria, if initial number of bacteria and number of generation is known where as Eq 9.4 can directly been used to calculate number of generations.

Bacterial growth in a liquid media is given in Figure 9.3 and it has 4 distinct phases:

1. Lag Phase-The single cell inoculation into the liquid media doesn't start dividing as per its generation time. During this phase bacteria gets adjusted to the new media and grow in size instead of dividing into daughter cells. In this phase, bacteria synthesize the most crucial enzymes or co-enzyme present in traces and required for optimal growth and multiplication. In addition, cell is metabolically active and be busy in synthesizing large amount of protoplasm. At the end of this phase, each bacterial cell divides and enter into the next phase of active multiplication.

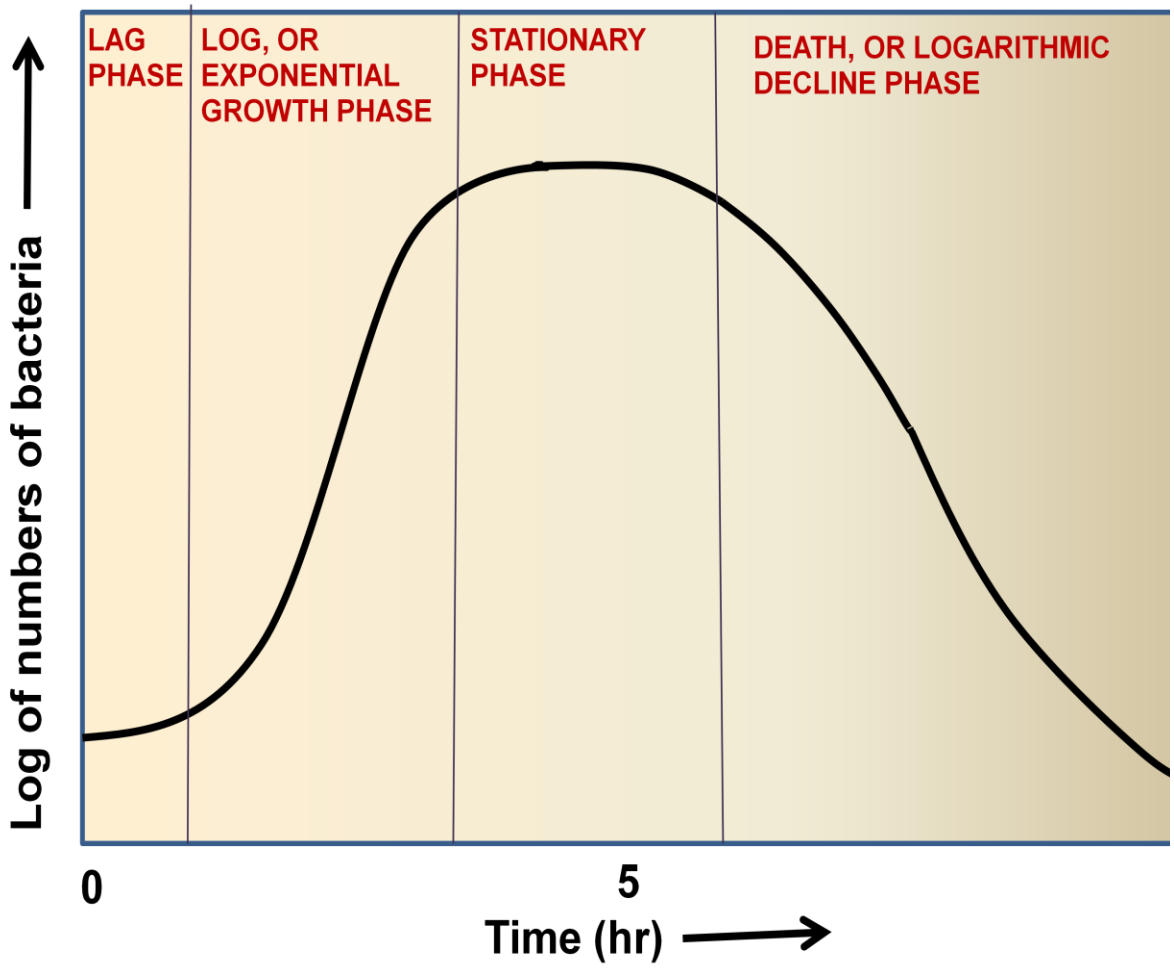


Figure 9.3: kinetics of Growth of bacteria.

2. Log Phase-In this phase, bacterial cell population is involved in active division and whole cell population is more or less homogenous in terms of chemical composition, physiology and metabolic activity. A plot of number of cell (in log scale) against time gives straight line. The growth of bacterial cell population is increasing at a constant rate and continues until substrate concentration is not limiting.

3. Stationary Phase-Once substrate is limiting, the logarithmic phase of growth begins to decline gradually with a constant number of cells to give a staright line. The population remains constant because number of divisions are equal to the number of death events. As substrate is limiting, death of old cell provides enough nutrient for remaining cells to grow and multiply to maintain the constant number.

4. Death Phase-When substrate is not sufficient from dying cells, death rate of bacteria superseed rate of growth and as a result number of bacteria declines sharply.

Quiz

Q1: In a culture of bacteria, a sample is taken at 10:00AM and contains 1000 cells per ml. A second sample at 8:00PM has 10,000 cells per ml. what is the generation time ?

Q2: A scientist wants 20,000/ml *E.Coli* cells for his molecular biology experiment, he has inoculated 1000 cells at around 8:00PM. The generation time of *E.coli* is 20 mins. Please tell when he should harvest the culture?

Q3: What is the significance of lag phase in bacterial growth curve?

Q4: Which mode of cell division is most common in bacteria?

Q5: The unit of measurement in the turbidimetric method of measuring bacteria growth ?