Chapter 1

CELL AND MARKER ENZYMES

Cell is defined as structural and functional unit of life.
Cell membrane has two major components Lipids and Proteins
Lipids can be Phospholipids, Glycolipids, Cholesterol, fatty acids. All are Amphipathic in nature
Proteins can be Integral proteins or Peripheral proteins
Fluidity of cell membrane increases on addition of unsaturated fatty acid with cis double bonds
Major phospholipids of cell membrane Glycerophospholipids
The aminophospholipids in cell membrane are located in the inner leaflet, while Choline containing phospholipids are located on outer molecular layer
Most common steroid in membrane is Cholesterol. It is present in cell membrane on the outer leaflet
Filamentous material found on cytoplasmic pits which helps in Pinocytosis is Clathrin
Proteins synthesized on ER bound ribosomes are destined for ER membrane, Golgi membrane, cell membrane, Lysosomal enzymes, and secretory proteins
Proteins synthesized on free cytosolic ribosomes are destined for Nucleus, Mitochondria, Peroxisomes, and Cytosolic proteins.
Most of the glycosylation reactions occur in the Golgi apparatus
Zellweger's syndrome occurs due to deficiency of Peroxisomes.
Cytoskeleton (cell filaments) consists of Microfilaments Intermediate filaments and Microtubules
In the dividing cells spindle is formed by Microtubules
Exchange of cytoplasmic molecules between two cells are through regions known as Gap Junctions

Composition of cell membrane [% by weight]

Membrane	Protein %	Lipid %	Remarks
Myelin	20	75	Lipid = 75%
Erythrocyte	49	43	Protein to lipid around 50 %
Hepatocyte plasma membrane	54	39	Protein to lipid around 50
Outer mitochondrial membrane	50	46	Protein to lipid around 50
Inner mitochondrial membrane	75	23	Protein = 75%

Intracellular organelles, their functions and Marker enzymes

Organelle /Fraction	Marker Enzyme	Major Functions
Mitochondria	Glutamate dehydrogenase	Citric acid cycle, oxidative phosphorylation
Endoplasmic reticulum	Glucose-6-phosphatase	Membrane-bound ribosome RER is a major site of protein synthesis SER: Synthesis of various lipids Oxidation of many xenobiotics (cytochrome P-450)
Golgi apparatus	Galactosyl transferase	Intracellular sorting of proteins Glycosylation and Sulfating reactions
Lysosome	Acid phosphatase	Site of many hydrolases (enzymes catalyzing degradative reactions)
Peroxisome	Catalase Uric acid oxidase	Degradation of long chain fatty acids & amino acids Production and degradation of hydrogen peroxide
Nucleus	DNA	Site of chromosomes Site of DNA-directed RNA synthesis **(transcription)**
Plasma membrane	Na-K-ATPase 5'-Nucleotidase	Transport of molecules in and out of cells Intercellular adhesion and communication
Cytoskeleton	Nil (Not applicable)	Microfilaments, microtubules, intermediate filaments – impart strength to the cell
Cytosol	Lactate dehydrogenase	Site of glycolysis, fatty acid synthesis, HMP

Chapter 2

FREE RADICALS AND ANTIOXIDANTS

Any chemical species with a single unpaired electron is called free radical. It is a highly reactive species. There are two highly reactive species or free radicals produced in the human body

Oxygen based free radicals (ROS)	Nitrogen based free radicals (RNI)
Singlet oxygen, Superoxide, hydroxyl radical, hydrogen peroxide, hypochlorite	NO, nitrates and nitrites
Produced in various metabolic reactions	NO Produced by NO synthase, which in turn is rapidly converted to nitrates and nitrites
Result in: • DNA damage • Protein damage • Membrane lipid peroxidation Free radical mediated membrane lipid peroxidation is a pathogenic mechanism in: • Aging • Cancer • Inflammation • Atherosclerosis	NO synthase is present in: • Endothelium (eNOS): NO produced by this is responsible for vasodilatation. hence NO is also called endothelium derived relaxing factor (EDRF) • Cholinergic neurons (nNOS) • Induced at the site of inflammation (iNOS)
Antioxidants: take care of normal inevitable production of ROS	
Antioxidant Enzymes: SOD Peroxidase Catalase	
Antioxidant molecules: Tocopherol: 1st line of antioxidant defense Ascorbate: 2nd line of antioxidant defense in all cells except RBC Glutathione: 2nd line of antioxidant defense in RBC Selenium Beta carotene Urate	

Xenobiotic Metabolism

Xenobiotics are foreign molecules. They are not a part of human metabolism. We acquire these xenobiotics from environment in the form of:

1. Pollutants
2. Carcinogens
3. Food additives
4. Drugs

These Xenobiotics have to be effectively detoxified. It is done in liver in two phases

Phase I reaction (Hydroxylation): Catalyzed by cytochrome P450 Monooxygenase. A hydroxyl group is added to the xenobiotic. In this process the iron in cytochrome P450 gets oxidized to ferric. It is reduced back to ferrous by cytochrome P450 Reductase. These 2 enzymes Cytochrome P450 Monooxygenase and Cytochrome P450 Reductase are present in liver endoplasmic reticulum and hence called microsomal enzyme system.

Phase II reaction (Conjugation): Hydroxylated Xenobiotic is conjugated by

1. Glycine
2. Glutathione
3. Glucuronate: UDP Glucuronate is the donor
4. Acetylation: Acetyl CoA is the donor of acetyl group
5. Methylation: S – Adenosyl Methionine is the donor of methyl group
6. Sulfation: PAPS (3 – Phospho Adenosyl – 5 Phospho Sulfate) is the donor of sulfate group

Chapter 3
CARBOHYDRATES: Chemistry and metabolism

Aldehyde or Ketone are derivatives of polyhydric alcohols or compounds which yield these derivatives on hydrolysis.

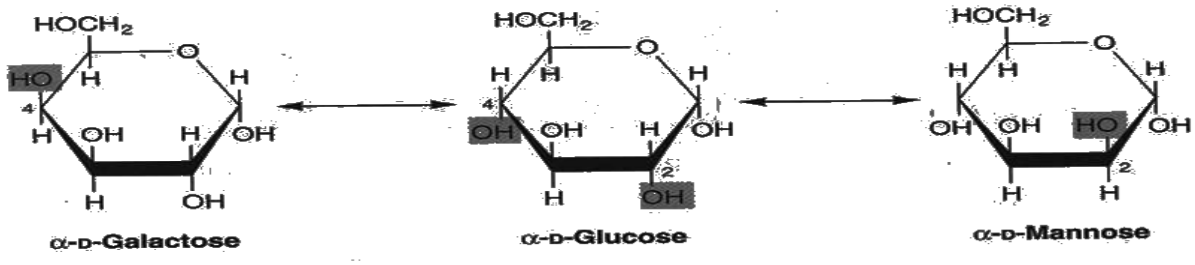
Classification
Monosaccharides :
Contain only one Sugar unit ; cannot be hydrolysed to more simple sugars.
Depending on the functional group they can be Aldose or Ketose

	Aldose	**Ketose**
Triose	Glyceraldehyde	Dihydroxy acetone
Tetrose	Erythrose, Arbinose	Erythrulose
Pentose	Ribose, Xylose	Ribulose, Xylulose
Hexose	Glucose, Galactose, Mannose	Fructose

D-Fructose has optical rotation of (minus) - 92° and therefore it is laevorotatory

The predominant sialic acid found in glycoproteins and gangliosides is the 9 carbon sugar is N Acetyl Neuraminic acid and Neuraminic acid is derivative of N Acetyl mannose and pyruvic acid

Isomerism in Carbohydrates
Anomerism: Difference in the orientation of H and OH around anomeric carbon (C1 for aldoses and C2 for ketoses) . e.g. Alpha and beta anomers
D and L forms: Based on orientation of OH around penultimate carbon. Also for cyclic structures the orientation of ring.
Epimerism: Difference in the orientation of H and OH around a single carbon other than anomeric and penultimate carbon



- Mannose is the epimer of glucose with respect to C2 while Galactose is C4 epimer;
- Similarly Allose is C3 epimer and L Idose is C5 epimer
- d and l forms- Depending on the direction of rotation of polarised light

Important derivatives of monosaccharides are :

Class	**Examples**	**Functions**
1. Glycosides	Digitalis	Cardiac drugs
2. Amino sugars	Glucosamine	Glycosaminoglycan (GAG)
3. Deoxy sugar	Deoxyribose,	DNA, mucin
4. Uronic acid	Glucuronic acid, Iduronic acid	GAG
5. Fucose	Mucin Glycoprotein	

Disaccharides:

Contains 2 monosaccharide units joined together by a glycosidic linkage

Eg:- Maltose = Glucose + Glucose in α 1, 4 linkage

Lactose = Galactose + Glucose in β 1, 4 linkage

Sucrose = Glucose + Fructose in α, β 1, 2 linkage

They can be Reducing or Non reducing

Different disaccharides of glucose: Glucose + Glucose

Cellobiose → β 1,4

Maltose → α 1,4

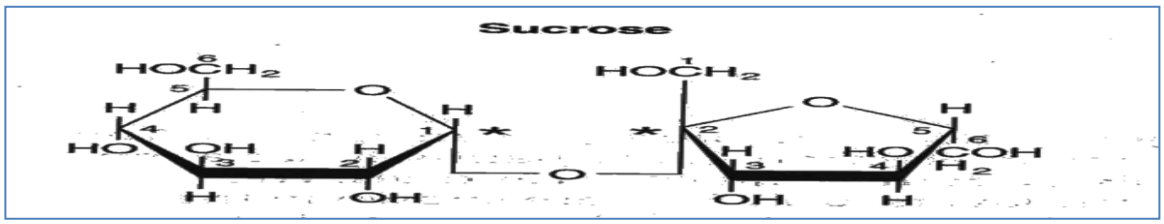
Isomaltose → α 1,6

Trehalose → α 1, 1

Note: intestinal brush border contains enzymes: For disaccharides (Disaccharidases): *Lactase (β – galactosidase), Sucrase, Maltase, Isomaltase (α 1, 6 Glucosidase), Limit Dextrinase, Trehalase*

For Peptides: *Entero peptidases, Amino peptidases, Carboxy peptidases, Endo peptidases, Di peptidases*

For nucleotides and Nucleosides: *Phosphatases, Nucleosidases*



Oligosaccharides : Contain 3-10 monosaccharide units

Polysaccharides : More than 10 monosaccharide units

Can be Homoglycans or (Homopolysaccharide) (Contain same type of monosaccharide units) or Heteroglycans or (Heteropolysaccharide) More than one type of monosaccharide units

Homoglycans: Starch, Glycogen, Cellulose, Inulin, Chitin

Heteroglycans: Mucopolysaccharides

Starch: Amylose + Amylopectin

Amylose: Soluble: Glucose units in α 1, 4 linkages: No branches

Amylopectin: Insoluble: Glucose units in α 1, 4 linkage: Branches with α 1,6 linkage

Glycogen: Same as amylopectin but more branched and compact. Spherical molecule with 21 ηm diameter & 12 concentric layers. 13 Glucose residues in each chain with α 1.4 linkage. Generally branching at every 5th glucose residue with α 1, 6 linkage.

Cellulose: Glucose in β-1, 4 linkages; Extensive H bonds between and within the chains → rigid fibrils

Inulin: Fructose in β-1, 2 linkages. If taken orally it is not hydrolyzed & constitutes dietary fiber. If given I.V it is not metabolized or degraded or taken up by cells and exclusively filtered. Used in clearance test

Chitin: N-acetyl Glucosamine (GluNac) in β-1, 4 links; Extensive H bonding; Seen in Exoskeleton of insects and crustaceans

Dextran: α Glucose units in 1→6, 1→4 and 1→3 linkages; Plasma volume expanders

Agar: polymer of galactose

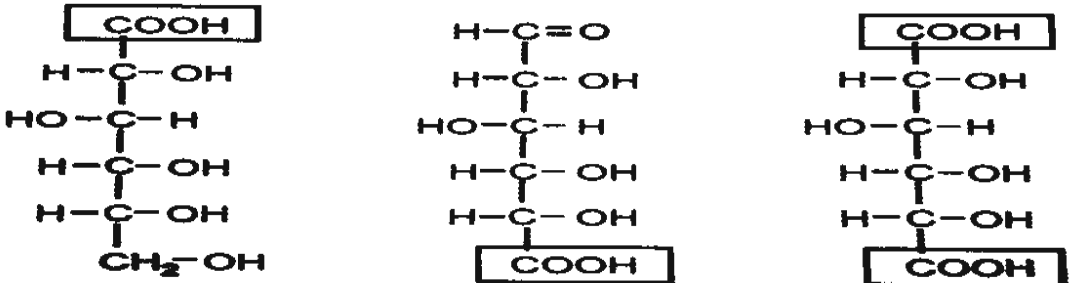
Heteroglycans

Mucopolysaccharides (GAG): Contains aminosugars and uronic acids

Glycosaminoglycans	Sugars	Location
Hyaluronic acid	GlcNAc,GlcUA	Synovial fluid, vitreous humor, loose connective
Chondroitin SO4	GalNAc,GlcUA	Cartilage, bone, cornea
Keratan SO4	GlcNAc, Gal	Cornea Loose connective tissue
Heparin	GlcN,IdUA	Mast cells Present in liver, lung, spleen, monocytes
Heparan sulfate	GlcN, GlcUA	Skin fibroblasts, aortic wall
Dermatan sulfate	GalNAc,IdUA, (GlcUA)	Wide distribution

OXIDATION AND REDUCTION REACTIONS AND TESTS IN CARBOHYDRATES:

Oxidation of sugars



Oxidation at C1→ Gluconic acid (aldonic); Oxidation at C6→ Glucuronic acid (uronic)
Oxidation at both C1&C6→Glucaric (Aldaric)

Reduction of carbohydrates

Glucose→ Sorbitol. Sorbitol is one of the reasons for long term complications of diabetes
Fructose can be reduced to either Sorbitol or Mannitol because of the keto group at C2
Galactose→ Dulcitol (galactitol). Responsible for premature cataract in galactosemics
Non reducing sugars: Polysaccharides, aminosugars and glycosides are non reducing
Sucrose and trehalose are non reducing disaccharides
Mannose → Mannitol. Mannitol is :

- Mild osmotic diuretic. Maintains GFR in ARF, Used for forced diuresis in poisonings.
- Reduces cerebral edema, given in Head injury, Stroke, before and after brain surgery.
- Reduces intraocular tension, given in Acute congestive glaucoma, before and after ocular surgery

Different tests for carbohydrates

Molisch test- General test for carbohydrates
Benedict's test and **Fehling's test** : for reducing sugars
Barfoeds test, Moore's test- to differentiate between monosaccharides and disaccharides
Feulgen stain- for deoxy sugars: used to detect DNA – Reaction of deoxy sugars with Schiff reagent

COMPLEX CARBOHYDRATES WITH PROTEINS

1. **Glycoproteins:-**

- Protein molecule on which oligosaccharide chain (upto 15 subunits) is attached by N or O glycosidic bond
- This process is called glycosylation or glycation and the complex is called glycosylated or glycated or glyco protein
- It is most common post translational modification and almost every protein is glycated
- The oligosaccharides are:
    - Hexoses

- Mannose
- Galactose

o Acetyl Hexosamines (amino sugars)
- Gal Nac
- Glu Nac

o Sialic acids – NANA (predominat sialic acid)
o Pentoses
- Arabinose
- Xylose

o Methyl pentoses
o L – Fucose (6 deoxy - β-L-galactose)

2. GAGs – Glycosaminoglycans (Mucopolysaccharides)

- Heteropolysaccharides containing
  i. amino sugars
     1. Glucosamine
     2. N Acetyl Glucosamine
     3. Galactosamine (Chondrosamine)
     4. N Acetyl Galactosamine
     5. Galactose residues may be sulfated
  ii. Uronic acids
     1. Glucuronic acid
     2. Iduronic acid

If attached to a protein core: - Proteoglycans (mucoproteins)

- Large No. of OH groups & negative charge hold large qty of water & occupy space.
- Negative charges also repel & keep the chains apart.
- Due to above 2 properties proteoglycan chains can easily slide over each other and have cushioning & lubricating functions
- Provide ground (packing) substance of connective tissue
- Due to high viscosity, also present in mucus and synovial fluid

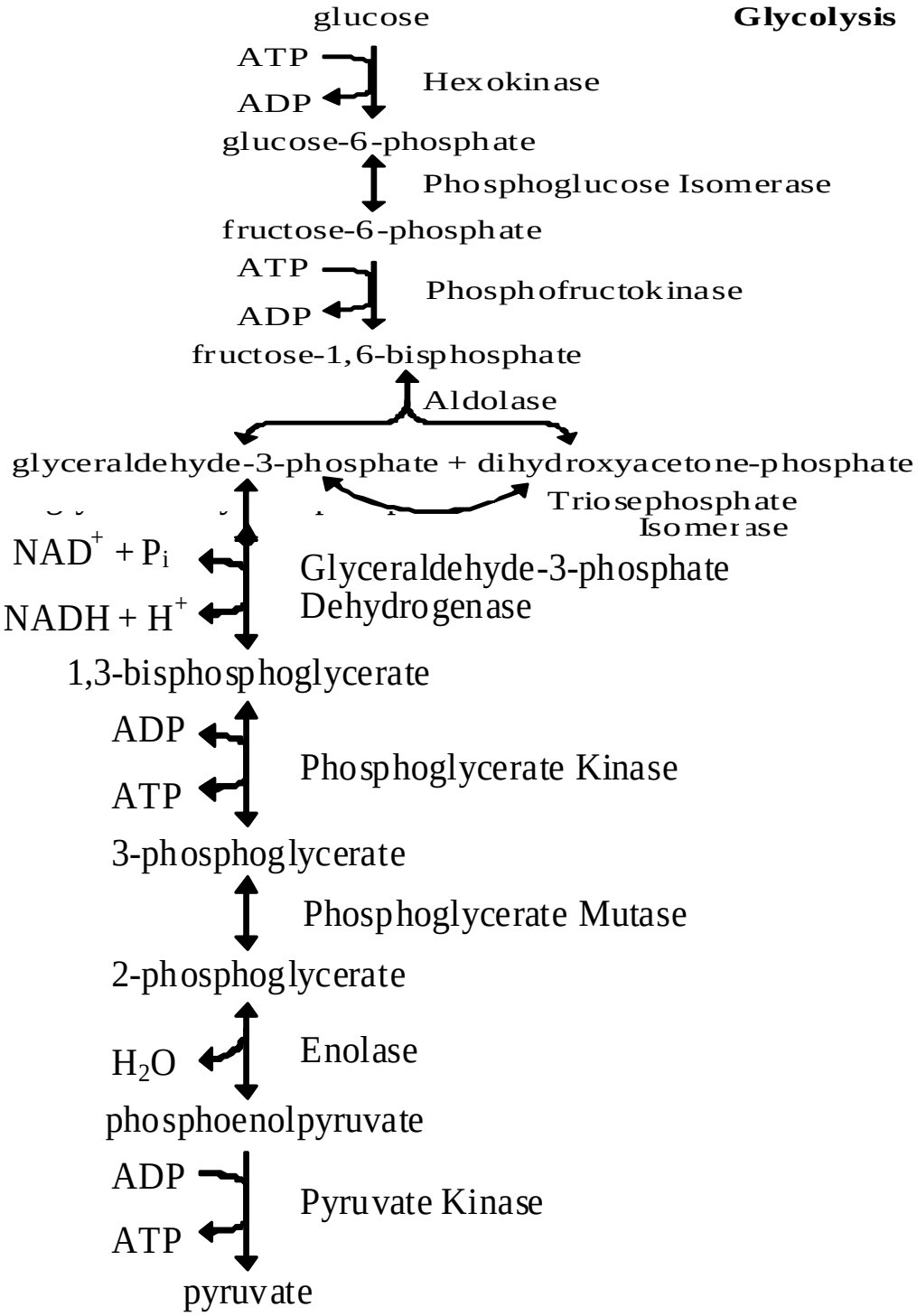
GLUCOSE TRANSPORTERS

Facilitative Bidirectional Transporters

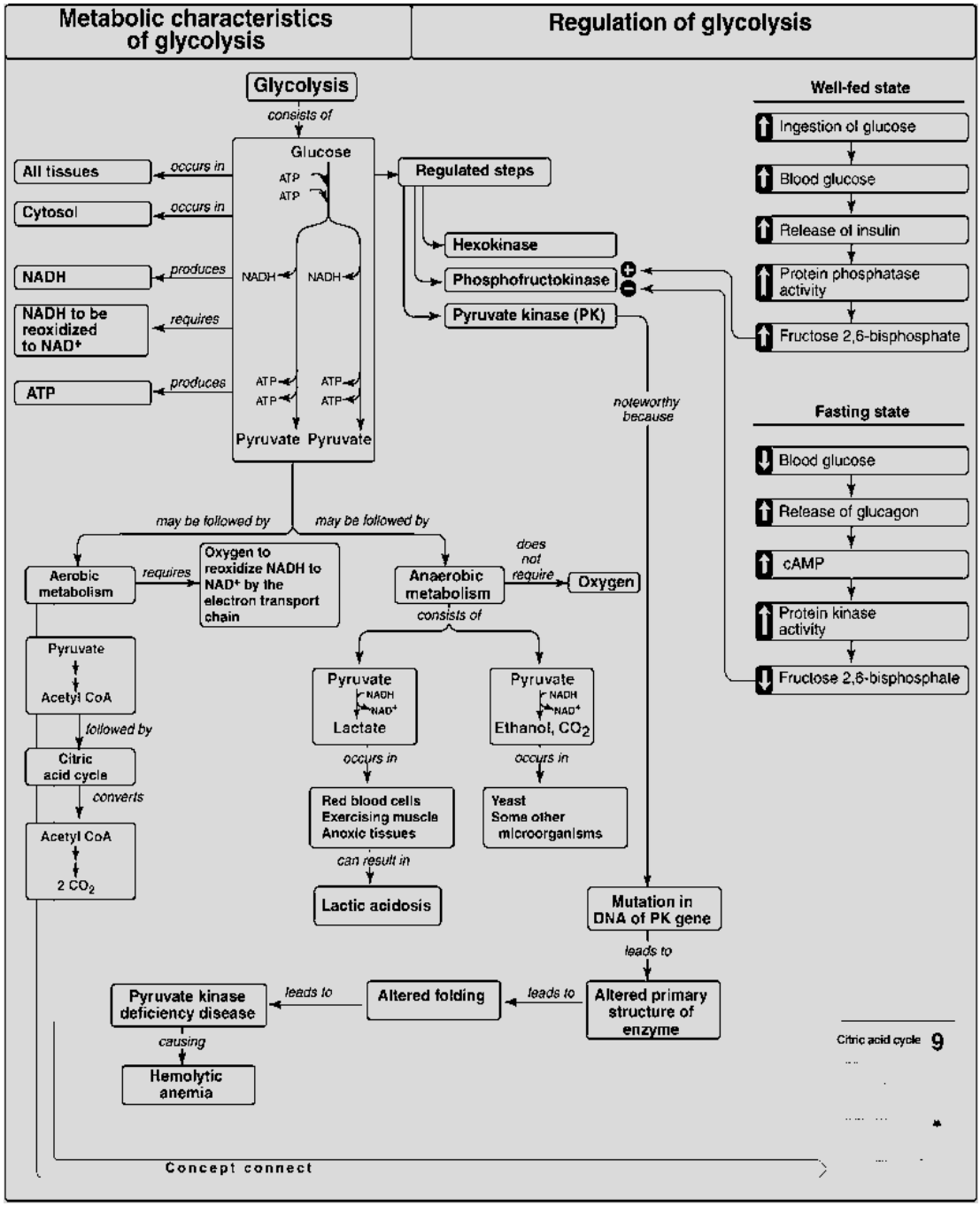
Transporter	Specified Tissue location	Functions
GLUT 1	Brain, Kidney, Colon, Placenta, RBCs, Retina	Uptake of Glc
GLUT 2	Liver, β Cell of pancreas, serosal surface of intestinal cells	Km high. Low affinity for Glc.
GLUT 3	Brain, Neurons, Placenta	High affinity for Glc.
GLUT 4	Heart and Skeletal muscle, adipose tissue	Insulin dependent uptake
GLUT 5	Small intestine, testis sperms, kidney	Fructose transporter- Low affinity to Glc

Sodium dependent unidirectional Transporter

SGLT 1 SGLT 2	Small intestine- High affinity Kidney- Low affinity	Active uptake of Glc


glucose
Glycolysis
ATP
ADP
Hexokinase
glucose-6-phosphate
Phosphoglucose Isomerase
fructose-6-phosphate
ATP
ADP
Phosphofructokinase
fructose-1,6-bisphosphate
Aldolase
glyceraldehyde-3-phosphate + dihydroxyacetone-phosphate
Triosephosphate Isomerase
NAD+ + Pi
NADH + H+
Glyceraldehyde-3-phosphate Dehydrogenase
1,3-bisphosphoglycerate
ADP
ATP
Phosphoglycerate Kinase
3-phosphoglycerate
Phosphoglycerate Mutase
2-phosphoglycerate
H2O
Enolase
phosphoenolpyruvate
ADP
ATP
Pyruvate Kinase
pyruvate

Glycolysis- key concepts



BPG shunt

- Rapaport-Leubering cycle
- No net ATP production
- Occurs in RBC
- For production of 2,3 BPG

Important inhibitors: **Iodoacetate: Glyceraldehyde 3 Phosphate DH;**

Arsenate: Phosphoglycerate Kinase step; Fluoride: Enolase step

Regulation of glycolysis

Enzyme	Activator	Inhibitor
HK		G-6-P
GK	Insulin	Glucagon
PFK	Insulin, AMP, F-6-P, F-2,6-BP	Glucagon, ATP, Citrate, Low pH, cAMP
Pyruvate kinase	Insulin, F-1,6-BP	Glucagon, ATP, cAMP, Alanine
PDH	CoA, NAD+	Acetyl CoA, NADH

Energy yield- Anaerobic glycolysis

Enzyme	Source	ATP per Glucose
HK		-1
PFK		-1
1,3 BPG kinase	ATP	1x2=2
Pyruvate kinase	ATP	1x2=2
	Total	2 ATP

Energy yield- Aerobic glycolysis

Enzyme	Source	ATP / glucose
HK		-1
PFK		-1
Glyceral-3P DH	NADH	3x2=6
1,3 BPG kinase	ATP	1x2=2
Pyruvate kinase	ATP	1x2=2
	Total	8 ATP

Energetic of Aerobic glycolysis+TCA cycle

- Glycolysis → 8 ATP
- PDH → 6 ATP (2 x NADH)
- TCA cycle → 24 ATP (2x 12)
- Total = 38 ATP

TCA cycle

Oxidation of Pyruvate to acetyl CoA

Multienzyme complex (Pyruvate Dehydrogenase Complex)

Present in inner mitochondrial membrane. Made up of

3 enzymes: PDH, DHL Transacetylase , DHL Dehydrogenase

5 Coenzymes: TDP (Thiamine, Vitamin B_1), Lipoamide (lipoic acid), CoASH (Pantothenate vitamin B_5), FAD (Riboflavin vitamin B_2), NAD (Niacin vitamin B_3)

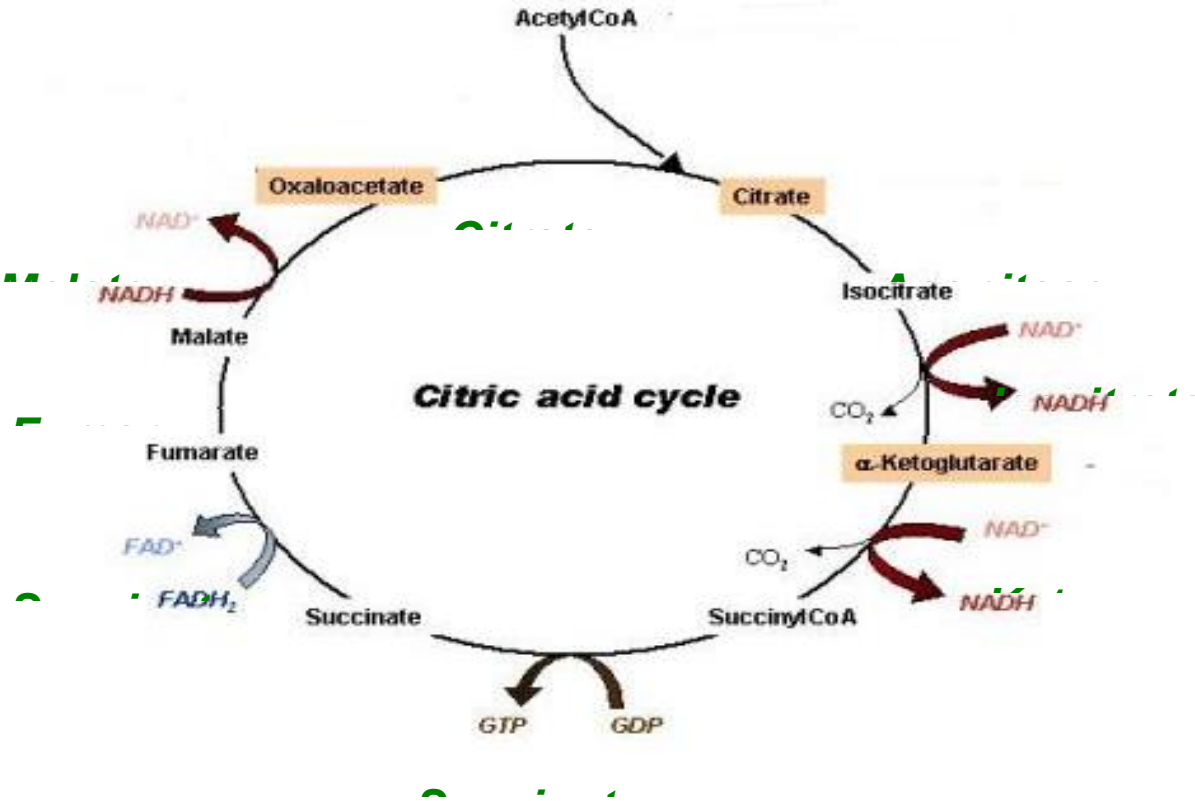
Analogous to αKG DH complex of TCA cycle & αKA Decarboxylase complex in branched chain amino acid metabolism

All the three are thymine dependent enzymes and carry out oxidative decarboxylation.

Other example of multienzyme complex fatty acid synthase complex

Overall reaction: Pyr + CoASH + NAD+ → CO2 + Acetyl CoA + NADH + H+

Final common oxidative pathway- occur in mitochondria

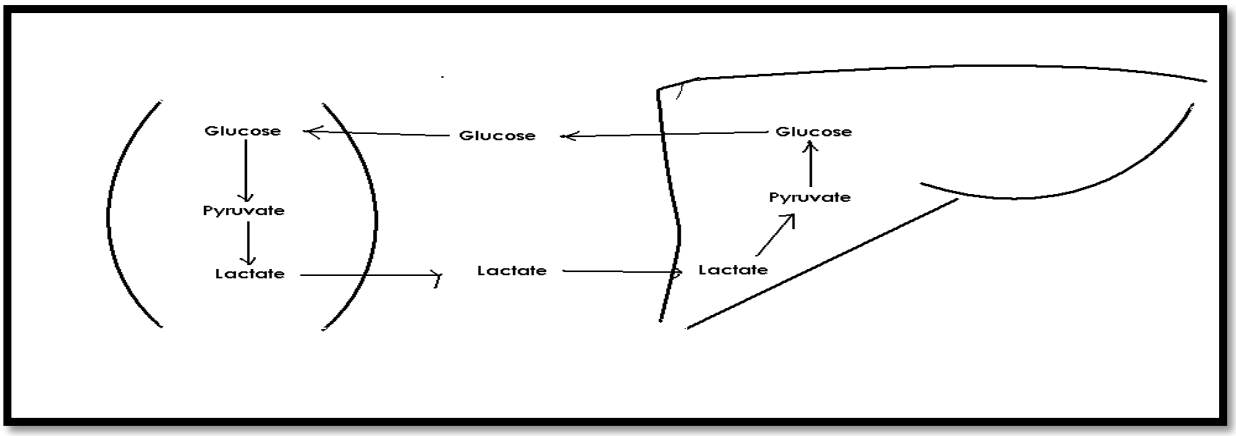


Energy yielding steps in TCA cycle
Succinate dehydrogenase - $FADH_2$
Isocitrate dehydrogenase, α ketoglutarate dehydrogenase and Malate dehydrogenase - NADH
Succinate thiokinase – GTP

(1 NADH = 3 ATP; 1 FADH = 2 ATP; 1 GTP = 1 ATP)

Isocitrate dehydrogenase and α ketoglutarate dehydrogenase removes CO_2 α ketoglutarate dehydrogenase reaction is irreversible

CORI's lactic acid cycle: for effective utilization of lactate formed during muscle contraction



HMP shunt pathway: Other names are

- Pentose phosphate pathway; Phosphogluconate pathway
- Dickens-Horecker pathway
- Warburg-Lipmann- Dickens-Horecker shunt

Sites

- Liver, Adipose tissue, RBC, Adrenal cortex, Mammary glands, Testes, ovary, Lens of the eye

2 phases

- Oxidative phase: Irreversible; Produce NADPH
  Operates in those organs where NADPH is required for fatty acid, cholesterol or steroid synthesis
  Also in tissues where NADPH is required to prevent oxidative damage
- Non-oxidative phase: Reversible; Produce pentoses
  Operates in all tissues
  Pentoses can be produced in all tissues

Oxidative phase Steps are irreversible

- 2 steps produce NADPH
  - G-6-PD (Glucose-6-phosphate dehydrogenase)
  - 6-phosphogluconate dehydrogenase
- First pentose formed is RIBULOSE-5-Phosphate
- Rate limiting enzyme is G-6-PD

Non-oxidative phase

Produces pentoses

'Transketolase reaction'

- (Transketolases transfer a 2 C unit [glycolaldehyde]from a ketose to an aldose)
- Transketolase **requires thiamine pyrophosphate (TPP)**, a derivative of **vitamin B_1**. as a coenzyme

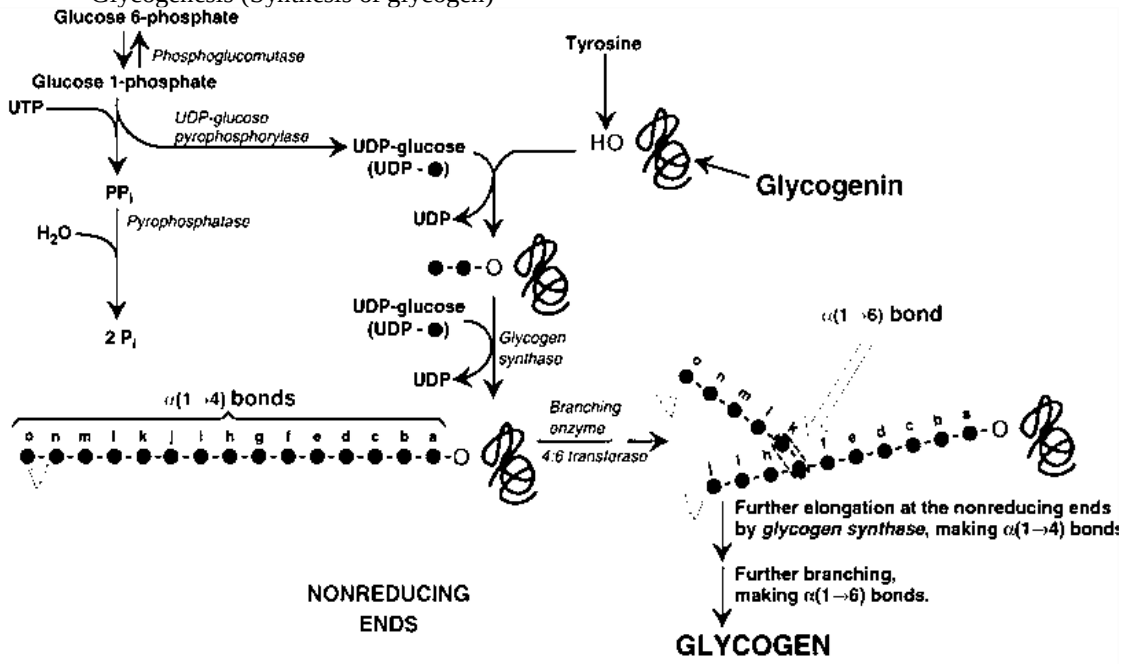
G-6-PD deficiency

- Most common enzyme deficiency
- Trasmitted as X-linked recessive
- Hemolysis when exposed to certain drugs [Eg: Antimalarials, Sulpha drugs etc]
- Ingestion of Fava beans also precipitate hemolysis

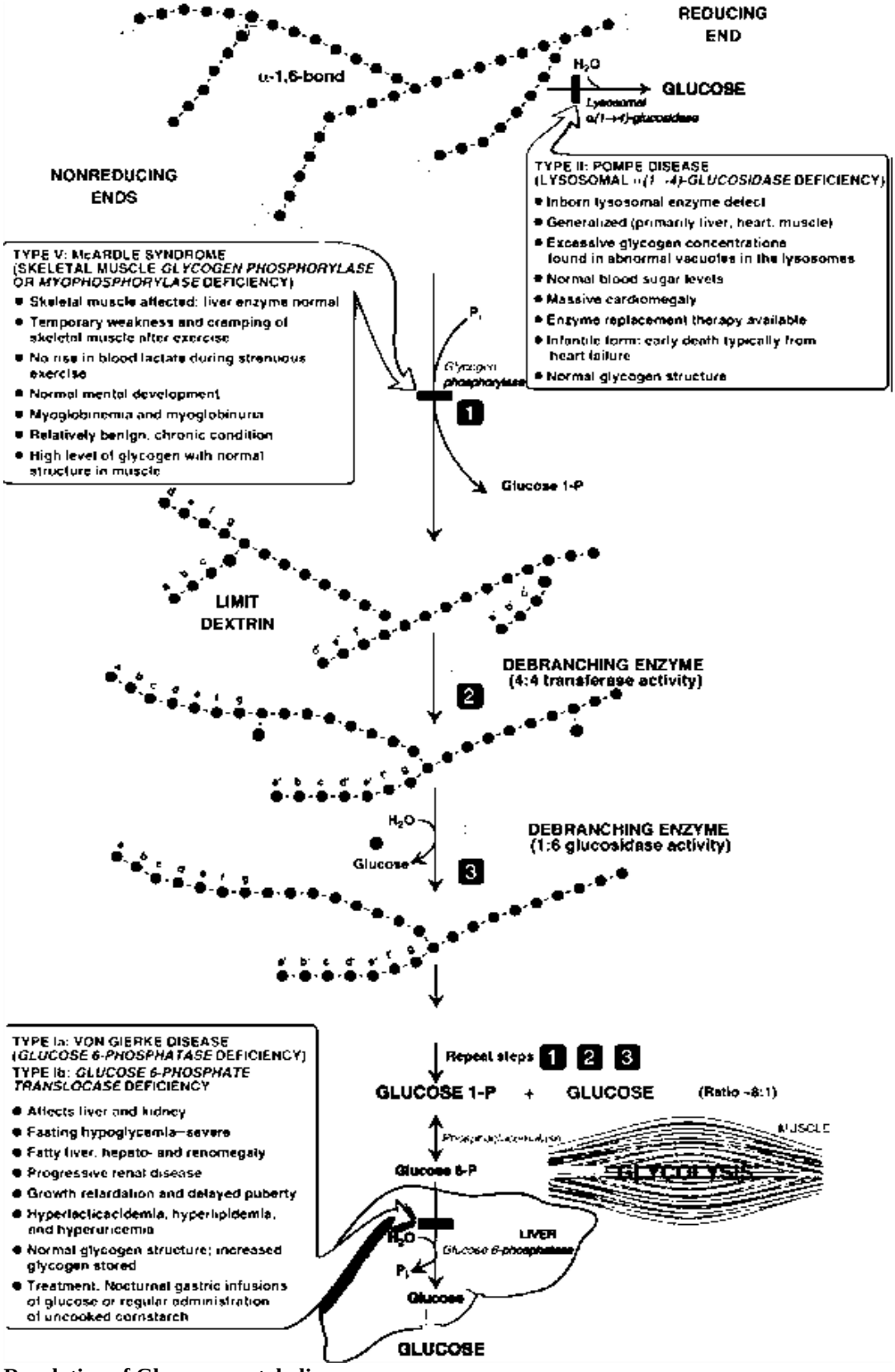
- Met hemoglobinemia

Metabolism of glycogen

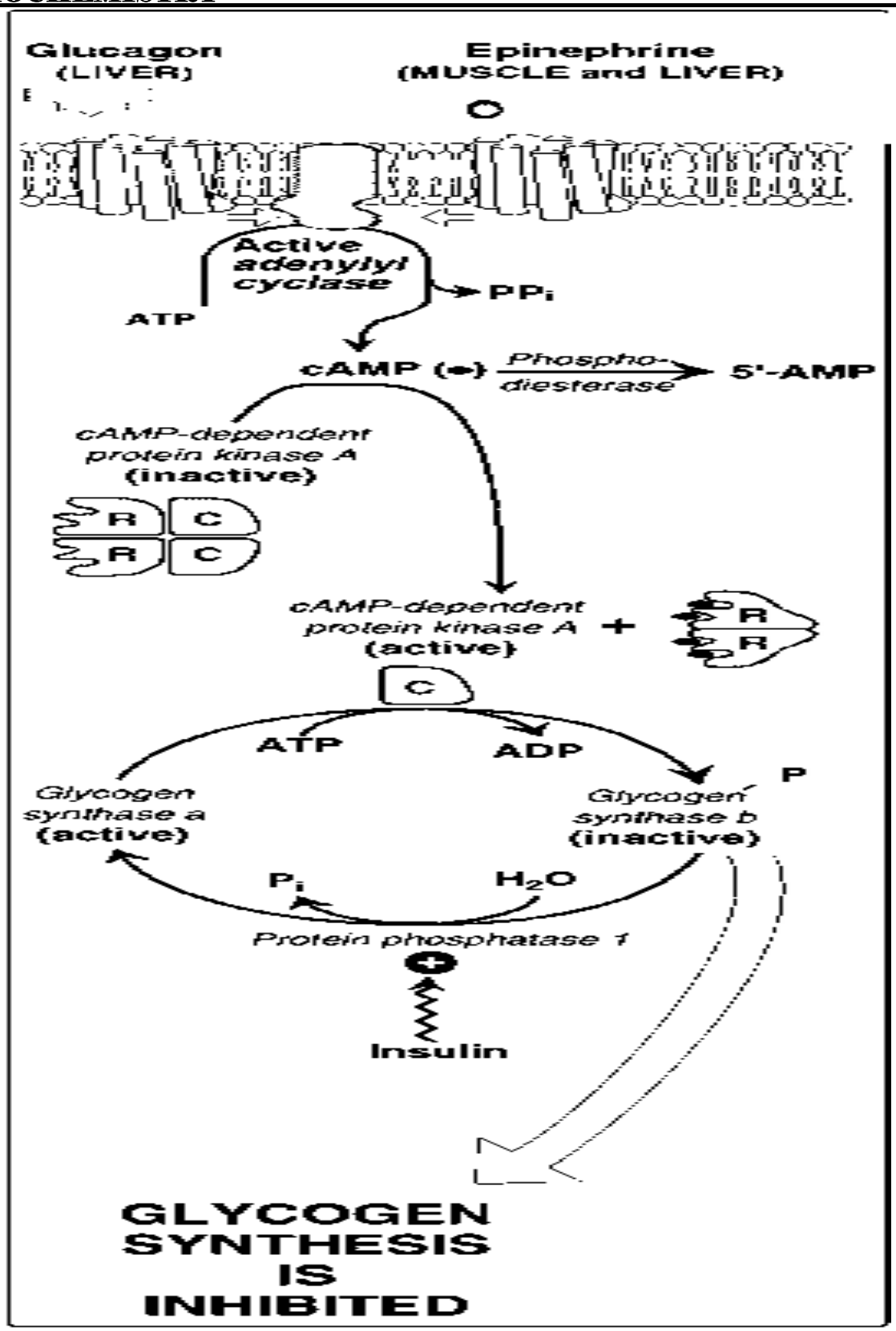
Glycogenesis (Synthesis of glycogen)



Degradation of Glycogen



Regulation of Glycogen metabolism


Glucagon
(LIVER)
Epinephrine
(MUSCLE and LIVER)
Active adenylyl cyclase
ATP
PPi
cAMP (+)
Phospho-diesterase
5'-AMP
cAMP-dependent protein kinase A (inactive)
R
C
R
C
cAMP-dependent protein kinase A (active)
+
R
R
C
ATP
ADP
P
Glycogen synthase a (active)
Glycogen synthase b (inactive)
Pi
H2O
Protein phosphatase 1
Insulin
GLYCOGEN SYNTHESIS IS INHIBITED

Importance of glycogen

1. Major storage form of glucose
   Liver – 5% of tissue concentration (1/4 of total body glycogen)
   Muscle – 0.7 % of tissue concentration (3/4 of total body glycogen)
2. Muscle glycogen – Provides glucose for its own use (lacks Glucose 6 Phosphatase)
   Depletion occurs after prolonged vigorous exercise
3. Liver glycogen – Maintains Blood glucose for 10 – 12 hours in between meals.
4. Highly branched structure of glycogen provides multiple sites for glycogenolysis.

↓

Rapid release of glucose in need

Glycogen Storage Diseases

Glycogen storage disorders are inborn errors due to defect in enzmes concerned with metabolism of Glycogen. Glycogen gets deposited in Liver, spleen, muscles etc.

Characteristic features are hepatosplenomegaly, fasting hypoglycemia, decreased exercise tolerance.

Glycogenosis	Name	Cause of Disorder
Type 0		**Deficiency of Glycogen Synthase**
Type 1	**Von Glerke's disease**	**Deficiency of glucose – 6 phosphatase**
Type II	**Pompe's disease (1-3% glycogen is degraded in lysosomes)**	**Deficiency of lysosomal α-1$\rightarrow$4 and 1 $\rightarrow$6-glucosidase (acid maltase)**
Type III a	**Limit dextrinosis, Forbes' or cori's disease**	**Deficiency of debranching enzyme in Liver and Muscle**
Type III b	**Limit dextrinosis**	**Deficiency of debranching enzyme only in liver**
Type IV	**Amylopectinosis, Andersen's disease**	**Deficiency of Branching Enzyme**
Type V	**Myophosphorylase deficiency, McArdle's syndrome**	**Deficiency of muscle phosphorylase**
Type VI	**Her's disease**	**Deficiency of liver phosphaorylase**
Type VII	**Tarui's disease**	**Deficiency of phosphofructokinase in muscle and erythrocytes**
Type VIII		**Deficiency of liver phosphorylase kinase**
Type IX		**Deficiency of liver & muscle phosphorylase kinase**
Type X		**Deficiency of cAMP dependent Protein Kinase A in both muscle and liver but affects only liver**

Type 0

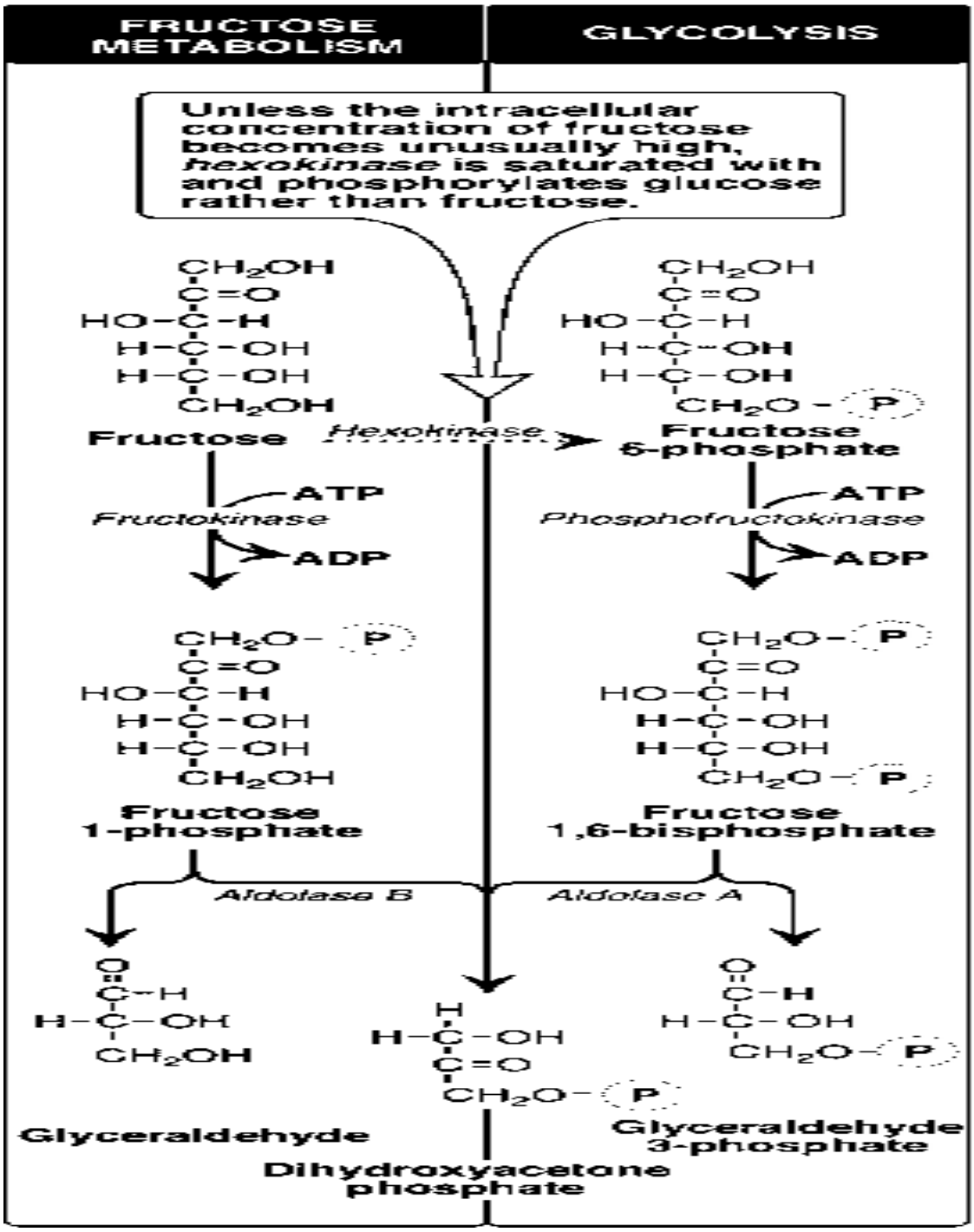
- Glycogen synthase deficiency
- Hypoglycemia, hyperketonemia, early death

Type I

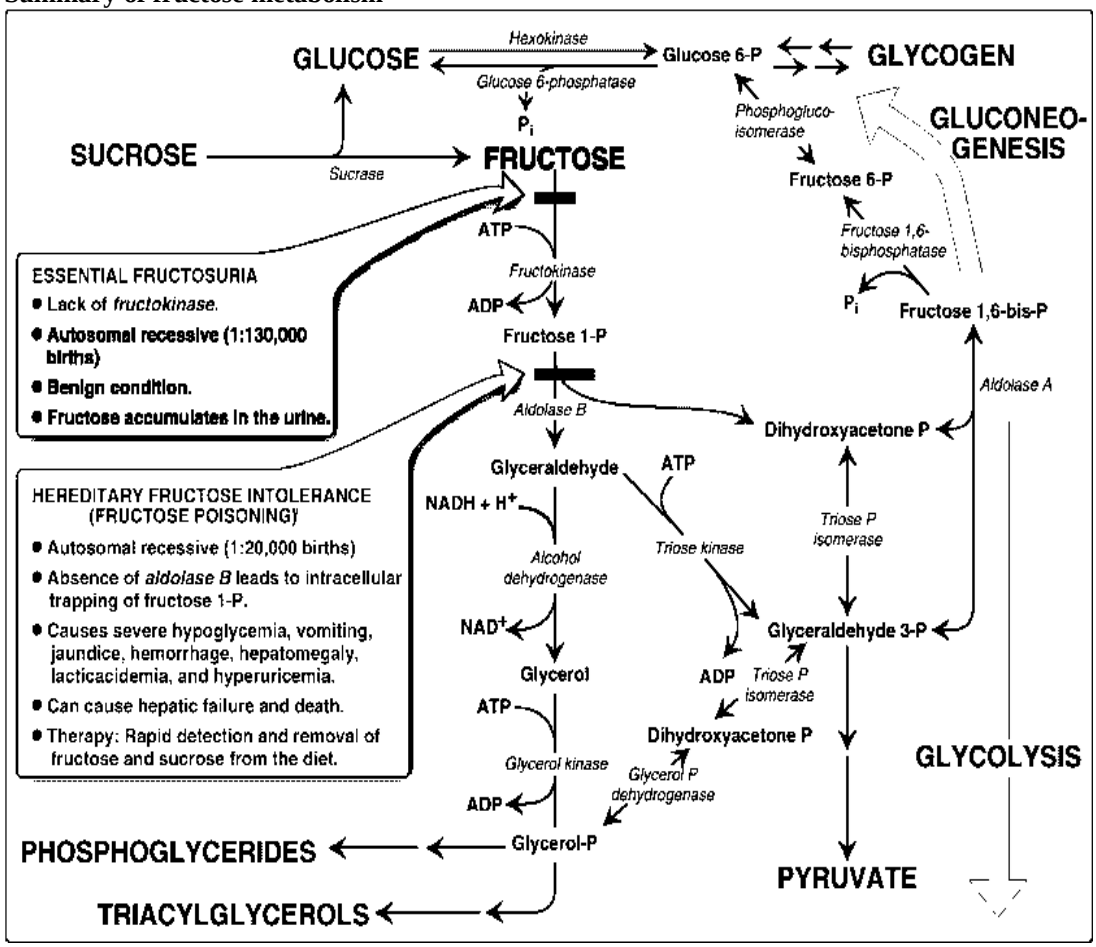
- I a – von Gierke's
- I b – E.R glucose 6 PO_4 transporter

Fatal GSD: **Cardiac failure in GSD- Pompe's disease, Anderson's disease**

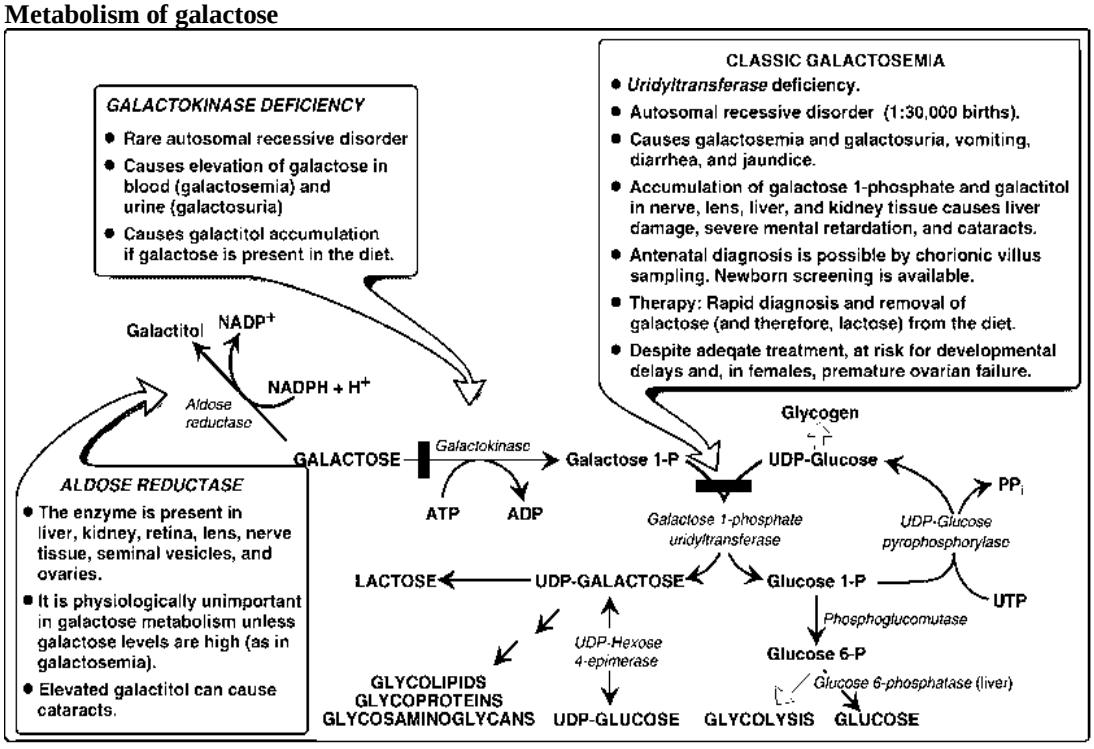
Fructose metabolism



Summary of fructose metabolism



Metabolism of galactose



Galactosemia

Gal-1-P uridyl transferase deficiency
Cataract is due to accumulation of dulcitol (from galactose)
Other features due to toxic effects of Galactose-1-P

- MR, Jaundice, Hepatomegaly, Renal damage
- Hypoglycemia
- Galactosuria, aminoaciduria

Galactokinase deficiency

- Gal-1-P not formed : No toxic effects
- Galactose accumulates: → cataract

•Social smile not developed

MUCOPLOYSACCHARIDOSIS

Disease	Defective enzyme	GAG in urine
Hurler	L Iduronidase	DS, HS
Hunter	Iduronate Sulfatase	DS, HS
Sanfilippo	NAc Glucosaminidase	HS
Morquis	NGal Sulfatase	KS,CS
Scheie's	L Iduronidase	DS
Maroteaux	NAc Gal 4 sulfatase	DS
Slys	Beta Glucuronidase	DS, HS

Insulin

Secreted as Pre-pro insulin (109 a.a)
Coverted to Pro insulin (86 a.a) in E.R
B chain-C peptide-A chain
Cleaved in Golgi apparatus
C peptide 33 a.a

Insulin

Composed of 51 amino acids arranged in two polypeptide chains, designated A and B, which are linked together by two disulfide bridges. The insulin molecule also contains an intramolecular disulfide bridge between amino acid residues of the A chain
2 chains: A (21 a.a) B (30 a.a)
One intrachain (A6→A11) S-S bonds
2 interchain (A7→B7 and A20→B19)
Species variation is restricted to a.a 8,9,10 of A chain and C terminal of B chain
Number of a.a in C-peptide also varies according to species

Metabolic effects of insulin

- **Carbohydrates:** In the liver and muscle, increases glycogen synthesis. In the muscle and adipose, increases glucose uptake by increasing the number of glucose transporters (GLUT-2).. In liver, insulin decreases the production of glucose through the inhibition of glycogenolysis and gluconeogenesis.
- **Lipids:**
  - Inhibit hormone sensitive lipase by dephosphorylation.
  - Increased triacylglycerol synthesis**:** increases the transport and metabolism of glucose into adipocytes, providing glycerol 3-phosphate for TAG synthesis. Also increases the *lipoprotein lipase* in adipose tissue by increasing the enzyme's synthesis, thus providing fatty acids for esterification
  - In liver, insulin promotes the conversion of glucose to triacylglycerols
- **Protein:** stimulates the entry of amino acids into cells, and protein synthesis.

Metabolic effects of Glucagon

- **Carbohydrate:** increase the breakdown of liver (not muscle) glycogen and increase gluconeogenesis.
- **Lipids:** Glucagon activates lipolysis in adipose. So glucagon is ketogenic.
- **Proteins:** Glucagon increases uptake of amino acids by the liver, resulting in increased availability of carbon skeletons for gluconeogenesis. Decreases plasma levels of amino acids

Metabolic syndrome (X) , Reaven syndrome: NCEP criteria

Central obesity, Elevated BP

High TG, Low HDL, Elevated FBS

Chapter 4
LIPIDS

Important points to remember

Neutral lipids: Triglycerides, Cholesterol and Cholesteryl esters are uncharged and thus neutral lipids
All phospholipids are derived from simplest phospholipid **Phosphatidic acid**
Phospholipids contain in addition to fatty acids and alcohol A phosphoric acid residue
Based on whether the alcohol is glycerol or sphingosine, the phospholipids are classified respectively as Glycerophospholipids or Sphingophospholipids
Plasmalogens which constitute 10% of total phospholipids of brain and muscle has **Ether linkage instead of ester linkage at Sn_1**
Neonatal Respiratory distress syndrome results from **deficiency of Dipalmitoyl lecithin (surfactant)**
Cardiolipin, a major lipid of the inner mitochondrial membrane is Diphosphatidyl glycerol
The alcohol present in sphingophospholipids and glycolipids is Sphingosine (Glycerol is not present)
Ceramide is formed by combination of Sphingosine and fatty acid
Sphingomyelins consist of Ceramide (Sphingosine + fatty acid), phosphoric acid and choline
Cerebrosides are glycolipids containing Ceramide and either glucose or galactose
Gangliosides are composed of Sphingosine, a long chain fatty acid, and oligosaccharide chain containing sialic acid and glucose
The **simplest ganglioside** present in human body **GM_3**
Fatty acids, glycerol and steroids & other alcohols eg. Cetyl alcohol, Dolichol are called Derived lipids
Most of the naturally occurring unsaturated fatty acids have double bonds in Cis configuration
Oleic acid with 18 carbons, one double bond at 9^{th} position from carboxyl carbon can be represented as 18 :1; 9 or Δ^9, 18 : 1 (belongs to ω_9 series)
Linoleic acid and Arachidonic acid belong to the ω_6 series
α Linolenic acid is an example of ω_3 fatty acid
Eicosanoids (derived from C_{20} PUFAs) are Prostaglandins, Thromboxanes, Leukotrienes & Lipoxins
Common feature of all prostaglandins is Cyclopentane ring
Common to all thromboxanes is Cyclopentane ring interrupted with an oxygen atom
Common feature of Leukotrienes & Lipoxins is Absence of cyclopentane ring and presence of 3 & 4 conjugated double bonds respectively
All steroids contain Cyclo Pentano Perhydro Phenanthrene ring (CPP ring)
Chemical features of cholesterol are Cyclopentano perhydro phenanthrene ring; 27 Carbon atoms; OH at position 3; Double bond between C_5 and C_6
Lipids that contain both hydrophilic & hydrophobic groups are called Amphipathic lipids
Examples of amphipathic lipids are Phospholipids, cholesterol, fatty acids, bile salts
Examples of totally hydrophobic lipids are Triacylglycerol and cholesteryl ester

Lipids- Classification

Simple lipids: Esters of fatty acids with an alcohol
- Esters of fatty acids with glycerol- Fats
- Fats which exist as liquid at room temperature- Oils
- Esters of fatty acids with higher molecular weight monohydric alcohols- waxes

Compound lipids: Contain groups in addition to f.a and alcohol
- Phospholipids, Glycolipids, Sulfolipids etc

Derived lipids: Derived from any of the above:

Fatty acids

Saturated fatty acids

Acetic	(2)		SCFA
Butyric	(4)	←Milk, Butter	SCFA
Caproic	(6)	←Milk, Butter	SCFA
Caprylic	(8)		MCFA
Capric	(10)	← Coconut oil	MCFA
Lauric	(12)	← Coconut oil	MCFA
Myristic	(14)	← Coconut oil	MCFA

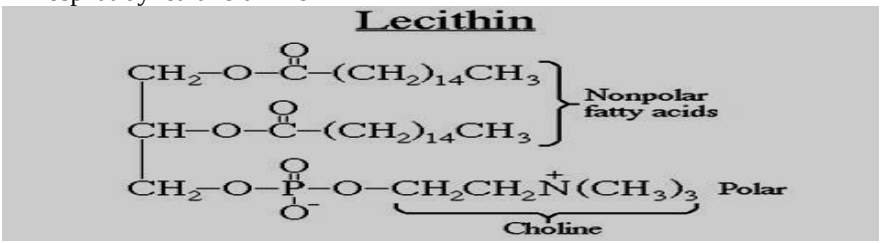
Palmitic (16) ← Body fat LCFA
Stearic (18) ← Body fat LCFA
(Very long chain= more than 24 C)

Unsaturated fatty acids

No of C and No & position of Double Bonds	Omega Number	Common name	Occurrence
Monoenoic acids (one double bond)			
16:1; 9	ω7	Palmitoleic	In nearly all fats
18:1; 9	ω9	Oleic	Possibly the most common fatty acid in natural fats
18:1; 9	ω9	Elaidic	Hydrogenated and ruminant fats
Dienoic Acids (two double bonds)			
18:2; 9,12	ω6	Linoleic	Corn, peanut, cottonseed, soybean, many plant oils
Trienoic acids (three double bonds)			
18:3; 6,9,12	ω6	γ-Linolenic	Some plants, eg. Oil of evening primrose, borage oil; minor fatty acid in animals
18:3; 9,12, 15	ω3	α-Linolenic	Frequently found with linoleic acid but particularly in linseed oil
Tetraenoic acids (four double bonds)			
20:4; 5,8,11,14	ω6	Arachidonic	Found in animal fats and in peanut oil, important component of phospholipids in animals
Pentaenoic acids (five double bonds)			
20:5; 5,8,11,14, 17	ω3	Timnodonic	Important component of fish oils, e.g. Cod liver, mackerel, menhaden, salmon oils
Hexaenoic acids (six double bonds)			
20:6; 4,7,10,13,16,19	ω3	Cervonic	Fish oil, phospholipids in brain

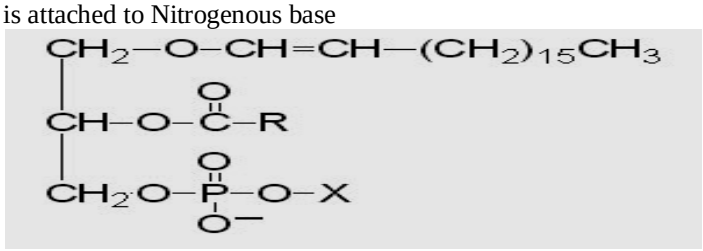
ω 9 fatty acids eg: Oleic, Elaidic **Note that Elaidic is a Trans fatty acid**

Phospholipids
Phosphatidates: those which contain phosphatidic acid [1, 2 diacyl glycerol to which phosphoric acid is added at 3rd position]
Nitrogenous base is attached to phosphoric acid
Cardiolipin is Diphosphatidyl glycerol
Lecithin- Phosphatidyl choline
Cephalin- Phosphatidyl ethanolamine



Plasmalogens

- Similar to phosphatidate; an aliphatic long chain unsaturated alcohol is attached in ether linkage to the first OH of glycerol
- Phosphoric acid is attached to Nitrogenous base



Plasmalogen

Sphingolipids

- They contain ceramide (fatty acid + sphingosine in amide linkage)
- Phosphosphingosides: have phosphoric acid Eg: Sphingomyelin
- Glycosphingolipids: Ceramide + carbohydrates Eg: Glucocerebroside

Gangliosides

- Ceramide-oligosaccharides with at least one NANA attached to them
- GM_3 is simpler than GM_1 G = Ganglioside M = monosialo
- 1,2,3 etc – based on electrophoretic migration

Antioxidants: Act either by preventing the chain of events which lead to damage from starting or by breaking the chain of events
Preventive: reduce the rate of chain initiation: catalase, peroxidase, Se, EDTA, DTPA (di ethylene triamine penta acetate)
Chain breaking: Interfere with chain propagation: SOD, Viamin E

Iodine number: No: of grams of Iodine taken up by 100g of fat. Directly proportional to the *content or* degree of un saturation.

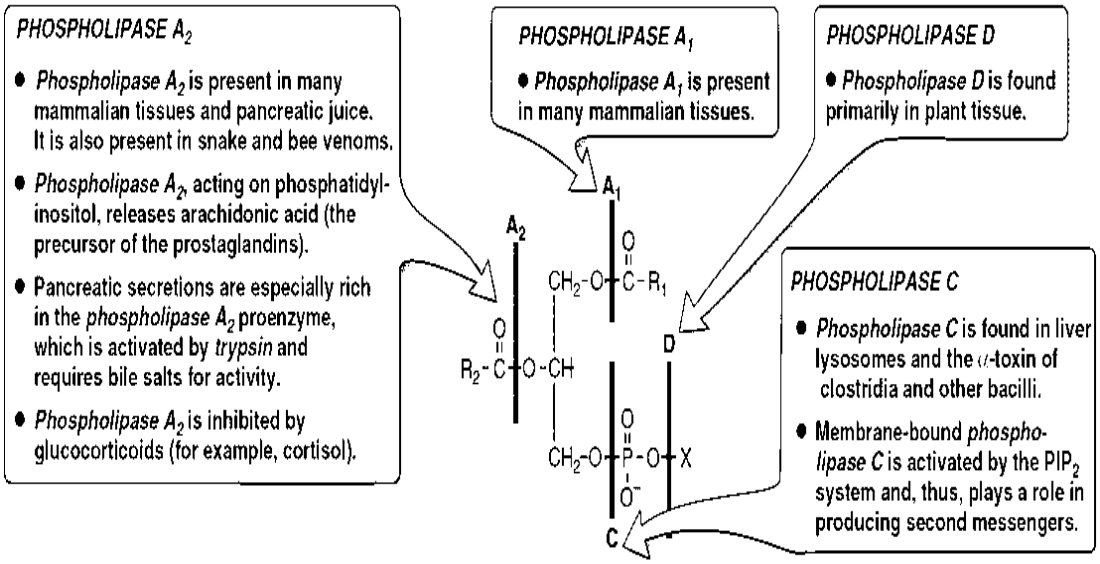
Saponification number : the number of mg of KOH required to saponify1g fat. Inversely proportional to molecular weight anf thus is measure of average molecular size of fatty acids
Higher Saponification number indicates Short chain fatty acids. butter (230 – 240; coconut oil (250-260)

Reichert – Meissl (RM) number defined as ml of 0.1 N KOH to neutralize soluble volatile fatty acids in 5 g fat is a measure of Volatile fatty acids like butyric, caproic and caprylic acid. RM number is highest for butter (range 25-30)

Acid number defined as mg of KOH to neutralize free fatty acids in 1 g fat is used to check decomposition and bacterial contamination of fats and oil

Essential fatty acids
Linoleic, α linolenic and Arachidonic acid
Linoleic and α linolenic acid cannot be synthesized in the body. Arachidonic acid can be formed from Linoleic acid

Action of phospholipases on Lecithin produces
PL A1 → Fatty acid + 2 acyl glycerophosphoryl choline
PLA2 → Lysolecithin + fatty acid
PL C → 1, 2 diacyl glycerol + Phosphoryl choline
PL D → Phosphatidic acid + Choline



OXIDATION OF FATTY ACIDS

Mainly β-oxidation

Fatty acids are "activated" first: Fatty acid → Fatty acyl CoA (enzyme: Acyl CoA synthetase)

Requires 2 high energy bonds (ATP→ AMP)

Only step in degradation of fatty acids which require energy

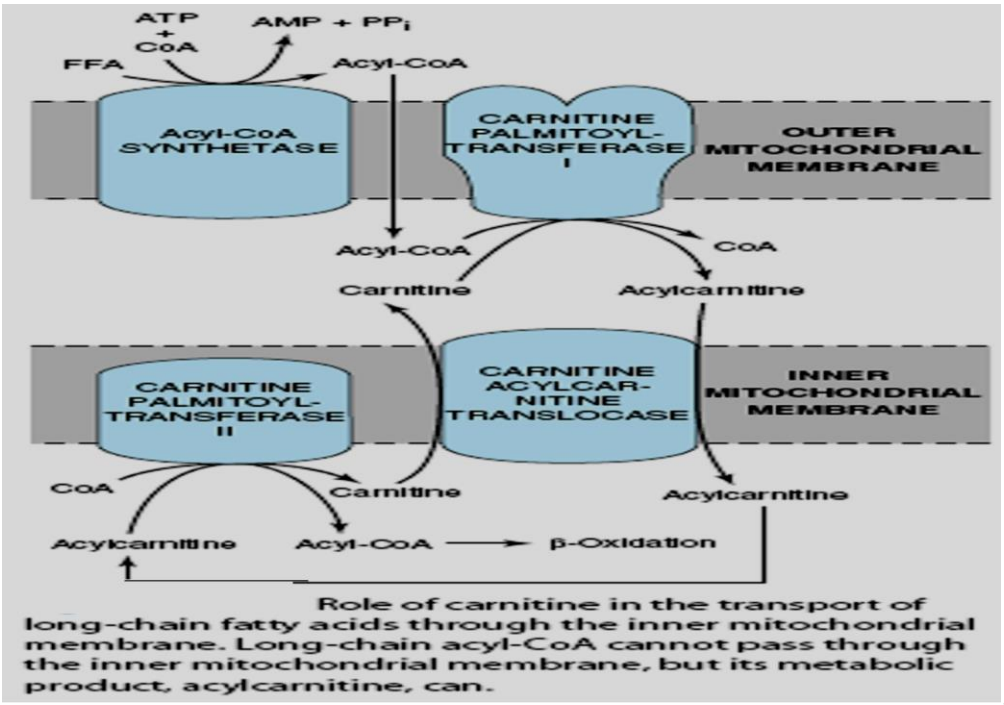
Takes place in E.R, Peroxisomes or on the outer mitochondrial membrane

Long chain fatty acyl CoA cannot penetrate IMM : So it combines with carnitine (Enzyme- CAT-I) to form acyl carnitine. Inside the mitochondria, acyl CoA is regenerated by CAT II

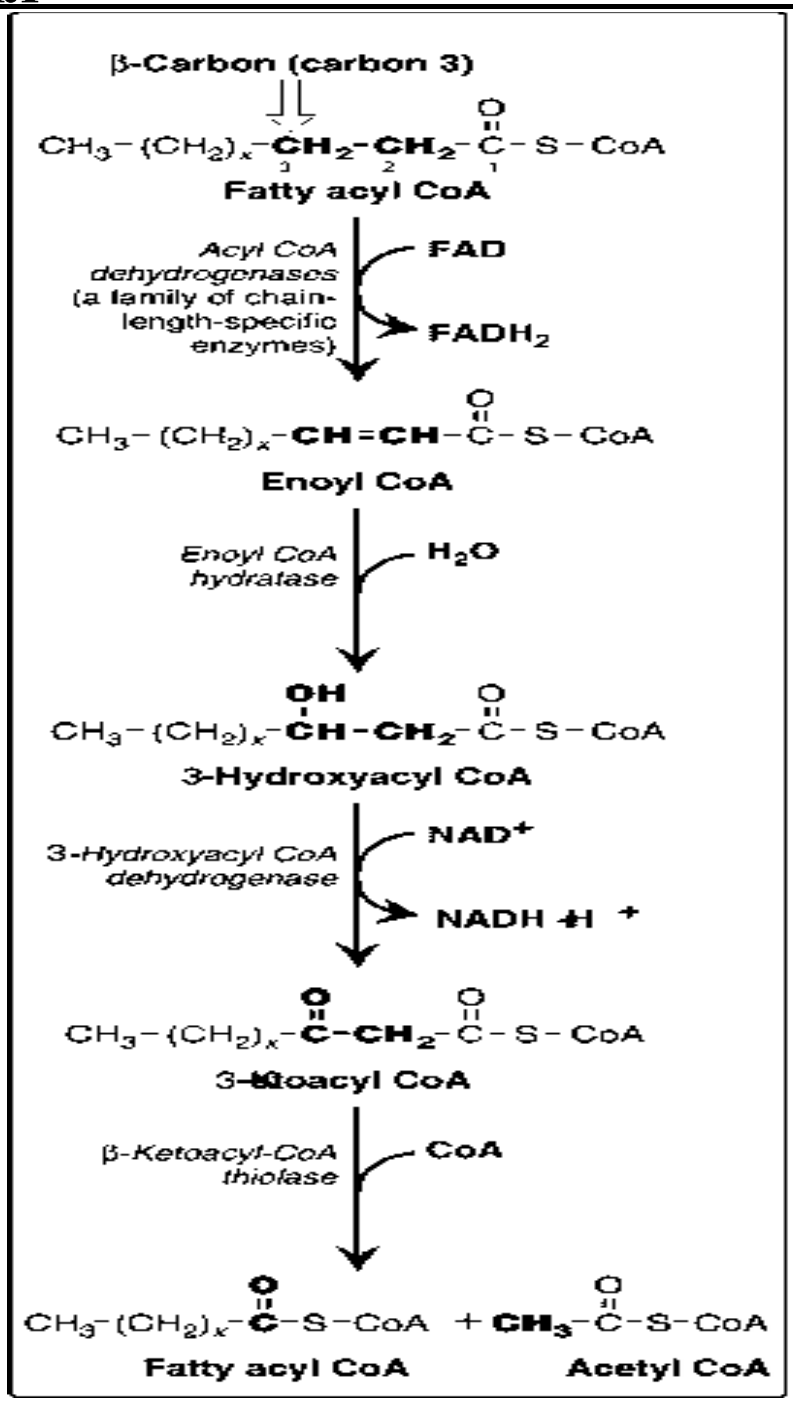
Carnitine : β hydroxyl γ trimethyl ammonium butyrate

Formed from Lys and Met I Liver and kidney

Note: Short chain fatty acids (SCFA) & Medium Chain Fatty acids (MCFA) < 12 carbons do not require carnitine system



Role of carnitine in the transport of long-chain fatty acids through the inner mitochondrial membrane. Long-chain acyl-CoA cannot pass through the inner mitochondrial membrane, but its metabolic product, acylcarnitine, can.

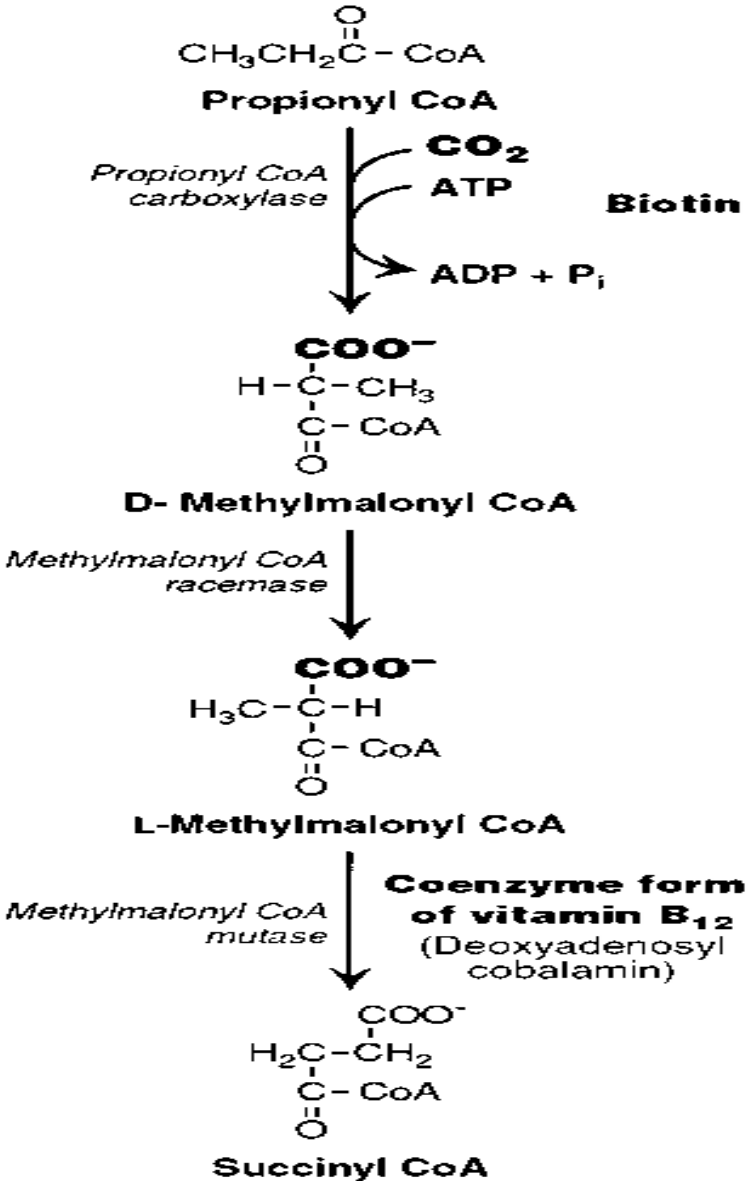


Beta oxidation of fatty acids with even number of C atoms produce acetyl CoA molecules
Energy yield from beta oxidation

Initial activation requires 2 ATP
Each cycle (which removes one acetyl CoA) produce 5 ATP (1 NADH + 1 $FADH_2$)
Each acetyl CoA will produce 12 ATP in TCA cycle

Oxidation of odd chain fatty acids

During beta oxidation of odd chain fatty acid, 2C units (Acetyl CoA) are successively removed till a 3C compound (Propionyl CoA) is formed



Oxidation of very long chain fatty acids

Peroxisomal beta oxidation: For very long chain fatty acids- does not produce ATP

β oxidation of unsaturated fatty acids occurs in mitochondria till $\Delta^{3 \text{ or } 4}$ cis acyl CoA forms. Then isomerases are required to convert it to a Δ^2 Trans enoyl compound

<u>α - Oxidation:</u>

For Phytanic Acid, which cannot be metabolized by β – oxidation. Phytanic acid is found in ruminant fat, meat and milk. Takes place in brain. Does not generate energy. End product is propionyl CoA

Refsum Disease: deficiency of Phytanoyl CoA Hydroxylase leads to defective α - oxidation & deposition of Phytanic Acid in Brain resulting in neurodegeneration

<u>ω Oxidation</u>

for MCFA due to deficiency of **MC acyl dehydrogenase** (1: 40000). Takes place in endoplasmic reticulum

Cytochrome P_{450} Monooxygenase introduces a COOH group at ω position. Di-carboxylic acid then undergoes β – oxidation from both ends forming Di-carboxylic acids (Succinate & Adipate) in the end. Deficiency of **medium chain acyl dehydrogenase** leads to dicarboxylic aciduria.

Defects in oxidation of fatty acids

Carnitine deficiency: Occur in preterm babies, hemodialysis
Hypoglycemia, lipid accumulation, muscle weakness
Treatment: oral carnitine

CAT –I (CPT-I) deficiency: Affects liver; Reduced ketogenesis, hypoglycemia

CAT-II deficiency: Affects skeletal muscles (affects liver in severe cases)

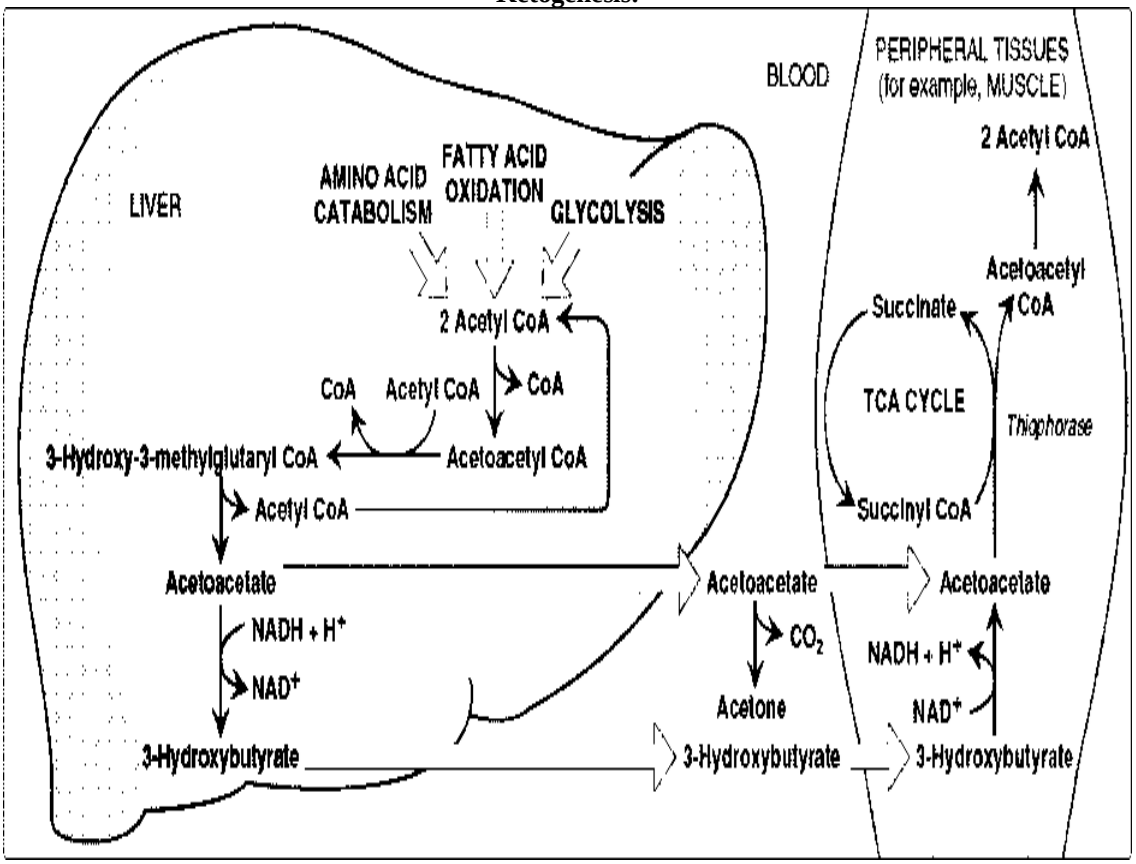
Medium chain acyl CoA DH deficiency : most common inborn error of fatty acid oxidation
Leads to non ketotic hypoglycemia, and dicarboxylic aciduria
Affects mainly infants

Jamaican vomiting sickness: Eating unripe fruit of akee tree: contains hypoglycin- Inactivates medium and short chain acyl CoA dehydrogenase

Refsum's disease: defective alpha oxidation of phytanic acid; leads to neurological disorder

Zellweger's syndrome: (cerebrohepatorenal syndrome)- Defective peroxisomal beta oxidation – due to defective protein targetting

Ketogenesis:



Acetoacetyl CoA+ Acetyl CoA → HMG CoA [HMG CoA synthase]
HMG CoA → Acetoacetate+ Acetyl CoA [HMG CoA lyase]
Acetoacetate is converted to acetone or β OH butyrate
Ketogenesis occurs in liver mitochondria only.

Ketogenesis

- Ketone bodies are produced in liver

- Due to increased breakdown of FA (Starvation & Diabetes mellitus)
- Acetoacetate is the 1st ketone body to be synthesized
- β – Hydroxybutyrate is present in maximum concentration
- both are organic acids causing metabolic acidosis (Ketoacidosis)
- all are excreted in urine (Ketonuria)
- Acetone is volatile, exhaled in breath and responsible for acidotic or ketotic breath
- Rothera's test detects ketone bodies in urine (acetoacetate and acetone)

Ketone bodies are utilized by : **Muscle, Heart, Brain**

Ketolysis occurs in extrahepatic tissues with the help of enzyme Succinyl CoA- Acetoacetate-CoA transferase. Rate of ketolysis increases with increase in blood level until it saturates the oxidative machinery.

Lipotropic factors:

Required for normal mobilization of fat from liver. Deficiency causes fatty liver
Eg: Choline, Lecithin, Methionine, Vitamin E, Se, Omega 3 fatty acids

Fatty acid synthase is a multienzyme complex having **7 enzymes** and acyl carrier protein. 7 enzymes are: Ketoacyl synthase, Acetyl transacylase, Malonyl transacylase, Hydratase, Enoyl reductase, Ketoacyl reductase and thiesterase
Acetyl CoA carboxylase is not its part
Acetyl CoA carboxylase converts acetyl CoA to **malonyl CoA**. It is inhibited by long chain acylCoA
Acetyl CoA is formed in cytoplasm. It cannot diffuse freely into cytosol. So it is converted to **citrate** and diffuses out with the help of **Tricarboxylate transporter**. And in cytosol, Acetyl CoA is released by the action of **ATP-Citrate lyase**

Microsomal fatty acid elongase system elongates saturated and unsaturated fatty acyl CoA: Malonyl CoA donates C (not acetyl CoA). Uses NADPH, but can also use NADH as coenzyme

Acetl CoA carboxylase (AcetylCoA→ Malonyl CoA) is the rate limiting step in de novo synthesis of fatty acids. Allosterically activated by citrate.
Inhibited by: Feedback← Long chain acyl CoA
Covalent modification← Phosphorylation

Prostaglandins
Originally isolated from prostate: Present in all tissues. Most potent biologically active substances: Local hormones
Considered to be derivatives of 20C cyclic saturated fatty acid:- Prostanoic acid
According to substituent groups in the ring, named as A, B, D etc
Numbering is given based on the number of double bonds in side chain (PGE_1, PGE_2 etc)
Series 2 is most common [C13-14 trans and C5-6 cis]

PG with Double ring structure is PGI and is known as Prostacyclin

Synthesis of prostaglandins
Series 1 ← Linoleic acid: Series 2 ← Arachidonic acid: Series 3 ← Eicosa penta enoic acid

COX: Activated by catecholamines: Inhibited by NSAIDs
COX-1 : Constitutive form → mediates gastric, renal and platelet functions
COX-2: Inducible form → Mediates inflammatory response
Corticosteroids inhibit the transcription of COX-2
Aspirin inhibits COX-1 and 2 : Coxibs selectively inhibit COX-2

Lipid storage diseases (Sphingolipidoses)
Due to defect in the **catabolism** of sphingolipids
All types except Fabry's disease have Mental retardation

Disease	Enzyme deficient	Lipid accumulating
Tay-Sach's disease	Hexosaminidase A	G_{M2} ganglioside
Fabry's disease	α galactosidase	Globotriaosylceramide
Metachromatic leukodystrophy	Aryl sulfatase A	3-sulphogalactosyl ceramide
Krabbe's disease	β galactosidase	Galactosyl ceramide
Gaucher's disease	β glucosidase	Glucosyl ceramide
Niemann-Pick disease	Sphingomyelinase	Sphingomyelin
Farber's disease	Ceramidase	Ceramide

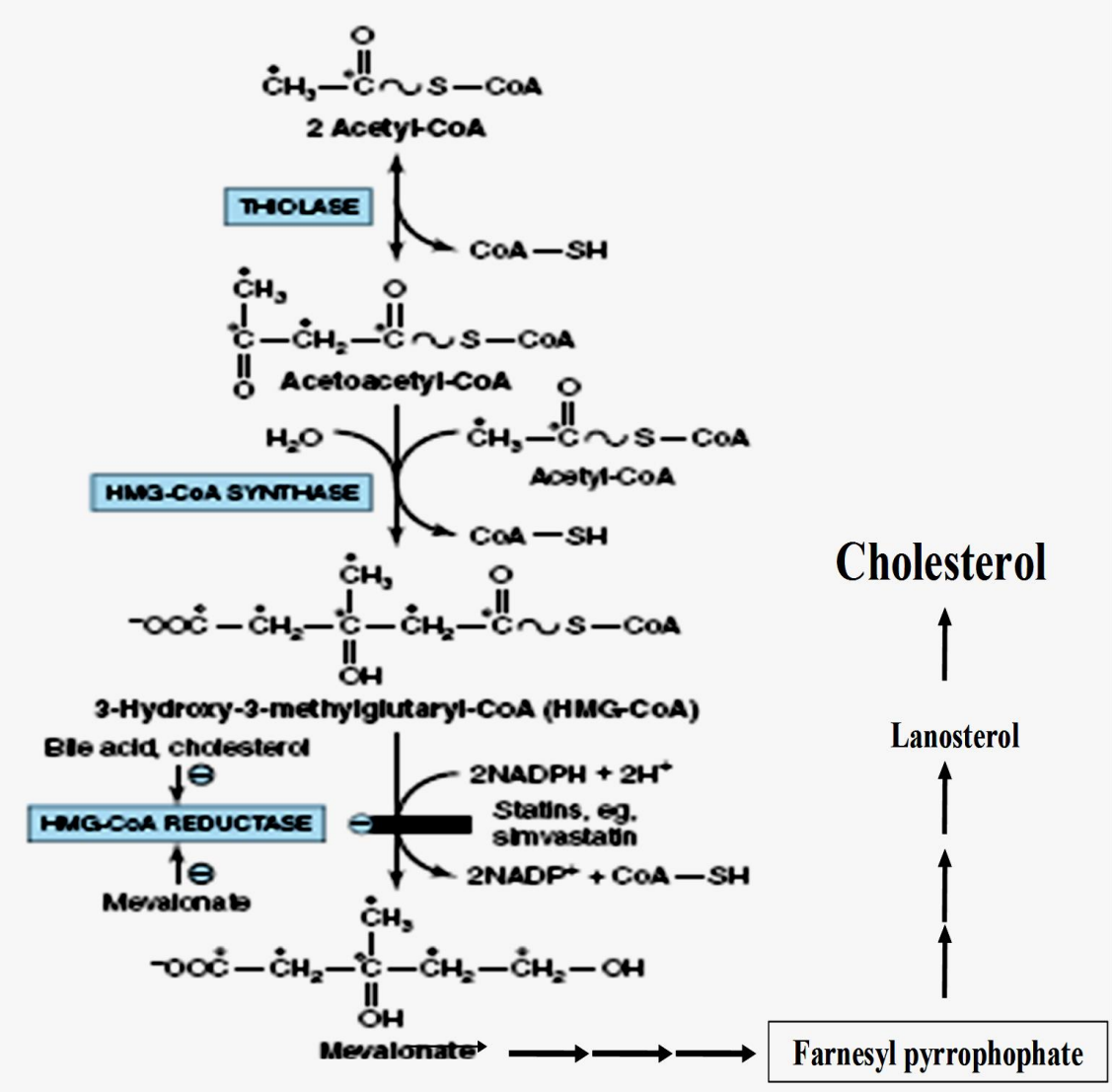
Multiple sulfatase deficiency : Combined deficiency of Aryl sulphatase A,B,C and steroid sulfatase: → accumulation of sulfogalactosyl ceramide, steroid sulfates and proteoglycans
Sandhoff's disease: Hexosaminidase A and B deficient→ accumulation of Globoside

Cholesterol Metabolism

Biosynthesis

It is synthesized from Acetyl COA in endoplasm reticulum (Microsomes)

All C- atoms derived from Acetyl CoA



Step I: Formation of mevalonate. Similar to formation of Ketone bodies in mitochondria

Main Regulatory enzyme: HMG CoA reductase

- Induced by insulin
- Stimulated by insulin and inhibited by glucagon by covalent modification
- Allosteric inhibitors:
  - Cholesterol
  - Bile acids
  - Mevalonate
- Competitive inhibitors
  - Hypocholesterolemic drugs i.e. statins

Rate limiting step in cholesterol synthesis: **HMG CoA reductase** (**HMG** CoA → Mevalonate)
Inhibited by mevalonate, cholesterol. Cholesterol and its metabolites causes repression of HMG CoA reductase via activation of sterol regulatory element binding protein (SREBP) transcription factor. Also regulated by covalent modification: Dephosphorylated form is active

Important products of cholesterol are steroids

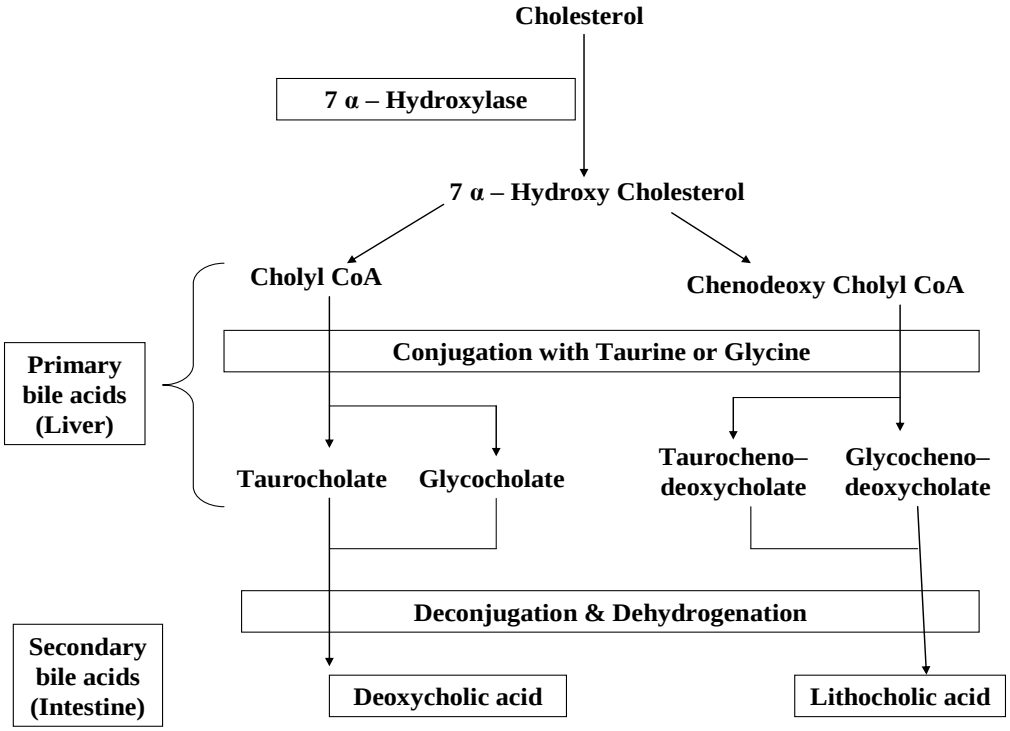
Cholesterol breakdown:

Cholesterol is excreted in Bile. It is a major constituent of bile stones

Total 1g/ Day

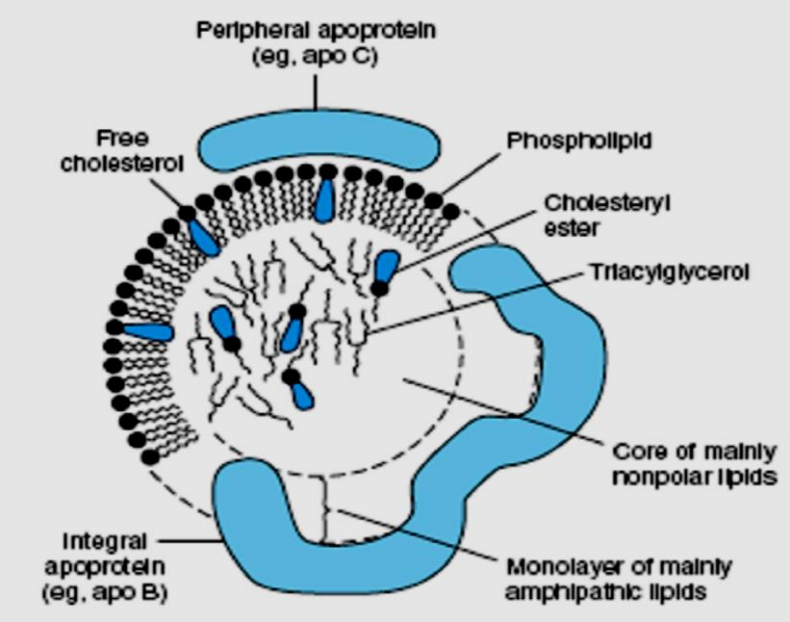
i. 600 mg as cholesterol (60 %) Coprostanol is the major end product formed from cholesterol by intestinal bacteria
ii. 400 mg as bile acids & salts (40 %)

Synthesis of bile salts



Lipoproteins

Lipids are non polar in nature and hence insoluble in water. They are transported in plasma (aqueous medium) as lipoproteins



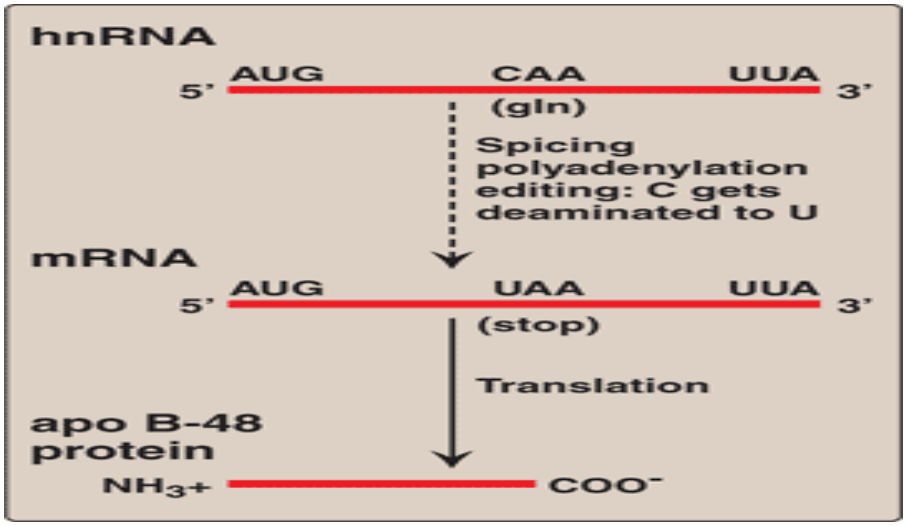
- Lipid component of lipoproteins:
  - Surface lipids
    - Amphipathic/ hydrophilic
      - Cholesterol
      - Phospholipids
  - Core lipids
    - Non polar/ hydrophophic
      - Cholesterol ester
      - Triglycerides
- Protein component of lipoproteins (Apoproteins)
  - Integral proteins: embedded in the surface membrane and cannot be separated from the lipoprotein
    - Apo A
      - A I: Cofactor for LCAT
      - A II: inhibit LPL
      - A IV

Note: Apo A is peripheral protein in Chylomicrons

    - Apo B
      - B 48
      - B 100: Ligand for LDL receptor
  - Peripheral proteins: present on the surface and can be interchanged between various lipoproteins
    - Apo C
      - C I : inhibits CETP
      - C II: activator (cofactor) for LPL
      - C III: Inhibit LPL
    - Apo E Ligand for LRP receptor and also LDL receptor
      - Recently Apo D has been identified

Apo B 48 is the integral apoprotein of chylomicron synthesized by intestinal cells
Apo B100 is the integral apoprotein of VLDL synthesized from Liver

The gene for both is same except that in intestine there is premature termination due to introduction of a stop signal by RNA editing enzyme converting CAA (codon) for glutamine to UAA (stop codon)
Apo B 100 is one of the longest single polypeptide chain known, having 4536 Amino acids. Apo B 48 is 48 % of Apo B 100



Lipoprotein	Source	Density	Protein %	Lipid %	Main lipid component	Electrophoresis on Agarose	Integral protein
Chylomicron	Intestine	↓	↓	↑	TG	Origin	B 48
VLDL	Liver				TG	Pre β	B 100
LDL	VLDL				Cholesterol	β	B 100
HDL	Liver + Intestine				Phospholipids Cholesterol	α	A
FFA/ Albumin	Adipose Tissue				FFA		

Note: Free fatty acid is a misnomer. Fatty acid is always associated with some protein
Plasma: Albumin
Plasma membrane: Membrane fatty acid transport protein
Intracellular: fatty acid binding protein (Z protein)
Non-esterified or un-esterified fatty acid is a more appropriate term

Chylomicron metabolism

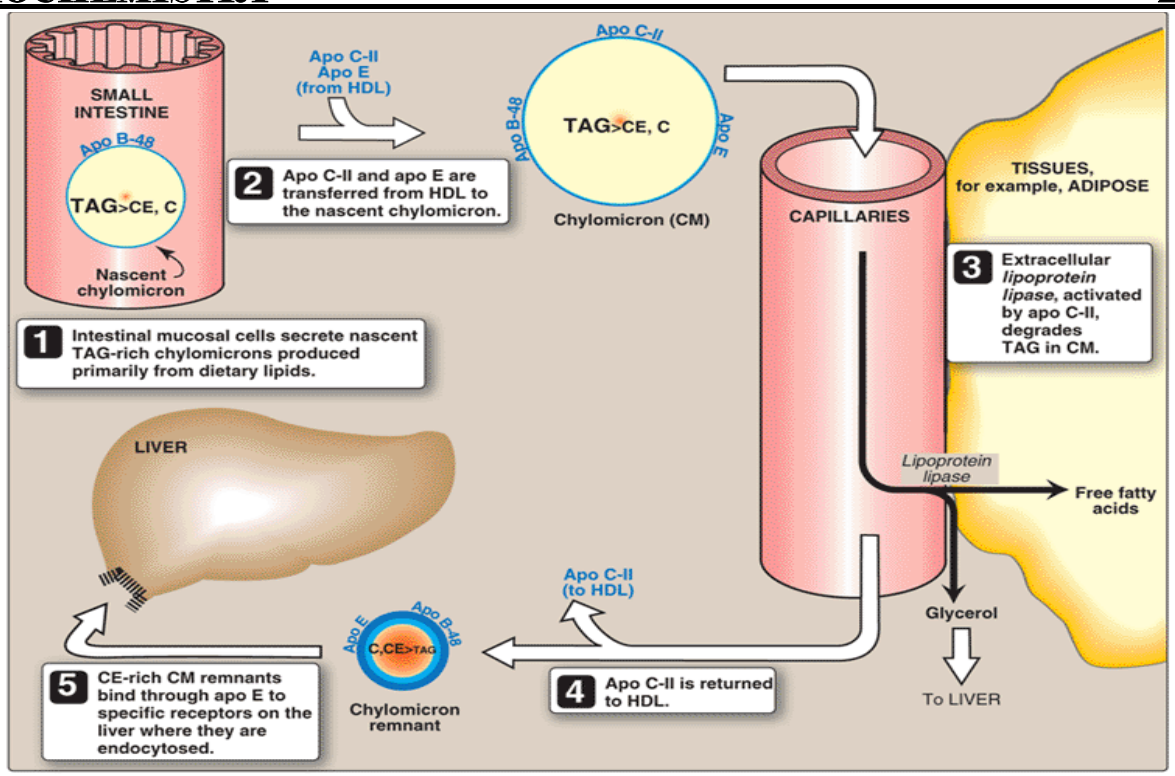
LPL

- Attached to the capillary membrane through heparin releasable heparan sulphate (HRHS)
- Hepatic lipase is also releasable by heparin (HRHL)
- Stimulated by Insulin and Apo CII
- Adipose Tissue (high Km)
- Skeletal muscle (low Km)
- Heart (maximum concentration of LPL)

LRP receptor:

- Also present in skeletal muscle apart from liver
- Binds Apo E
- Also releasable by heparin

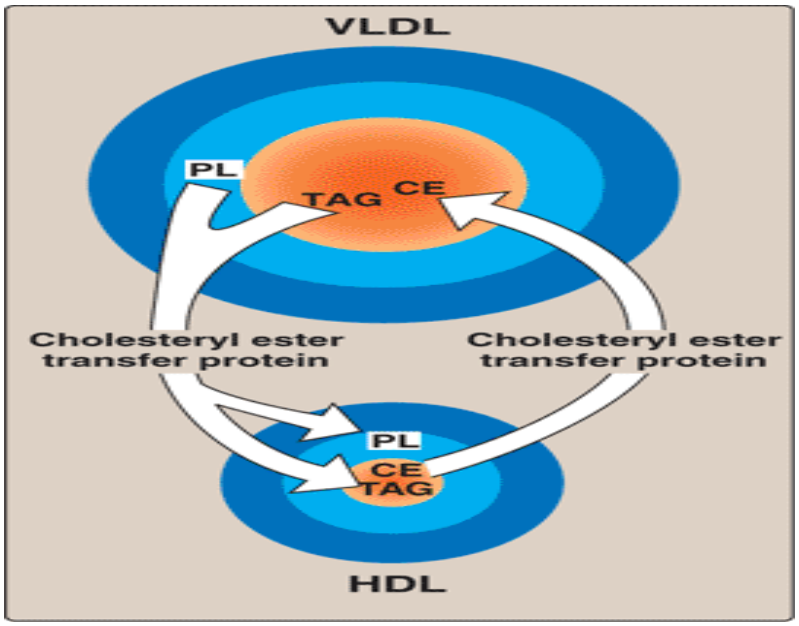
TG transfer protein required for assembly of B48/ B100 + phospholipids + Triglycerides
Chylomicrons deliver dietary lipids (TG, Cholesterol, cholesterol ester and fat soluble vitamins) to peripheral tissues

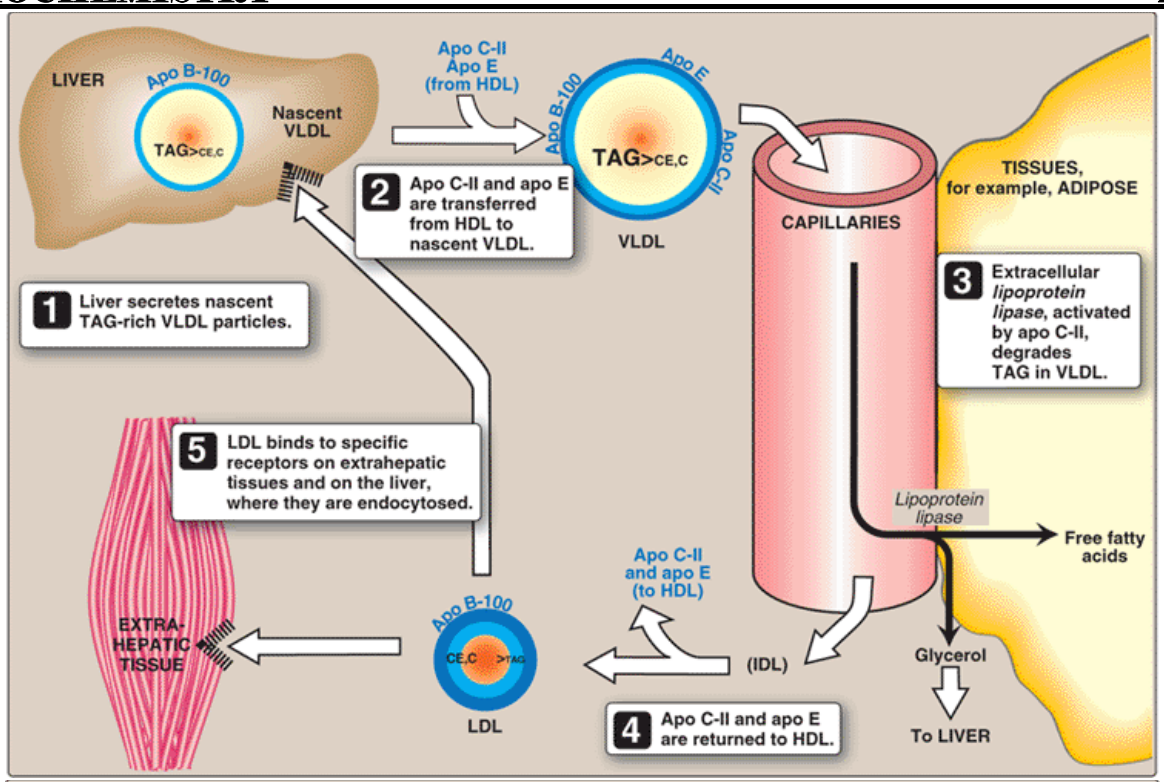


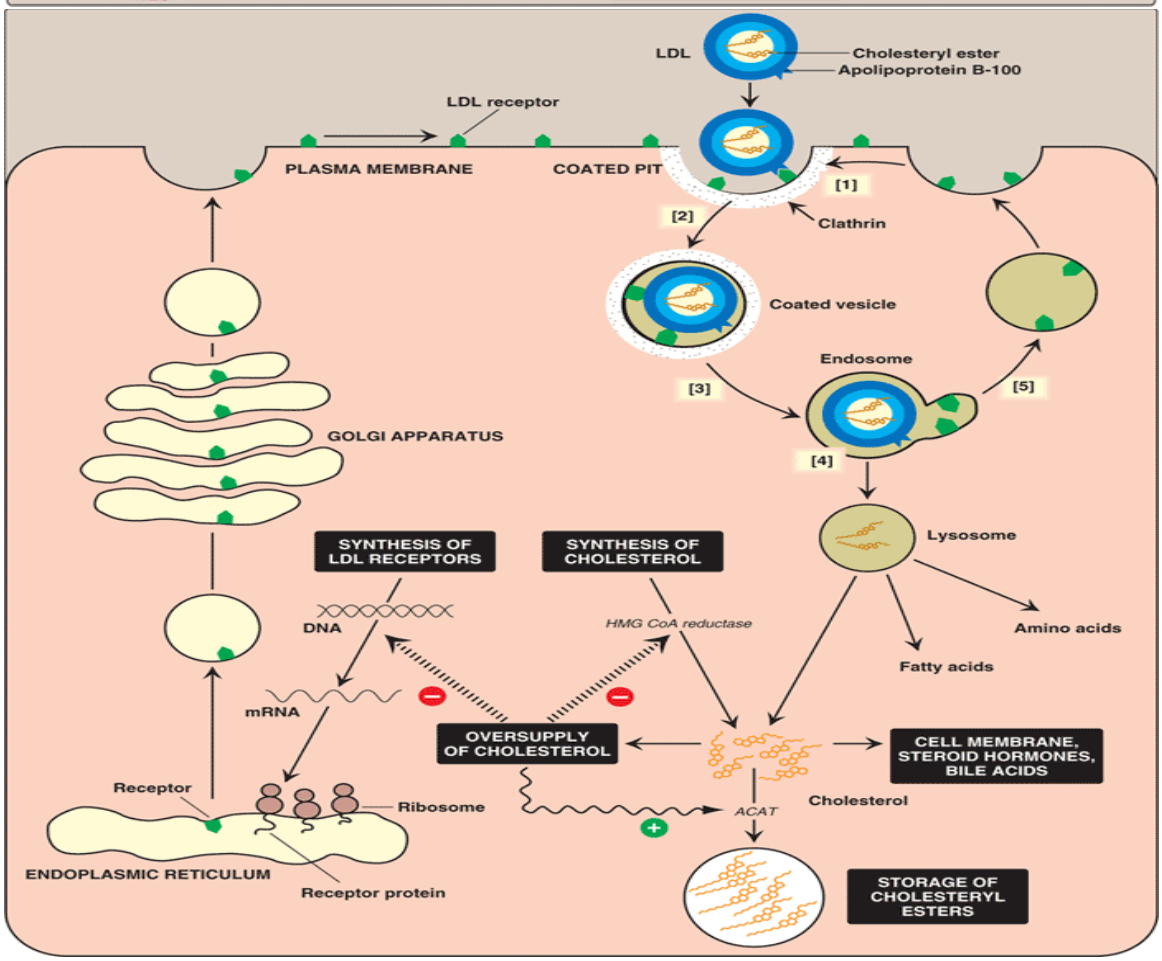
VLDL & LDL Metabolism

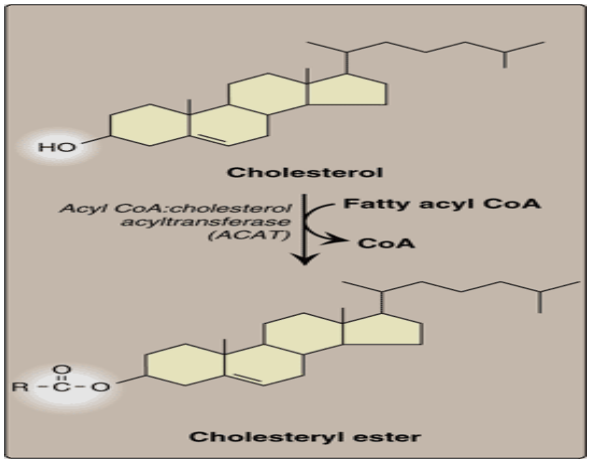
LDL receptor:
- Present in clathrin coated pits
- Recognizes & binds B100
- Also binds Apo E that is why it is called Apo B 100/ E receptor
- Carries out receptor mediated endocytosis

VLDL delivers TG from liver to peripheral tissues
There is transfer of TG and cholesterol ester between VLDL and HDL

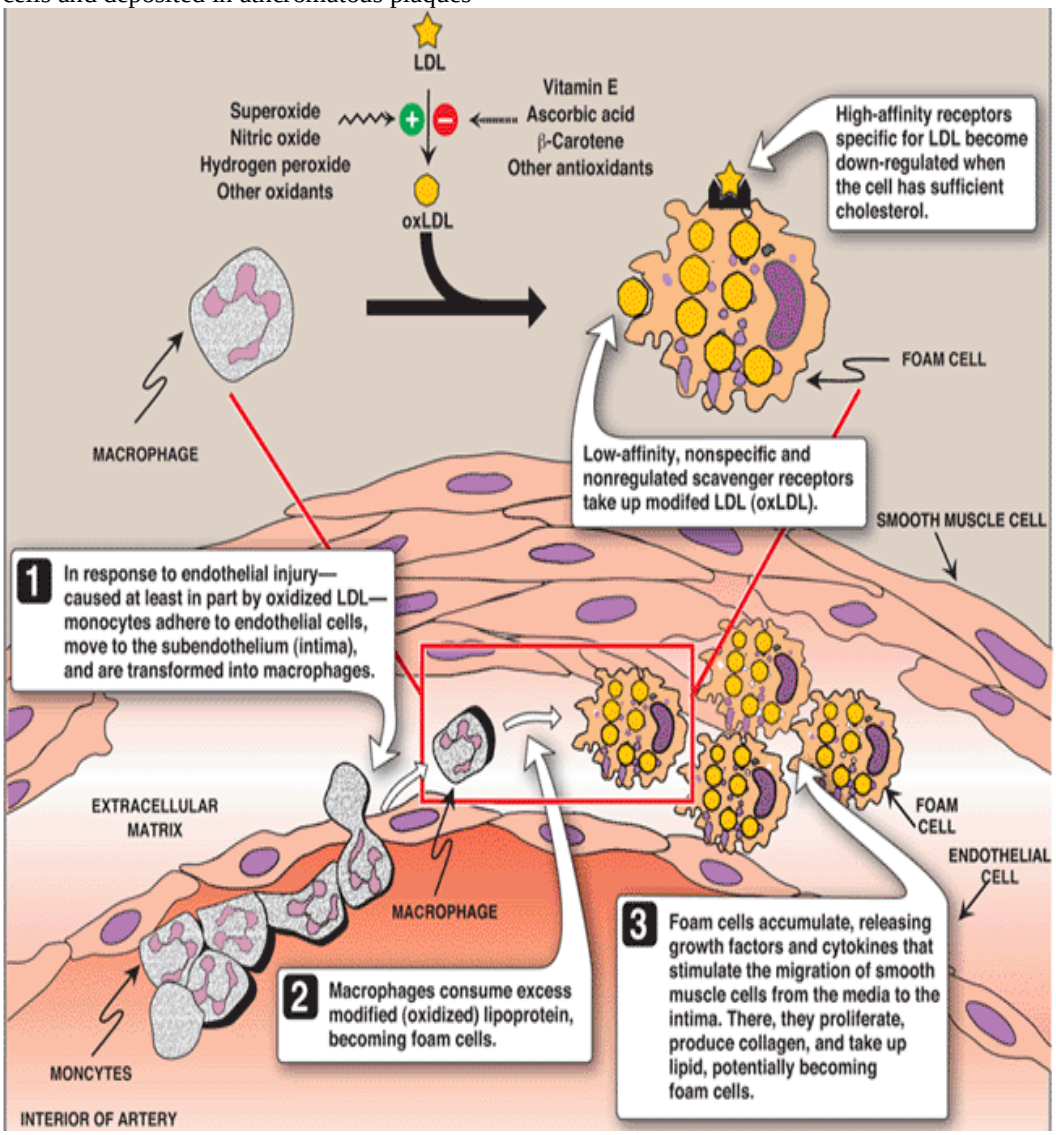



LIVER
Apo B-100
TAG>CE,C
Nascent VLDL
Apo C-II
Apo E
(from HDL)
Apo B-100
Apo E
Apo C-II
TAG>CE,C
VLDL
2 Apo C-II and apo E are transferred from HDL to nascent VLDL.
1 Liver secretes nascent TAG-rich VLDL particles.
CAPILLARIES
TISSUES, for example, ADIPOSE
3 Extracellular lipoprotein lipase, activated by apo C-II, degrades TAG in VLDL.
5 LDL binds to specific receptors on extrahepatic tissues and on the liver, where they are endocytosed.
Lipoprotein lipase
Free fatty acids
Apo C-II
and apo E
(to HDL)
EXTRA-HEPATIC TISSUE
Apo B-100
CE,C
>TAG
LDL
(IDL)
Glycerol
To LIVER
4 Apo C-II and apo E are returned to HDL.


LDL
Cholesteryl ester
Apolipoprotein B-100
LDL receptor
PLASMA MEMBRANE
COATED PIT
[1]
[2]
Clathrin
Coated vesicle
Endosome
[3]
[5]
[4]
GOLGI APPARATUS
Lysosome
SYNTHESIS OF LDL RECEPTORS
SYNTHESIS OF CHOLESTEROL
DNA
HMG CoA reductase
Amino acids
Fatty acids
mRNA
OVERSUPPLY OF CHOLESTEROL
CELL MEMBRANE, STEROID HORMONES, BILE ACIDS
Receptor
Ribosome
Cholesterol
ACAT
ENDOPLASMIC RETICULUM
Receptor protein
STORAGE OF CHOLESTERYL ESTERS

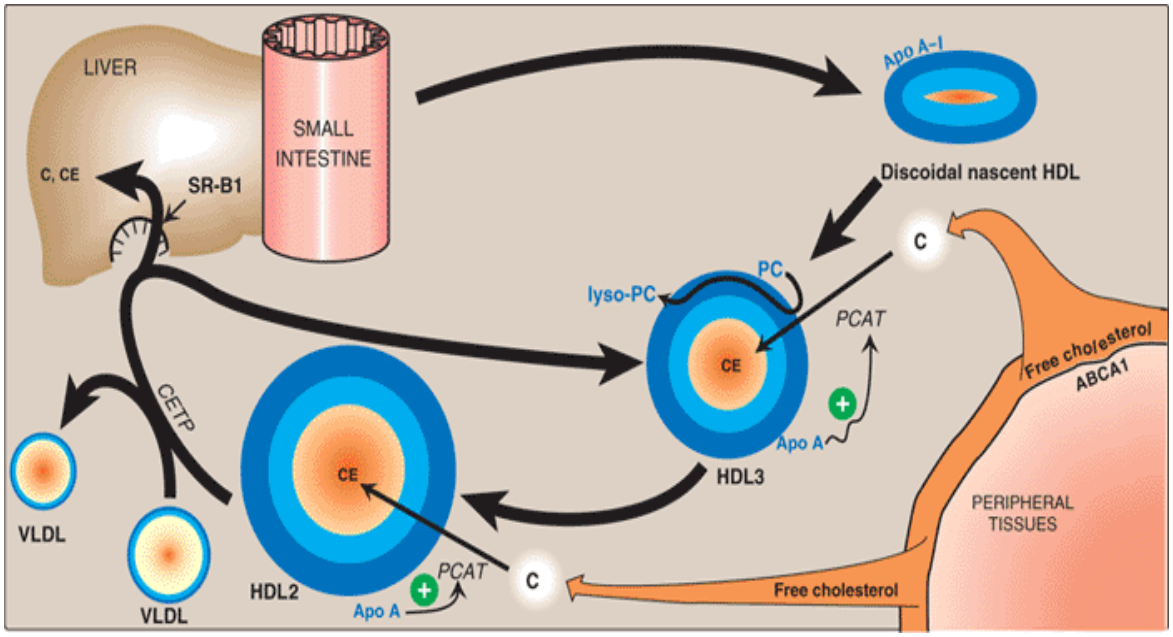


LDL which is not taken up by LDL receptor is phagocytosed by macrophages via scavenge receptor A (SRA) on their surface. Oxidized LDL is more readily taken up by macrophages which are subsequently converted to foam cells and deposited in atheromatous plaques



HDL Metabolism: HDL is:

- Reservoir of Apo C II & Apo E
- Contains **LCAT (PCAT)** with Apo AI as activator
- **Cholesterol scavenger**: takes up cholesterol from peripheral tissues through **ABC A–1 (ATP Binding Cassette Protein A1), ABC G–1 and SRB 1 (Scavenger receptor B1)**
- Carries **Reverse cholesterol transport** by delivering the cholesterol load to liver through **SR B–1 (Scavenger receptor B 1) (SRA is present on macrophages)**
- Up regulates LDL receptors
- A II inhibits LPL
- Deficiency of ABCA 1 **(Tangier's disease)**

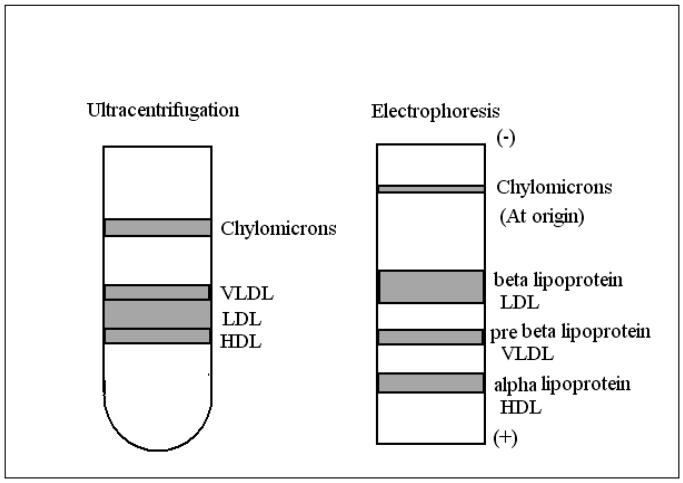


Lp(a) (Lipoprotein a):

- It is different from other categories of lipoproteins.
- It is made up of 1 molecule of LDL + 1 molecule of Apo (a)
- Apo(a) is different from Apo A
- Apo(a) contains domains that are very similar to **plasminogen** (PLG).
- **Plasminogen is anti clotting: normally plasminogen is converted to plasmin by tissue plasminogen activator which is secreted from injured endothelial cells. Plasmin causes fibrinolysis.**
- Lp(a) accumulates in the vessel wall and inhibits binding of PLG to the cell surface (or fibrin clots), reducing plasmin generation which increases clotting.
- This inhibition of PLG by Lp(a) also promotes proliferation of smooth muscle cells. These unique features of Lp(a) suggest Lp(a) causes generation of clots and atherosclerosis
- Lp(a) is an independent risk factor for atherosclerosis and its consequences i.e. coronary heart disease (CHD), cerebrovascular disease (CVD), thrombosis, and stroke,
- Lp-a concentrations may be affected by disease states, but are only moderately affected by diet, exercise, and other environmental factors
- Lipid-reducing drugs have no effect on Lp(a) concentration.
- Normal levels of Lp(a
  - Desirable: < 14 mg/dL
  - Borderline risk: 14 - 30 mg/dL
  - High risk: 31 - 50 mg/dL
  - Very high risk: > 50 mg/dL

During electrophoresis, free fatty acids move to the position of Albumin: Chylomicrons remain at the origin. Other fractions move in the region of globulins

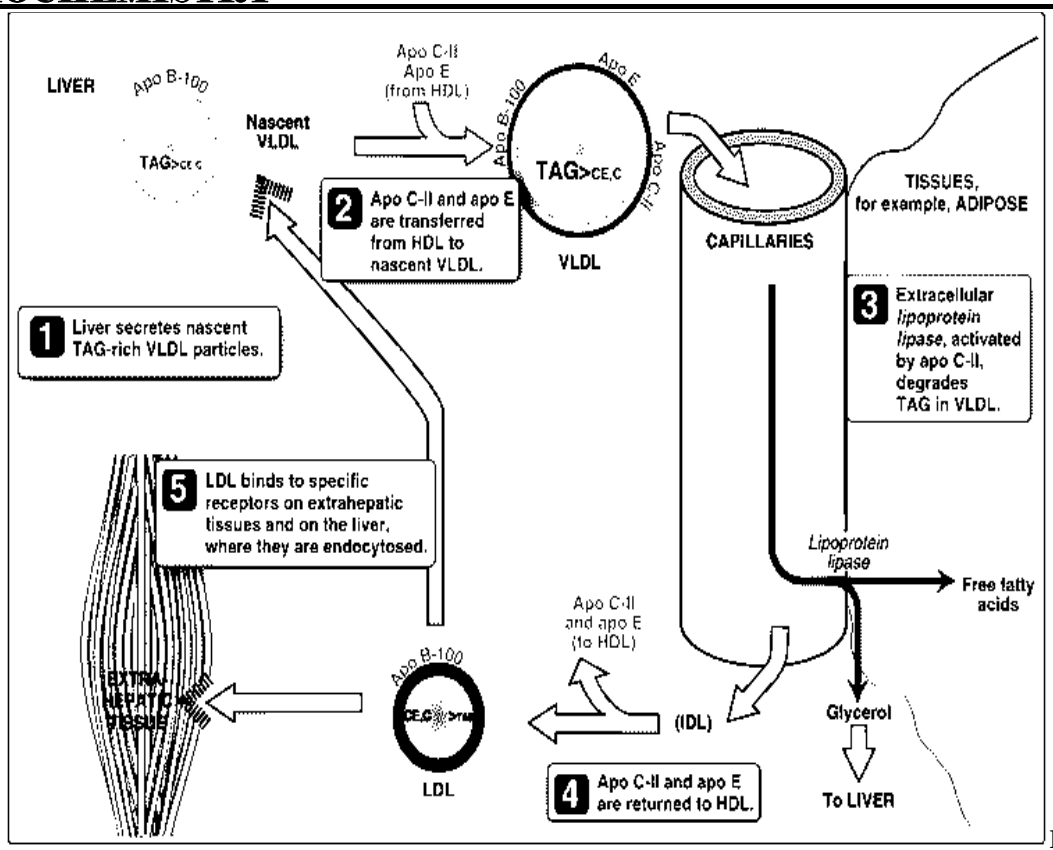
HDL – α lipoprotein
VLDL- Pre β lipoprotein
{IDL- Broad β lipoprotein}
LDL- β lipoprotein



Lipoprotein	**Apolipoproteins**
Chylomicron-Nascent	B-48, A
Chylomicron- circulation	B-48, A, E,C
Chylomicron remnant	B-48, E
VLDL	B-100, E,C
VLDL remnant (IDL)	B-100, E
LDL	B-100
HDL	A-I

Apo C-II acts as cofactor for lipoprotein lipase; Apo A-II and Apo C-III inhibits lipoprotein lipase
Apo A-I acts as cofactor for LCAT
Apo C-I inhibits CETP

Dietary triglycerides are transported as chylomicrons



Lipoprotein lipase

Hydrolyse TAG → Glycerol +fatty acids
Attached to capillary endothelium by chains of heparin sulphate
Active in many tisuues, not in liver
Heart lipoprotein lipase has a low Km
Not normally found in blood, but may be released after injecting heparin
Phospholipids and apo C-II are required as cofactors

Hepatic lipase: Bound to sinusoidal surface of liver cells, released by heparin. Involved in metabolism of chylomicron remnants and HDL.
LDL receptors are most abundant in hepatocytes. Binds apo B100 and apo E. They are located in Clathrin coated pits. Can take up LDL and Chylomicron remnants which is then internalized for lysosomal degradation. Free receptors return to membrane surface.

Lipoprotein (a) Lp (a)
Attached to apo B100 by S-S
Homology with plasminogen: Interferes with plasminogen activation
Inhibits fibrinolysis: Strong association with MI

Hyperlipoproteinemias

Type	LP elevated	Metabolic defect	TG	Cholesterol
Type I Familial LPL def	CM	LPL deficiency or Apo CII	↑ ↑	N
Type II A Familial hypercholesterolaemia	LDL	LDL receptor defect	N	↑ ↑
Type II B	LDL,VLDL	Excess of apo B	↑	↑ ↑
Type III Familial hypolipoproteinaemia	Broad beta VLDL &CM	Abnormal apo E	↑ ↑	↑
Type IV Familial hypertriglycerolaenaemia	VLDL	Over production of VLDL. Associated with glucose intolerance & hyperinsulinaemia	↑ ↑	↑ ↑ ↓LDL, ↓ HDL
Type V	LP elevated	Metabolic defect	TAG	Cholesterol
Familial hyper alpha lipoproteinemia	HDL	Not known	N	↑ ↑
Hepatic lipase deficiency	TG rich HDL & VLDL remnants	LPL deficient	↑ ↑	N
Familial LCAT deficiency	Nascent discoidal HDL	Absence of LCAT	N	↑ ↑
Familial lipoprotein (a) excess	LP(a)	Abnormal LP (a)	↑	↑

HYPERLIPOPROTEINEMIAS

Type II A (Primary familial hypercholesterolemia)

- Most common
- Due to LDL receptor defect
  - LDL receptor deficiency
  - Defective binding of B-100 to LDL receptor
    - Eg: 3500th Arg → Gln (familial defective apo-B or B-3500)
  - Defective internalisation of receptor-LDL complex
- LDL very high; Strong predisposition to develop MI;Death in second decade

TYPE II B

- Excess production of Apo-B (LDL and VLDL are elevated) thus Increased cholesterol and TG
- Risk of MI (usually from 3rd decade)

SECONDARY HYPERLIPIDEMIAS

Cholesterol and triglycerides increased: occurs in Diabetes mellitus, Nephrotic syndrome, Hypothyroidism

Increased cholesterol, Normal TG: Biliary obstruction

Hypertriglyceridemia with normal cholesterol: Alcoholism, Pregnancy, Oral contraceptive

WOLMANN'S DISEASE

- Lysosomal storage disease: Lysosomal acid lipase (Cholesterol ester hydrolase) is deficient

- Autosomal recessive
- Late onset form is called Cholesteryl Ester Storage Disease (CESD)

Perilipin

Protein involved in the formation of lipid droplets in adipose tisse. Inhibts lipolysis by preventing access of lipases to the stored TAG. When needed, it targets Hormone sensitive lipase to the lipid droplets

METABOLIC SYNDROME (SYNDROME X) (REAVEN SYNDROME)

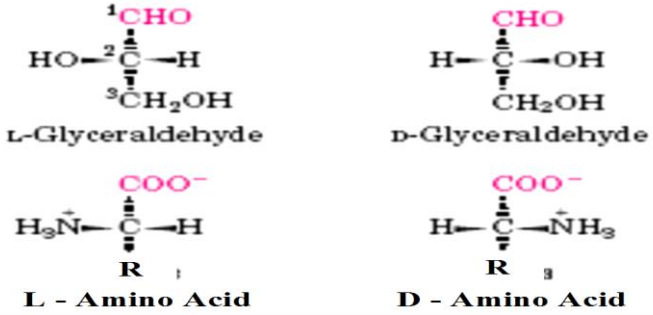
Diagnostic criteria

- Central obesity: Waist 40 (m)/ 35 (f) or above
- Elevated BP.> 130/85 mm Hg
- High TG > 150 mg/dl;
- Low HDL < 40 (m); <50 (f) mg/dl
- Elevated FBS > 100 mg/dl

3 out of 5 criteria is diagnostic

Chapter 5
AMINOACIDS AND PROTEINS

Proteins are formed by polymerization of amino acids. Human amino acids are present as **L α – amino acids** All proteins contain only the same 20 L α amino acids



All amino acids are structurally similar to L-Glyceraldehyde & amino group is attached to ∝–Carbon

All amino acids are in L form *(all carbohydrates are in D form except L- Fucose and L - Iduuronate).* They are optically active and may be d (dextrorotatory) or l (levorotatory) at pH 7, **EXCEPT** Glycine, which is optically inactive

∝ – Carbon is asymmetric (chiral) to which are attached:-

i. H atom;.
ii. Carboxyl group (–COOH)
iii. Amino group ($–NH_2$):

There are certain **non ∝ amino acids** e.g.

a. GABA (Gamma Amino Butyric Acid): It is an inhibitory neurotransmitter
$NH_2 – CH_2 – CH_2 – CH_2 – COOH$

b. β – Alanine: $NH_2 – CH_2 – CH_2 – COOH$

iv. R – Group: the 4th group which is different in different amino acids

Properties of R group

1. Determine **physical properties** of amino acids e.g. polarity, acidity, basicity etc.
2. R group undergoes various **post translational modifications.**
3. R groups determine **proteins folding & confirmation**.

Important points to remember

All amino acids:

- **Except glycine** have at least 1 asymmetric carbon and thus exhibit optical activity
- Have no net charge at isoelectric pH (PI) , therefore exists as zwitterions
- Have positive charge at pH less than PI and have negative charge at pH greater than PI
- Do not absorb visible light and therefore give colourless solutions

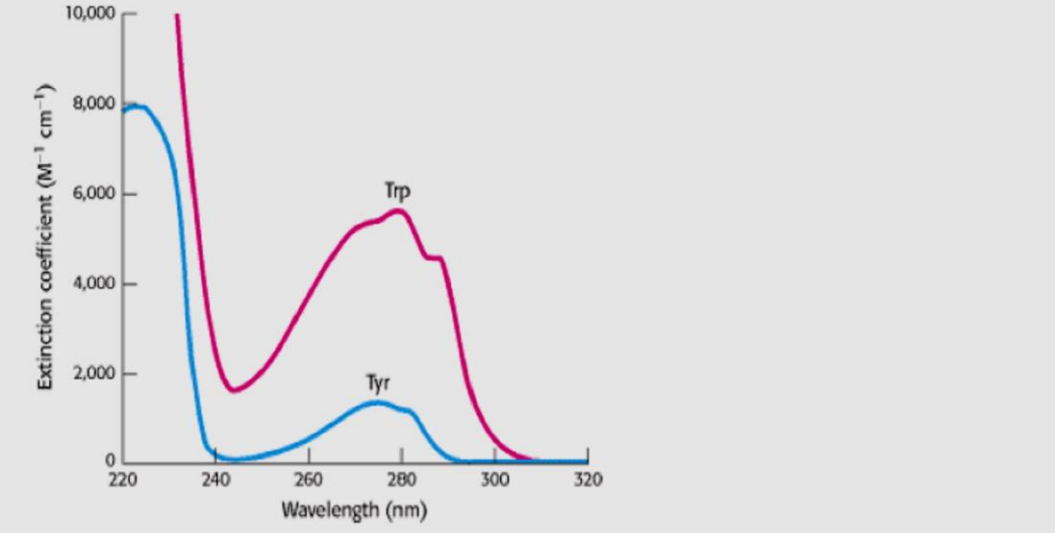
All amino acids absorb light of wave length less than 240 nm except aromatic amino acids which absorb light of 280 nm

Most effective amino acid that can act as a buffer at physiologic pH is Histidine due to its imidazole group

At Isoelectric pH, the amino acids exists as Zwitter ions .The amino acid has both positive and negative charges same (Electrically neutral)

Classification of amino acids: Based on chemistry

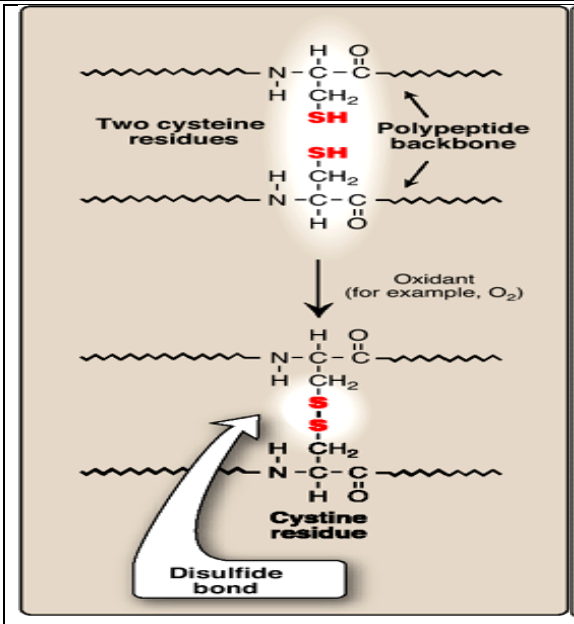
Amino Acid	R Group	Importance
Aliphatic Amino Acids		
Glycine	H	Smallest Amino Acid Easily fits the bends in proteins Only amino acid which is optically inactive
Alanine		Major transport form of Amino Nitrogen from muscle to liver Glucose Alanine cycle • Between muscle and liver • Effectively transports amino nitrogen (ammonia) from amino acid catabolism due to muscle protein turnover to liver where it is converted to urea and excreted • Provides liver with a glucogenic substrate so that liver can maintain blood glucose concentration.
Valine		All the three are called branched chain amino acids
Leucine		
Isoleucine		
Aromatic Amino Acids		
Histidine	Imidazole	Also comes under basic amino acids
Phenylalanine	Phenyl	Tryptophan is particularly responsible for absorption of UV light by proteins at 280 nm. Other amino acids which absorb UV light are phenylalanine and tyrosine
Tyrosine	Hydroxyphenyl	
Tryptophan	Indole	

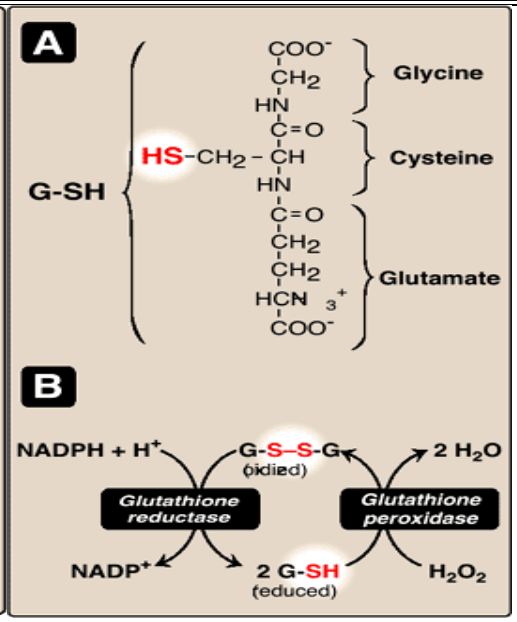


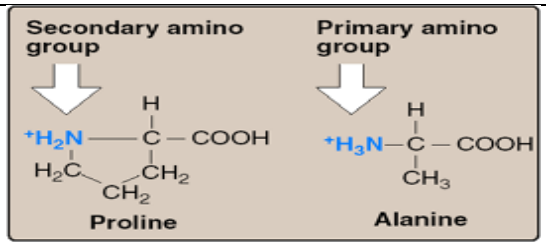
Absorption Spectra of the Aromatic Amino Acids Tryptophan (Red) and Tyrosine (Blue)

Basic Amino Acids

Histidine	Imidazole	also comes under aromatic amino acids **R groiup of Histidine** can act as acid or base at pH 7.0. This helps histidine residues in protein to have **buffering action**.
Lysine	Epsilon (ε) amino group	ε NH2 group forms Schiff base with aldehyde group in: • Retinal (Vitamin A) • Collagen cross linkages It also forms amide bond with carboxyl group of: • Biotin in various carboxylases
Arginine	Guanidium/ Guanido	Strongest basic amino acid
Acidic Amino Acids		
Glutamate	γ – carboxyl	• Acidic & basic R groups form **salt bridges** in hemoglobin & other proteins • Vitamin K dependent γ – carboxylation of glutamate residues in factor II, VII, IX and X is responsible for their activation.
Aspartate	β – carboxyl	
Amides of Acidic Amino Acids		
Glutamine (Amide of Glutamate)		Aspargine and glutamine are neither acidic nor basic
Aspargine (Amide of Aspartate)		
Amino Acids with OH groups		
Serine		OH group of serine, Threonine & tyrosine get phosphorylated or dephosphorylated changing the activity/ confirmation of protein. This is called **covalent modification** of proteins Serine and threonine also get glycosylated in various proteins.
Threonine		
Tyrosine		
Sulphur containing amino acids		
Cysteine	Sulfhydryl	2 molecules of cysteine form cystine, which is:- • 2nd most common post translational modification • Responsible for formation of various inter-chain and intra-chain disulfide bonds or linkages or bridges • Responsible for glutathione acting as an antioxidant.





Methionine	Thioether	
Imino acid		
Proline	Imino group Instead of amino	Contains secondary amino group Produces a bend in the polypeptide Important in formation of fibrous structure of collagen but disrupts alpha helix
		

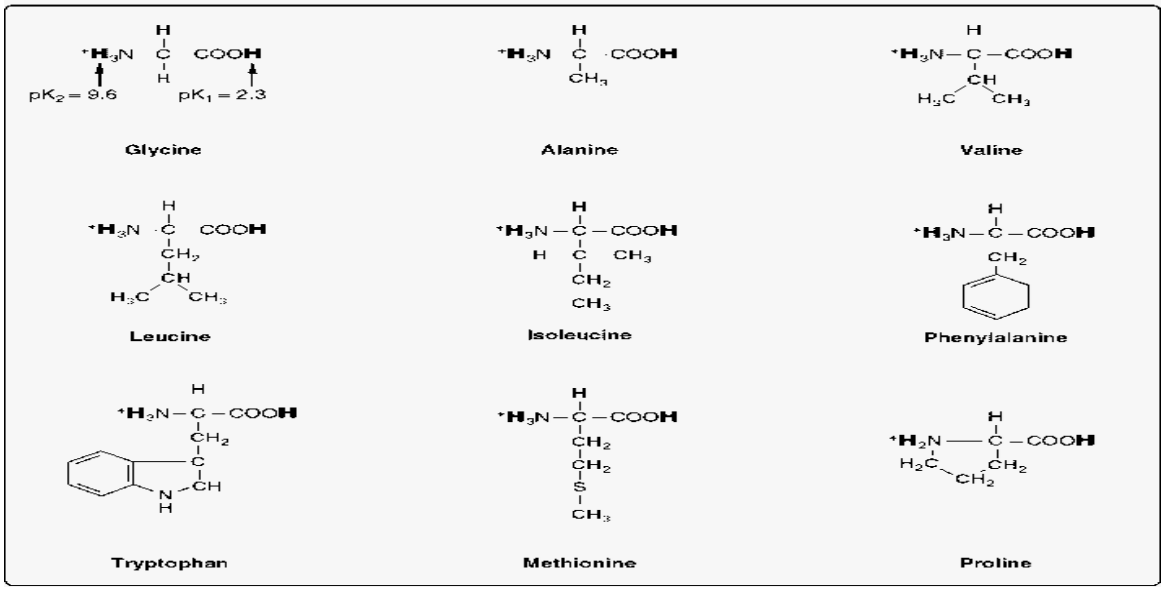
Classification of amino acids: based on polarity of R groups

Hydrophilic And Hydrophobic Amino Acids

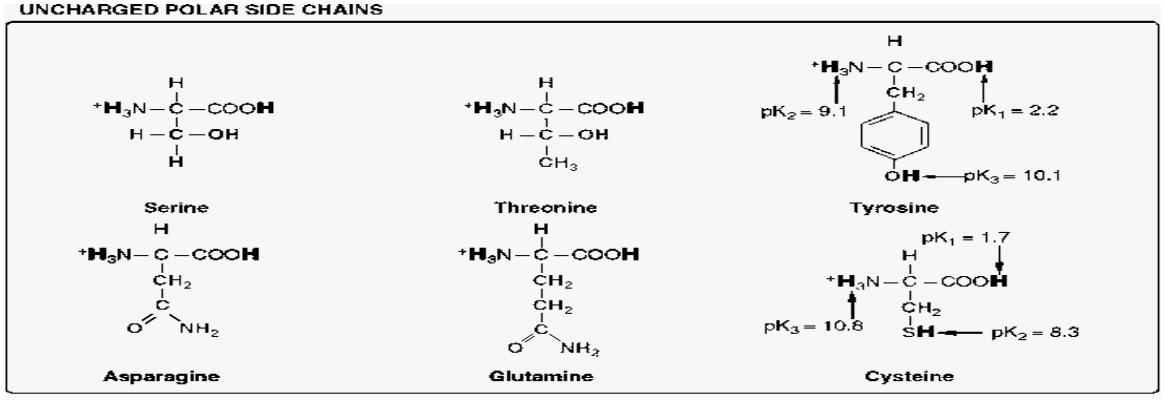
POLAR (HYDROHILIC)		NON-POLAR (HYDROPHOBIC)
Charged	**Uncharged**	
Aspartic acid Glutamic acid Arginine Lysine Histidine	Asparagine Glutamine Serine Threonine Tyrosine Cysteine	Glycine, Alanine Leucine, Isoleucine Valine Methionine Phenylalanine Tryptophan Proline

Amino acids with ring in their structure:
Phen : Benzene ring; Tyr: Phenyl ring; Trp: Indole; His: Imidazole

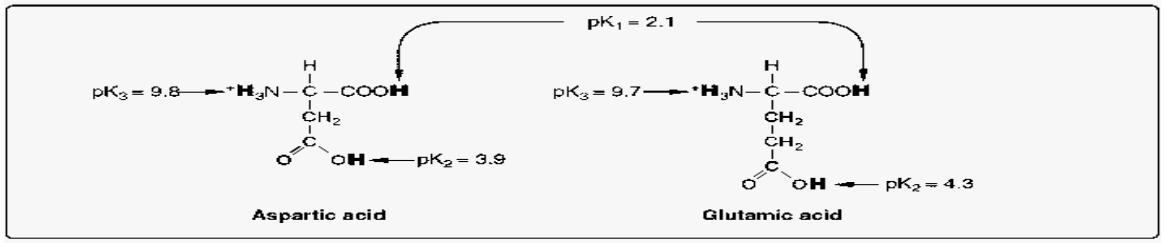
NONPOLAR SIDE CHAINS



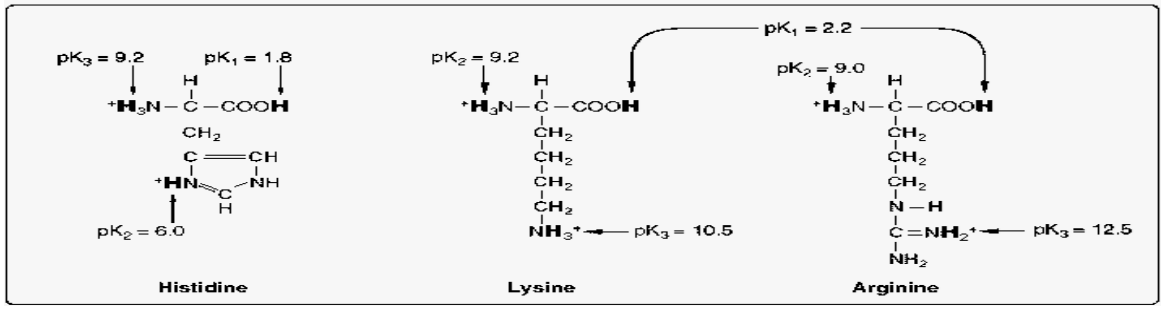
UNCHARGED POLAR SIDE CHAINS



ACIDIC SIDE CHAINS



BASIC SIDE CHAINS



Dicarboxylic acids have more than one COOH and Diamino (basic) amino acids- more than one NH2

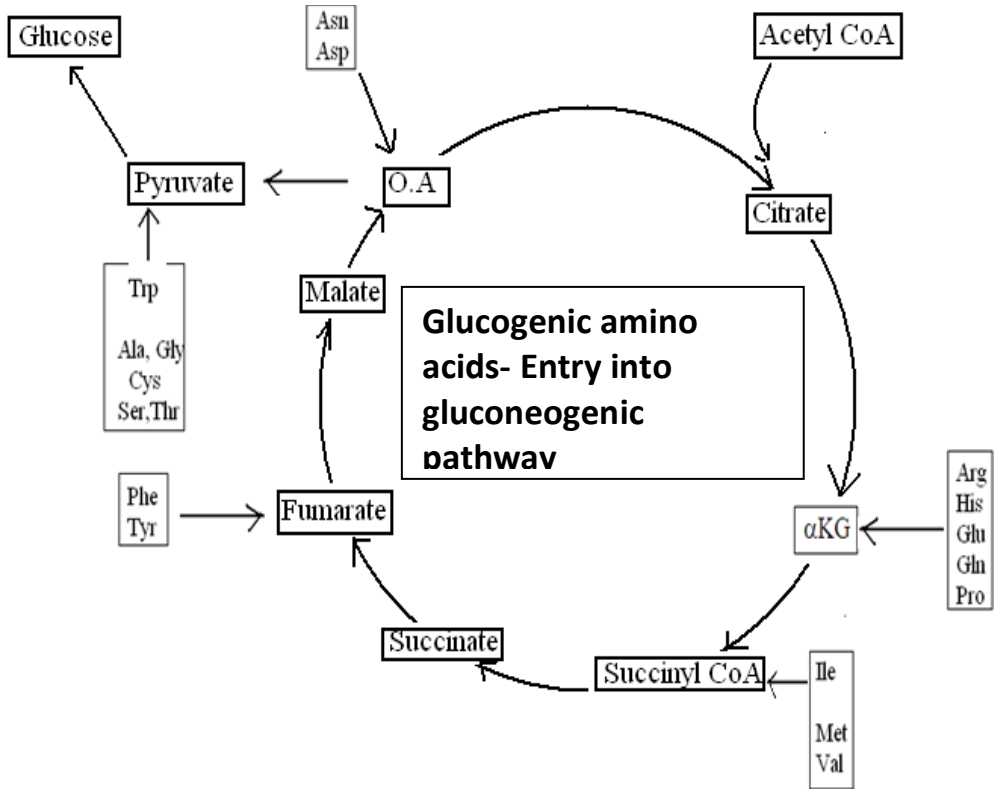
Dicarboxylic : In Aspartic acid, the additional COOH is attached to β carbon; In Glutamic acid , this is attached to γ carbon

Basic amino acids

Arginine- Complicated structure/ Extra amino group attached to δ carbon
Lysine-Extra is ε amino group
Most basic amino acid- Arginine

Classification of amino acids based on metabolism of carbon skeleton of amino acids

1.	Pure Glucoogenic : Converted to Glucose or any substance that can be converted to glucose	All remaining 14 amino acids except those mentioned below
2.	Pure ketogenic : Converted to ketones (ACoA) that is lipids	Leucine, Lysine
3.	Both Glucogenic and ketogenic	Tryptophan, Isoleucine, Phenylalanine, Tyrosine



Classification based on nutritional requirement

1.	Essential amino acids: Not synthesized in the body; should be supplied in the diet	Valine, leucine, isoleucine, phenylalanine, tryptophan, lysine, methionine, threonine
2.	Semi essential : Synthesised in small amount; this is not sufficient in the growing period	Histidine, Arginine
3.	Non essential amino acids	All 12 others

Non essential amino acids are usually formed by transamination of corresponding alpha keto acids
Most are formed from amphibolic intermediates **EXCEPT**
Cys, Tyr and Hydroxylysine : these are formed from essential amino acids
(Cys←Met; Tyr←Phe; Hly←Lys)

Abbreviations of amino acids

Name	3 letter	Single letter
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Cysteine	Cys	C
Methionine	Met	M
Aspartic acid	Asp	D
Asparagine	Asn	N
Glutamic acid	Glu	E
Glutamine	Gln	Q
Arginine	Arg	R
Lysine	Lys	K
Histidine	His	H
Phenyl alanine	Phe	F
Tryptophan	Trp	W
Proline	Pro	P

Acid base properties of amono acid

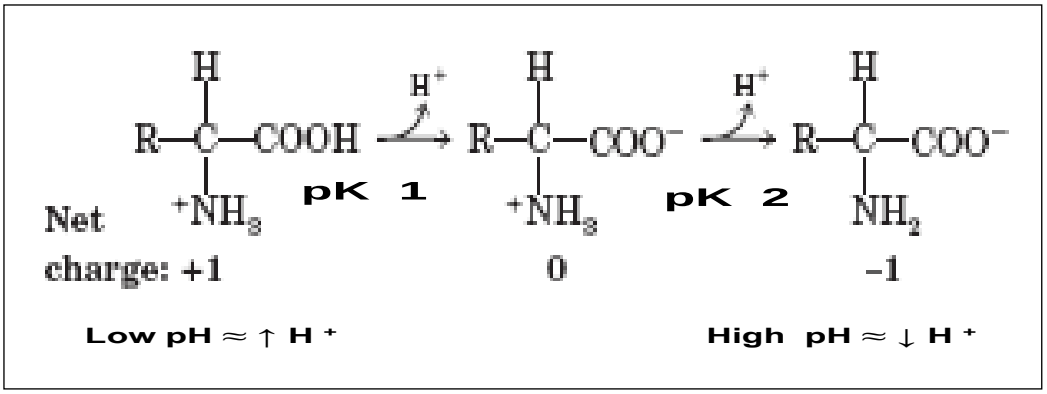
Amino acids are amphoteric molecules (Ampholytes) i.e. they can act either as acid or a base depending on pH of surrounding medium

Each amino acid has at least 2 ionizable groups & each ionizable group has its own pK value

pK1 is that of carboxyl and pK2 is that of amino group

Each amino acid has a fully protonated (undissociated) form, a fully deprotonated (dissociated) form and an isoelectric form

- The species which has zero net charge does not move in the electrical field is called isoelectric species.
- Isoelectric species is also called zwitterion
- The pH at which an amino acid exists as isoelectric species (zwitterion) is called isoelectric pH
- Isoelectric pH is denoted by pI.



$$pI = \frac{pK_1 + pK_2}{2}$$

- Each amino acid has its unique isoelectric pH
- Each amino acid has maximum buffering capacity at pK1 and pK2 and least buffering capacity at pI

PI of Histidine = $\frac{9.3+6}{2}$ = 7.6

a. At physiological pH free histidine has zero charge.
b. When combined in proteins, its R- group at physiological pH is ½ dissociated so acts as buffer.

Isoelectric point – important feature

pH at which the amino amino acid has NO NET charge
Applicable to proteins also
At isoelectric point,
Does not migrate in electric field
Solubility is minimum
Buffering capacity minimum
Precipitability is maximum

Non Standard amino acids

GABA (Gamma Amino Butyric Acid)

- **β Alanine**

Only naturally occuring β amino acid. Present in
Pantothenic acid-- CoA
β Alanyl dipeptides
Carnosine (skeletal muscle) – His+β alanine
Anserine (skeletal muscle) – N-methyl carnosine
Homocarnosine (brain)- GABA+Histidine
Formed from Pyrimidines, Carnosine or Anserine
Transamination produces malonate semialdehyde
Metabolized into-Acetic acid

- **Ornithine, Citrulline, Arginosuccinate**
- **Hydroxyproline and hydroxylysine**

21st and 22nd amino acids

Selenocysteine (Sec)

21st amino acid. Formed in the body from **Serine**
Coded by UGA. Incorporated into protein with the help of SECIS element (Selenocysteine insertion sequence element)

Present in

– Thioredoxin reductase; Glutathione peroxidase; Deiodinase;
–Selenoprotein-P (an antioxidant glycoprotein present in mammalian blood)

Pyrrolysine (Pyl)

22nd amino acid. Coded by UAG (amber codon). Incorporated with the help of PYLIS element.

Detection of amino acids & proteins

- Ninhydrin- can detect microgram quantities of amino acids

–Forms Ruhemann's purple colour with all amino acids EXCEPT Proline which gives yellow colour
–Used to detect fingerprints

- **Fluorescamine- detect picogram quantities of amino acids- Most sensitive method**

Colour reactions of proteins and amino acids

Xanthoproteic test: Aromatic amino acids
Millon's test : Tyrosine, Phenylalanine
Aldehyde test: Tryptophan (Hopkins-Cole test)
Acree-Rosenheim reaction : Tryptophan
Sakaguchi's test : Arginine

Pauly's test : Histidine, Tyrosine
Sulphur test: Cysteine, Cystine
Biuret test: general test for proteins. At least 2 peptide bonds required

STRUCTURE OF PROTEIN - Different levels of protein structure

Primary structure
Includes a.a sequence and position of disulfide bonds. Maintained by the covalent bonds of peptide linkages. Peptide bond- partial double bond character. Distance 1.32 A^o
Secondary structure
Relationship between residues which are 3-4 a. a apart
H bonds
α helix, β pleated sheet, collagen helix

Alpha helix
Commonly right handed. Abundant in Hb and Mb
Absent in chymotrypsin
Pro and Hyp do not allow its formation
Collagen: Triple helix
Glycine induces bends in the alpha helix.
β pleated sheet
Chains fully extended.Stabilised by H bonds.

Tertiary structure
-AA far apart. Bonds- Hydogen, Hydrophobic, Ionic, van der Waal's
Quarternary – only when protein has 2 or more subunits
Stabilized by non-covalent interactions Domain- Compact globular functional unit of a protein

Amino acid residues which favor the secondary structure of proteins

Alpha helix	Beta pleated sheet	Turns
Ala, Cys, Leu, Met, Glu, Gln His, Lys, Arg	Ile, Val, Phe, Tyr, Trp, Thr, Arg	Gly, Ser, Pro Asp, Asn Arg

Protein sequencing- detecting the amino acid sequence : Sanger
Sanger's reagent
Fluoro DinitroBenzene.Used for identification of N-terminal amino acid
End group analysis
N-terminal a.a identified by Sanger's reagent
C-terminal by Carboxypeptidase A and B

- Carboxypeptidase A will not act if C-terminal is Arg, Lys or Pro
- Carboxypeptidase B will act only if penultimate residue is Pro

Edman's reagent
Phenyl isothiocyanate. Used for Sequential analysis of amino acids from N-terminal

Dansyl chloride
Combines with N-terminal amino acid. Used to assess the number of polypeptide chains
Cyanogen bromide
Hydrolyses peptide bonds following Mehionine
"Sequenator": Automated instrument for protein sequencing

Ingram's technique
Protein finger printing
Helps to identify qualitative abnormalities in proteins
Digestion by Trypsin followed by Chromatography and peptide mapping

SOME IMPORTANT PROTEINS:

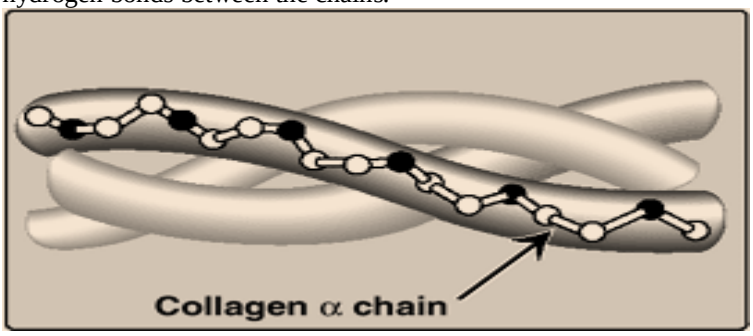
Chaperones

Heat shock proteins which help in proper folding of proteins
Different classes (hsp 70kDa, hsp 60 kDa)
Prevent misfolding
Unfold misfolded regions
Keeps protein unfolded to pass through membranes
Prevents inappropriate interactions with other proteins

Chaperonins are hsp60 chaperones

Collagen

Most abundant protein in body
3 polypeptides (referred to as "α chains") are wound around one another in a rope-like triple helix: held together by hydrogen bonds between the chains.



Variations in amino acids in the alpha chains forms different types of collagen

Formed as procollagen
Every 3rd amino acid is glycine.
Cross links are by lysine (hydroxy lysine)

Collagen diseases:

Ehlers-Danlos syndrome (EDS): result from a deficiency of collagen-processing enzymes (eg: *lysyl hydroxylase* deficiency or *procollagen peptidase* deficiency), or from mutations in the amino acid sequences of collagen types I, III, or V. The most clinically important mutations are found in the gene for type III collagen
Vascular defects, fragile, stretchy skin and loose joints
Osteogenesis Imperfecta (Brittle bone syndrome): Brittle bones, delayed wound healing, humped back.
Type I : Osteogenesis imperfecta tarda. Due to decreased production of α1 and α2 chains. Presents in early infancy with pathologic fractures and may be suspected if prenatal ultrasound detects bowing or fractures of long bones.
Type II : Osteogenesis imperfecta congenital; more severe, and patients die of pulmonary hypoplasia in utero or during the neonatal period.

Elastin

"Elastic" properties
Precursor- Tropoelastin (700 a.a)
Contains no hydroxylysine; Cross links by desmosine/isodesmosine
α1- Antitrypsin (α_1-AT), produced primarily by the liver but also by tissues such as monocytes and alveolar macrophages, prevents elastin degradation in the alveolar walls.

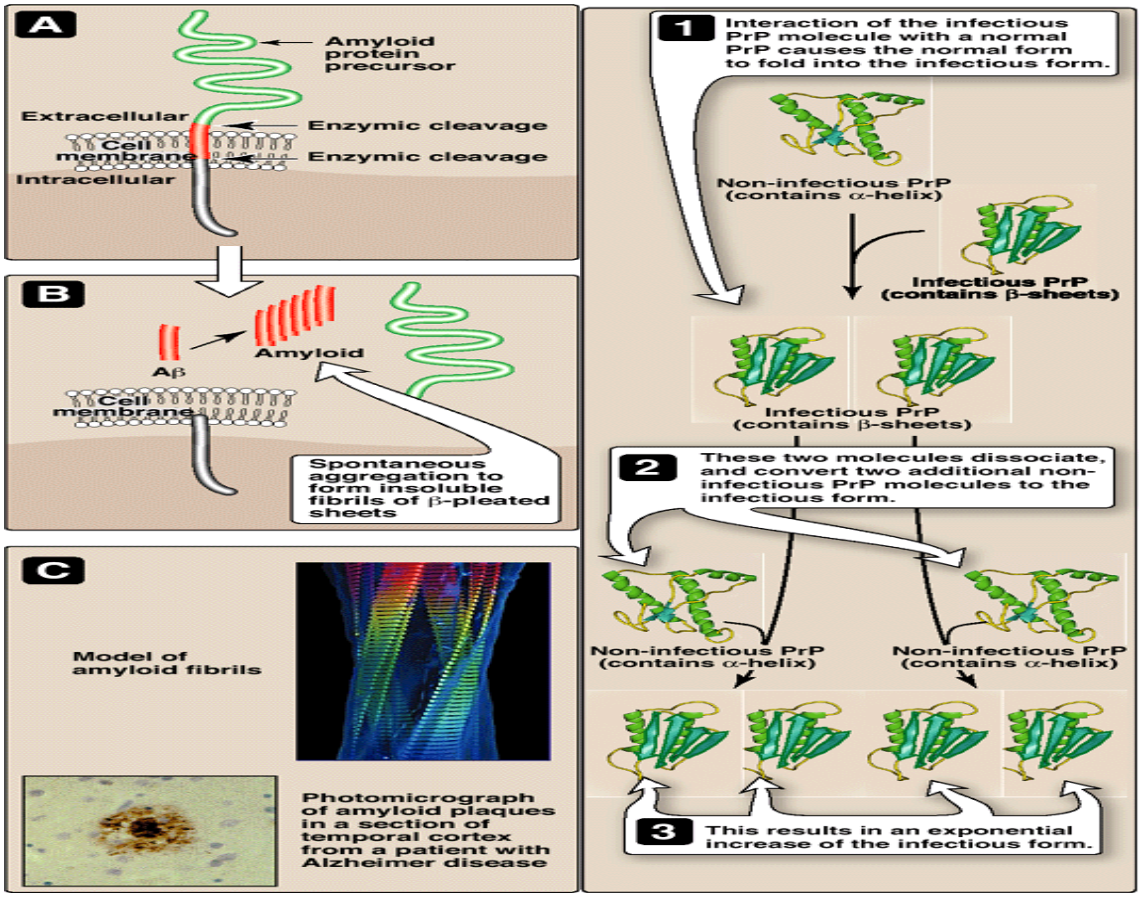
PROTEIN MISFOLDING

Misfolded proteins are usually tagged and degraded. If this system is not perfect, such abnormal protein are non functional, can form aggregates, cause cellular damage and result in diseases

1. **Amyloidosis**: Extracellular accumulation of β–pleated sheets forming long fibrils. If it occurs around neural cells, it is neurotoxic and is implicated in a neurodegenerative disease called Alzheimer's disease
   The dominant component of amyloid protein in Alzheimer's is amyloid β

   Another protein accumulating in Alzheimer's is abnormal tau (τ) protein as neurofibrillary tangles. Normal tau (τ) protein is required for microtubular assembly.

2. **Prion diseases**: these are caused by purely protein particles called prions. Normal prion particle (PrPc) is coded by a gene situated on chromosome 20 in neuroglial cells. It has a structure of α–Helix. Misfolded prion particle (PrPsc) has a predominant structure of β–pleated sheets. Misfolded prion:-
    a. Becomes infectious i.e. converts another PrP to infectious prion particle.
    b. Gets precipitated around neurons and glial cells causing fatal neurodegenerative disorders called Transmissible spongiform encephalopathies (TSE)
        i. Scrapie in sheep
        ii. Bovine spongiform encephalopathy (Mad Cow Disease) in cattle
        iii. Creutzfeldt–Jacob disease in humans
        iv. Kuru in cannibals
        v. Gerstmann–Straussler–Scheinker Syndrome (GSS)
        vi. Familial Fatal Insomnia (FFI)



METABOLISM OF AMINO ACIDS

Transamination

Transfer of amino group from one amino acid to a keto acid to form another pair of amino- and keto acids. No liberation of ammonia

Coenzyme is pyridoxal phosphate

All amino acids except Lysine, Threonine, Proline and Hydroxyproline

- Amino acids undergo transamination and finally concentrate nitrogen into Glutamic acid. Glutamate is the only amino acid that undergoes significant oxidative deamination to liberate free ammonia for urea synthesis

Deamination

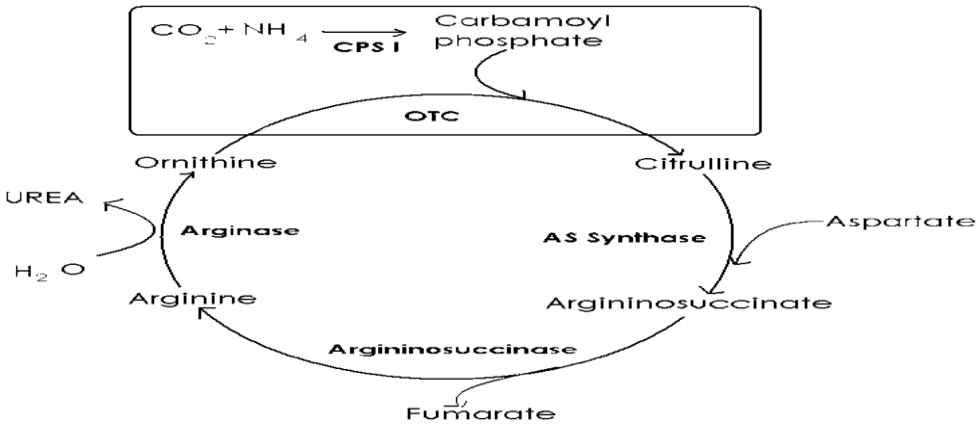
Removal of amino groups from amino acids as ammonia

- Oxidative and Non-oxidative

Oxidative deamination

All ammonia concentrated as glutamate. Oxidatively deaminated in liver and kidney. Enzyme is Glutamate dehydrogenase. Ammonia is liberated

UREA CYCLE and its defects



Summary of urea cycle

1. 5 enzymes
   - 2 Mitochondrial
   - 3 cytosolic
2. 6 Amino Acids Participate
   - 1 N-Acetyl Glutamate as activator of CPS I
   - 1 Aspartate Consumed
   - 4 as intermediates (Ornithine, citrulline, Arginosuccinate & Arginine)
3. 4 ~ P consumed per turn
4. One mole of NH_3 gives one N of urea, the other nitrogen comes from aspartate
5. 2 amino acids are catabolised in one turn of urea cycle

Hyperammonemias

- Primary – Urea cycle defects
- Secondary- defects in other pathways

Urea cycle defects

OTC deficiency: X- linked Recessive
Others Autosomal Recessive

–NAGS deficiency
–Transporter defects
Ornithine translocase deficiency- HHH syndrome
Mitochondrial Asp/Glu carrier def – Citrullinemia Type II
Dibasic a.a carrier def – Hyper dibasic aminoaciduria
(Lysinuric protein intolerance)

HHH syndrome

- Hyperammonemia-Hyperornithinemia-Homocitrullinuria syndrome
- Ornithine translocase deficiency
- Transport of ornithine to mitochondria defective

Arginine activates N-acetyl glutamate synthase: used in treatment of hyperammonemia

Differences between CPS-I and CPS-II

CPS-I	CPS-II

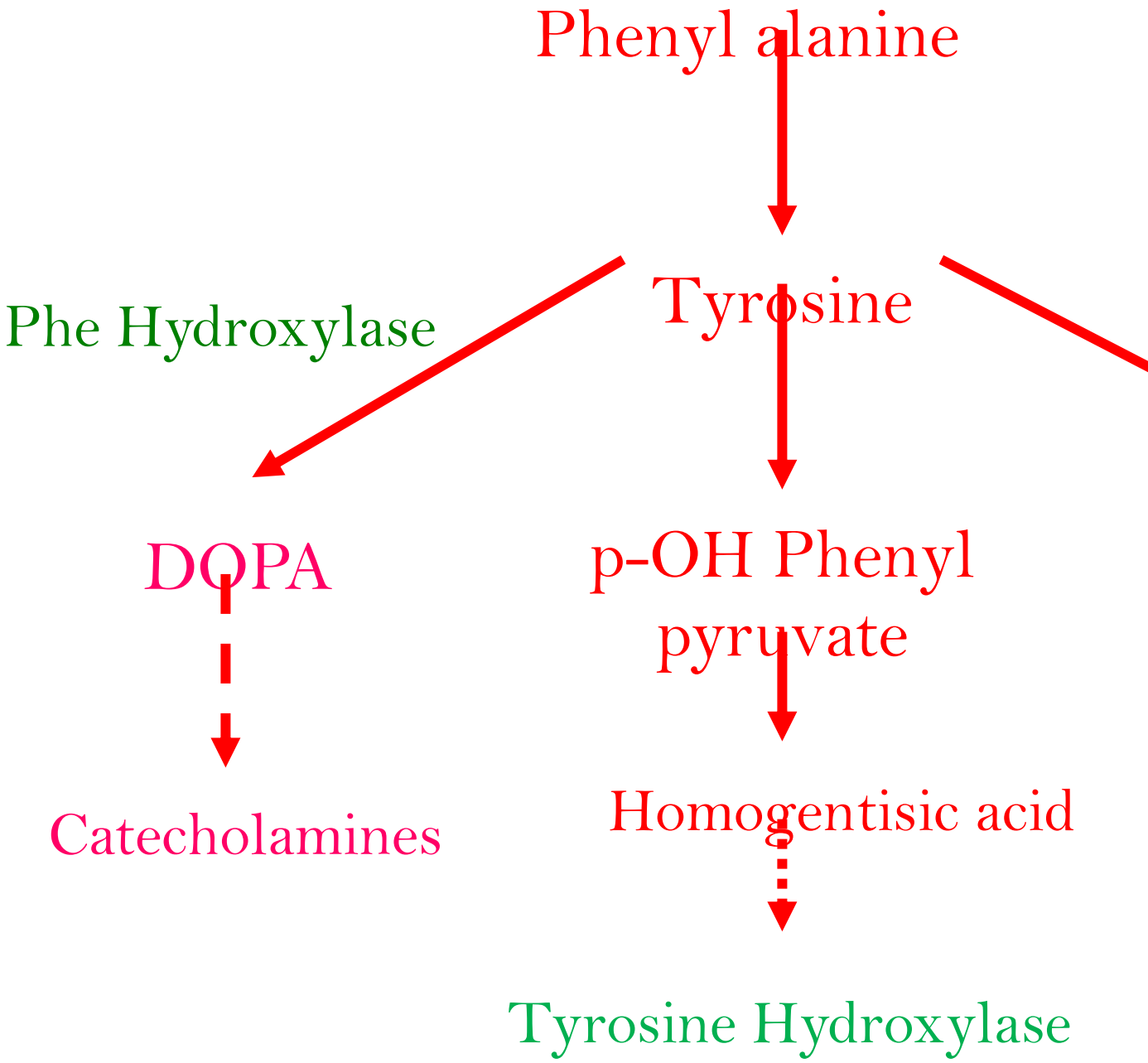
• Urea synthesis • Mitochondria • N donor is ammonia • Dependent on N-acetyl glutamate	• Pyrimidine synthesis • Cytosol • N donor is Gln • Not dependent

Biochemical Basis of Ammonia intoxication

Decreased energy charge due to: Decreased NADH/ NAD or NADPH/ NADP ratio
Decreased TCA cycle intermediate α – keto glutarate.
Decreased ATP
Synthesis of inhibitory neurotransmitter GABA
Local brain alkalosis

INDIVIDUAL AMINO ACID METABOLISM and their metabolic defects

A) AROMATIC AND HETEROCYCLIC AMINO ACIDS



Hawkinsinuria

- Hawkinsin is an unusual metabolite of Tyrosine
- Autosomal Dominant form of tyrosinuria
- Transient tyrosinemia
- Excretion of PHPPA,PHPAA, Hawkinsin

Hypertyrosinemias

Type I (hepatorenal)

Fumaryl acetoacetate hydrolase def
Hypoglycemia
Liver failure

Renal tubular dysfunction, rickets
Cabbage like odor
Early death

Type II (Oculocutaneous)

Tyrosine transaminase deficiency

- M.R, Palmar keratosis
- Corneal lesions, Photophobia

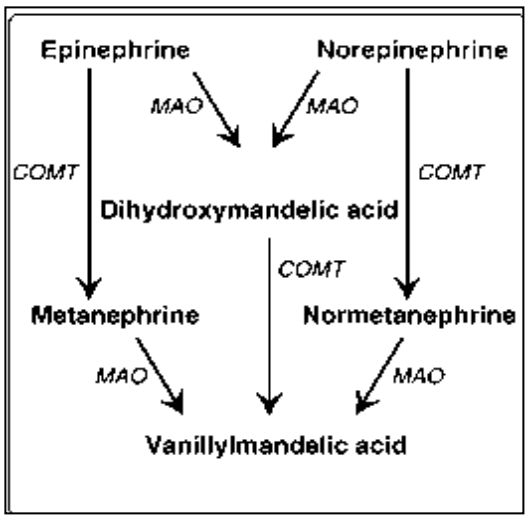
Type III

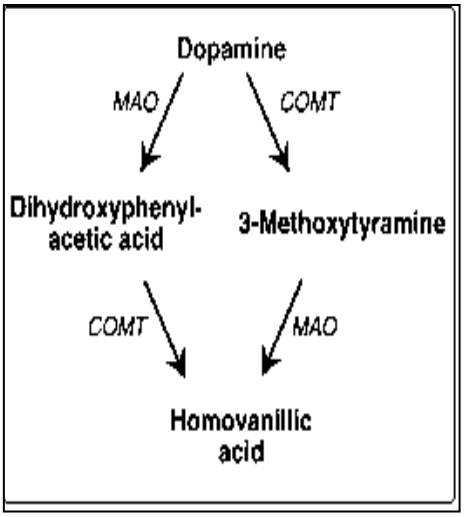
Deficiency of parahydroxy phenyl pyruvate hydroxylase
Normal liver function
Intermittent ataxia, Seizures
Drowsiness, mild M.R

All types have Autosomal Recessive inheritance

Catecholamines

Dopamine, Epinephrine, Norepinephrine
Formed from Tyrosine
DOPA is an intermediate in formation of Catecholamines and Melanin
Catecholamine synthesis: Tyrosine is converted to DOPA by tyrosine hydroxylase
Melanin synthesis: Tyr→DOPA by Tyrosinase





Phenylketonuria (PKU)

Enzyme: Phe hydroxylase
Metabolic abnormalities
Mental retardation, Hyopigmentation,Mousy odour
Tests: Guthrie test, Ferric chloride test, Phe load test, HPLC for Phe
Diet: Restriction to be started within 3 weeks
Continued throughout life

Alkaptonuria

Enzyme : Homogentisate oxidase
C/F : Blackening of urine, Ochronosis
Tests : Ferric chloride test (Transient blue-green), Benedict's test
Diet : Restrict Phe and Tyr
Drug : Nitisone [2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione]

Garrod's tetrad: Cystinuria, Alkaptonuria,Pentosuria, Albinism

Disorder of Histidine metabolism - Histidinemia

- AR due to deficiency of Histidase
- MR and delayed speech development
- Increased excretion of imidazole pyruvic acid in urine
- Sweat urocanic acid absent

Disorder of Tryptophan - Blue diaper syndrome

Trp malabsorption
Trp acted upon by bacteria: Trp→ Indican →→ Indicanuria
Indican in urine is oxidised to Indigo blue which stains diaper

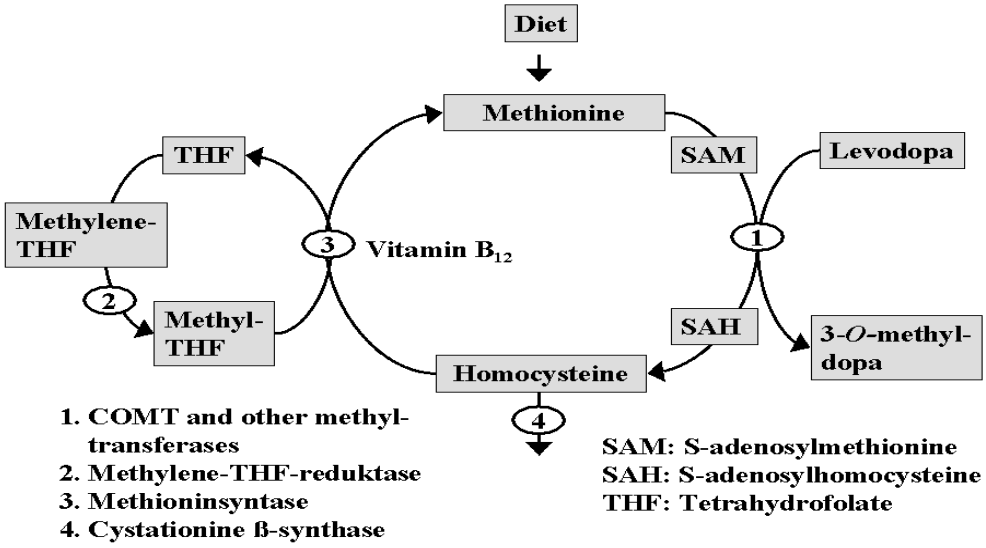
B) BRANCHED CHAIN AMINO ACID DEFECTS

Maple Syrup Urine Disease (MSUD)

Enzyme: Branched chain α keto acid dehydrogenase , Decarboxylation is defective
Mental Retardation, Convulsions, Acidosis, Coma , Death within 1 year
Tests : DNPH test, Rothera's test
Diet: Restrict branched chain amino acids

C) METABOLISM OF SULPHUR CONTAINING AMINO ACIDS:

METHIONINE



Homocystinuria

Note: Homocysteinuria is Defect of methionine metabolism not of cysteine

AR
C/F: M.R, Skeletal abnormalities, Ectopia lentis
Cyanide nitroprusside test – Magenta colour
Diet: Low Met
Normal HS in blood 5-15 μ mol/L
Increase of 5 μ mol/L in serum increases the risk of IHD equivalent to increase of 20 mg/dl of cholesterol
Thiolates LDL → Aggregation of LDL
Also activates Hageman's factor → intravascular thrombosis

CYSTEINE

Cystinuria

Cystinuria is a condition characterized by the buildup of cystine crystals or stones in the kidneys and bladder. Cystine is an amino acid, one of the building blocks of proteins. As the kidneys filter blood to create urine, cystine is normally absorbed back into the bloodstream. People with cystinuria cannot properly reabsorb cystine into their bloodstream and the amino acid accumulates in their urine. As urine becomes more concentrated in the kidneys, the excess cystine forms crystals. As these crystals become larger, they form stones that may lodge in the kidneys or in the bladder. Sometimes cystine crystals combine with calcium molecules in the kidneys to form larger stones. These crystals and stones can create blockages in the urinary tract and reduce the ability of the kidneys to eliminate waste through urine. The stones also provide sites where bacteria may cause infections.

Cystinuria affects approximately 1 in 10,000 people. Autosomal recessive

Genes related to cystinuria

Mutations in the SLC3A1 and SLC7A9 genes cause cystinuria. The SLC3A1 gene provides instructions for producing one part (subunit) of a protein made primarily in the kidneys. This subunit joins with another protein subunit, produced from the SLC7A9 gene, to form a transporter protein complex. (Location of SLC3A1 is at 2p16.3 while location of SLC7A9 gene is : 19q13.1)

The SLC3A1 and SLC7A9 genes (SLC3A1 stands for "solute carrier family 3, member 1.") provide instructions for making the two parts (subunits) of a transporter protein complex that is made primarily in the kidneys. Normally this protein complex controls the reabsorption of certain amino acids, including cystine, into the blood from the filtered fluid that will become urine. Mutations in either the SLC3A1 gene or SLC7A9 gene disrupt the ability of the transporter protein complex to reabsorb amino acids, which causes them to become concentrated in the urine. As the levels of cystine in the urine increase, the crystals typical of cystinuria form. The other amino acids that are reabsorbed by the transporter protein complex do not create crystals when they accumulate in the urine.

↑ **Cystine** in urine 30 times normal

+

↑ Excretion of **Cystine**, Ornithine, Lysine & Arginine (COLA)

↓

Cystine stones

Note: other aminoacidurias

- ***i) Dibasic aminoaciduria (Lysinuric protein intolerance): Mutations in the SLC7A7 gene (Location: 14q11.2) leads to defective transport of Ornithine, Lysine & Arginine but not of cystine.***
- ***ii) Dicarboxylic aminoaciduria: Glutamate and Aspartate reabsorption defect***
- ***iii) Hartnup disease: transport defect of neutral and aromatic amino (mainly Tryptophan) acids from intestine and renal tubules***
- ***iv) Iminoglycinuria: Glycine, proline and hydroxyproline reabsorption defect***

2. Cystinosis (Cys storage Disease)

Cystinosis is a condition characterized by accumulation of the amino acid cystine (a building block of proteins) within cells. Excess cystine damages cells and often forms crystals that can build up and cause problems in many organs and tissues. The kidneys and eyes are especially vulnerable to damage; the muscles, thyroid, pancreas, and testes may also be affected.

There are three distinct types of cystinosis. In order of decreasing severity, they are nephropathic cystinosis, intermediate cystinosis, and non-nephropathic or ocular cystinosis.

Nephropathic cystinosis begins in infancy, causing poor growth and a particular type of kidney damage (renal Fanconi syndrome) in which certain molecules that should be reabsorbed into the bloodstream are instead eliminated in the urine. The kidney problems lead to the loss of important minerals, salts, fluids, and many other nutrients. The loss of nutrients impairs growth and may result in soft, bowed bones (hypophosphatemic rickets), especially in the legs. The nutrient imbalances in the body lead to increased urination, thirst, dehydration, and abnormally acidic blood (acidosis). By about the age of 2, cystine crystals may be present in the clear covering of the eye (cornea). The

buildup of these crystals in the eye causes pain and an increased sensitivity to light (photophobia). Untreated children will experience complete kidney failure by about the age of 10.

The signs and symptoms of **intermediate cystinosis** are the same as nephropathic cystinosis, but they occur at a later age. Intermediate cystinosis typically becomes apparent in affected individuals in adolescence. Malfunctioning kidneys and corneal crystals are the main initial features of this disorder. If intermediate cystinosis is left untreated, complete kidney failure will occur, but usually not until the late teens to mid-twenties.

People with **non-nephropathic or ocular cystinosis** typically experience photophobia due to cystine crystals in the cornea, but usually do not develop kidney malfunction or most of the other signs and symptoms of cystinosis. Due to the absence of severe symptoms, the age at which this form of cystinosis is diagnosed varies widely.

Cystinosis affects approximately 1 in 100,000 to 200,000 newborns worldwide. The incidence is higher in the province of Brittany, France, where the disorder affects 1 in 26,000 individuals.

Genes related to Cystinosis

All three types of cystinosis are caused by mutations in the **CTNS gene**. Mutations in this gene lead to a deficiency of a transporter protein called cystinosin. Within cells, this protein normally moves cystine out of the lysosomes. When cystinosin is defective or missing, cystine accumulates and forms crystals in the lysosomes. The buildup of cystine damages cells in the kidneys and eyes and may also affect other organs.

CTNS gene

The official name of this gene is "cystinosin, lysosomal cystine transporter." CTNS is the gene's official symbol. The CTNS gene is located on the short (p) arm of chromosome 17 at position 13. (Cytogenetic Location: 17p13). More precisely, the CTNS gene is located from base pair 3,539,761 to base pair 3,566,396 on chromosome 17.

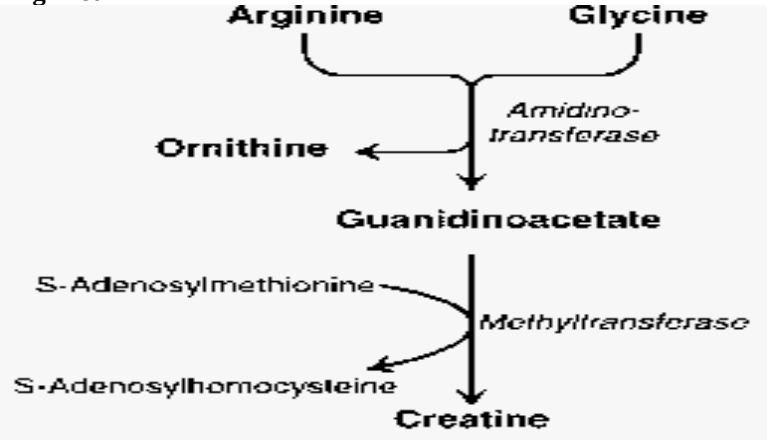
AMINOACIDURIAS AND THEIR ENZYME DEFECTS

Aminoaciduria	**Defective Enzyme**
Phenylketonuria	*Phenyl alanine Hydroxylase*
Alkaptonuria	*Homogentisate oxidase*
Tyrosinemia Type I	*Fumarylacetoacetate Hydrolase*
Tyrosinemia II	*Tyrosine Transaminase*
Albinism	*Tyrosinase*
Methyl malonic aciduria	*Methylmalonyl CoA mutase*
MSUD	*Branched Chain α keto acid decarboxylase*
Isovaleric aciduria	*Isovaleryl CoA DH (Leu)*
Histidinemia	*Histidase*

SOME IMPORTANT AMINO ACID COMPOUNDS:

Creatine

Glycine-Arg-Met



Glutathione

γ Glu-Cys-Gly

Important in membrane & Cellular integrity. Protection against free radicals
Prevents oxidation of SH groups at the active site of enzymes
Detoxification (with help of Glutathione-S-Transferase)
Important in the transport of a.a (Miester cycle)
N- Acetyl cysteine improves the levels of glutathione: used in treatment of injury by free radicals

Polyamines
Growth factors required for protein synthesis
Putrescine, Spermidine, Spermine
ODC is the key enzyme
DFMO- Kala-azar - Difluoro methyl Ornithine is a suicidal inhibitor of Ornithine decarboxylase.In Trypanosomiasis, DFMO is used as a drug because it inhibits ODC in parasites. Since half life of mammalian enzyme is very short, human cells are not affected.

Endothelium Derived Relaxing Factor(EDRF)- Nitric oxide

- Formed from Arg ; By N.O.Synthase – heme containing flavoprotein
- N of Guanidino group is incorporated to N.O
- 3 isoenzymes for NOS- inducible form in macrophages- induced by interleukins and TNF
- N.O activates guanylate cyclase
- cGMP = Smooth muscle relaxation -Vasodialatation

Glutathione synthesis inhibitor

- Buthionine sulfoximine has structural similarity with glutamic acid
- Used to keep Glutathione level low
- Low GSH level is useful for sensitization of tumor cells to radiations, sensitizing parasites to drugs etc

Amino Acid	Important Products
Glycine	❑ C 4,5,7 of purines ❑ Heme ❑ Creatine, Creatine phosphate and creatinine ❑ Conjugation of bile salts & xenobiotics
Phenyalanine & Tyrosine	❑ Catecholamines ❑ Melanins ❑ Thyroid hormones
Tryptophan	❑ 5 Hydroxy tryptamine (5 HT) (serotonin) ❑ Melatonin
Histidine	❑ Histamine
Arginine	❑ Nitric oxide ❑ Polyamines ❑ Creatine, Creatine phosphate and creatinine
Glutamate	❑ GABA
Serine	❑ Homocysteine ❑ Sphingosine ❑ C2 ,4 of Purines ❑ Methyl Group of Thymine
Cysteine	❑ CoASH ❑ Taurine
Methionine	❑ Homocysteine ❑ S- Adenosyl Methionine

Chapter 6
ENZYMES

Important points to remember
Enzyme classes that do not require coenzymes are Class 3 & 4 i.e. Hydrolases & Lyases
Coenzymes are Non–protein, low molecular wt., dialyzable and thermostable substances
Coenzymes act as 2nd substrates or group transfer agents
Coenzymes can be classified according to the group whose transfer they facilitate
Isoenzymes are physically distinct forms of the same enzyme
Isoenzymes differ in structure, properties, electrophoretic mobility, and Tissue of Expression
Isoenzyme has one common property in that they catalyze the same reaction

CLASSIFICATION OF ENZYMES: All enzymes are classified into following 6 classes as below
1. Oxidoreductase : Oxidation of one substrate with simultaneous reduction of another substrate/coenzyme.
Includes Oxidase, Reductase, Dehydrogenase, Catalase, Peroxidase, Oxygenases, (hydroxylases also)
2. Transferase: Transfer a group other than Hydrogen.
Includes Transaminases, Phospho transferases (Polymerases and kinase),Sulphotransferases, Methyl transferases, Acyl transferases, Glycosyl transferase,
3. Hydrolase: Splits the substrate into two by adding water.
Includes Esterases, Peptidases, Glycosidases etc
4. Lyase: splits the substrate into two without adding water: Eg Aldolase
5. Isomerase: Catalyzes intramolecular rearrangement.
Includes Epimerase, Racemase, Mutase, Cis-trans isomerase
6. Ligase: Bind together 2 substrates.
Includes Synthetases and Carboxylases

COENZYMES
Organic substances required by the enzyme for its proper action. (Cofactor: organic/inorganic)
Classified as
- Coenzymes taking part in the transfer of Hydrogen
  - NAD^+, $NADP^+$, FMN, FAD, Lipoic acid, Coenzyme Q
- Coenzymes taking part in the transfer of other groups
  - Coenzyme A – Transfer of Acyl groups
  - TPP – Transfer of Hydroxyethyl groups
  - PLP – Transfer of Amino group
  - Folate coenzyme – One carbon units
  - Biotin - Carbon dioxide

MECHANISM OF ENZYME ACTION
i) Lowering of activation energy
Activation energy is the energy required to bring a substrate from its ground energy state to the transition state. Enzymes reduce this energy
ii) Michaelis Menten theory : States that Enzyme combines with substrate to form as complex which dissociates into Enzyme and products.
iii) Fischer's template theory : "Active site" of the enzyme will have a structure complementary to that of substrate. Explains Enzyme specificity and competitive inhibition
iv) Koshland's theory: Binding of substrate to the active site induces a conformatonal change at the active site which facilitates further binding of substrate: explains allosteric regulation

RATE LIMITING ENZYME (KEY ENZYME)
These enzymes are easily regulated by physiological mechanisms
Rate limiting enzyme (Key enzyme) catalyses the slowest reaction in a pathway, (Km value high) so that regulatory mechanisms can exert their effect.
Key enzymes are allosterically regulated and Key enzymes are inducible enzymes

Km VALUE (MICHAELIS CONSTANT)

Substrate concentration at which half the maximum velocity is achieved

Significance

- Signature of the enzyme
- Inversely related to affinity
- To find out the natural substrate: Natural substrate has the lowest Km value
- To identify the key enzyme: Highest Km
- To find out the type of enzyme inhibition

SERINE PROTEASES:

Contain Ser at the active site.

Ser, His, Asp- FORMS THE CATALYTIC TRIAD

Mostly secreted as zymogens- undergoes "limited proteolysis

Serpin: Serine protease inhibitor

ENZYME INHIBITION

Various types as follows:

i) Competitive inhibition

Inhibitor is a substrate analogue; binds to the active site

Vmax is not affected; Km is increased

Reversible on adding excess substrate

Examples

- Sulphonamides as antibacterial drugs
- Methotrexate as anticancer drug
- Ethyl alcohol in treatment of methyl alcohol poisoning

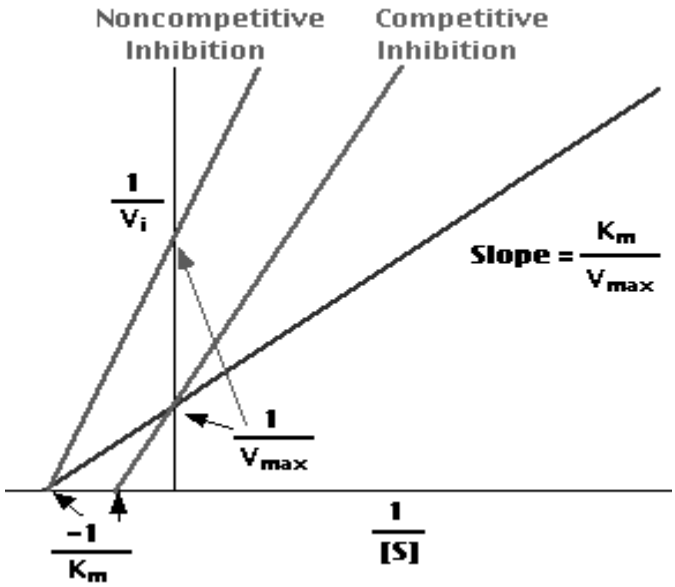
ii) Non-competitive inhibition

Inhibitor has no structural similarity with substrate

Inhibitor usually does not bind to the substrate binding site; acts by various other mechanisms

iii) Uncompetitive inhibition

Binding of substrate caused binding of inhibitor to the enzyme. Both Km and Vmax decreased



iv) Suicide inhibition

Enzyme catalyses a reaction involving the inhibitor. Product formed is a powerful inhibitor of the enzyme.

v) Allosteric inhibition

Allosterically regulated enzymes have substrate binding site and an allosteric site

Inhibitor binds to allosteric site; It induces a conformational change at the active site; So the substrate binding to active site is altered

ENZYME REGULATION- types

i. Induction and Repression
ii. Covalent modification
iii. Allosteric regulation

FUNCTIONAL AND NON FUNCTIONAL PLASMA ENZYMES

Functional enzymes: Those which have a specific function in plasma; Eg: Enzymes of coagulation pathway, Lipoprotein lipase, choleinestrase

Non-functional enzymes: Their function is not in plasma: they are present in plasma due to leakage from the organ where they are located (as a part of normal wear and tear). Their level increases in blood when there is damage to the tissue where it is located.Eg: CK is elevated in MI and ALT in hepatitis

DIAGNOSTIC APPLICATION OF ENZYMES AND ISOENZYMES IN VARIOUS DISEASES

Enzymes in Liver diseases:

ALT: Normal value 13-40 U/L; Elevated in liver cell damage; Hepatitis, Cirrhosis, Cancer

AST: Normal value 8-20 U/L; Elevated in Liver cell damage; not specific

Alkaline phosphatase: Has 6 isoenzymes; Normal value 3-13 KAU/dl; Very high levels are seen in obstructive liver diseases and bone diseases. High levels in children before closure of epiphyses

5'Nucleotidase: (Nucleotide triphosphatase); Elevated in obstructive liver diseases

Gamma Glutamyl Transferase: Very sensitive index of alcoholic liver disease

Enzymes in Heart disease:

Creatine kinase: (creatine phosphokinase); First enzyme to elevate after myocardial infarction; Rises within 2-3 hours. Remain elevated for 1-2 days only; For early diagnosis of MI

3 isoenzymes: CK-MB is to be measured for heart disease

Isoenzymes of CPK

CPK is a dimer – B and M subunits

CPK1 - BB – Brain : CPK2 - MB – Heart : CPK3 - MM – Muscles:

AST: Elevated in MI after 2 days; Remain elevated for 6-7 days

2 isoenzymes: one from heart; other from liver (Increased in liver diseases also)

Lactate dehydrogenase: Elevated after 2-3 days following MI; Remain elevated for 10-12 days

For late diagnosis of MI; 5 isoenzymes: LDH 1 is elevated in MI

(Normally LDH2 > LDH1- This is reversed in MI- "Flipped pattern"

Isoenzymes of LDH

LDH is a tetramer, 2 types of subunits - H and M

LDH1- HHHH – Heart;

LDH2 – HHHM – RBC

LDH3 – HHMM – Brain

LDH4 – HMMM – Liver

LDH5 – MMMM – Skeletal muscles

Other proteins in heart disease

Troponin: High sensitivity & Specificity

Myoglobin- Low specificity

Ischemia-modified albumin- Low specificity

Pro-brain natriuretic peptide: Elevated in CCF

Cardiac troponins

Not enzymes: Very sensitive index for MI: Elevated early in MI, remain elevated for 10-14 days

Considered "any time marker" of MI

ISOENZYMES OF ALP

α 1-	Biliary epithelium - Elevated in obstructive jaundice
α 2 HL -	Hepatocytes - Elevated in heaptitis
α 2 HS -	Placenta - Elevated in pregnancy, trophoblastic tumors
Pre β –	Bone - Elevated in Pagets disease, Rickets. Elevated values in children
γ –	Intestinal cells - Elevated in ulcerative colitis
LAP-	Leukocyte ALP - Elevated in myeloid leukemia

Chapter 7

VITAMINS

Fat soluble: A, D, E, K
Water soluble: B complex, C

Vitamin A: Active forms Retinal, retinol, retinoic acid
Provitamin – β carotene – Plants: Contains 2 β ionone rings; Cleaved by a dioxygenase: Produce 2 retinal
- β carotene has antioxidant properties
Absorbed with fat: **Stored in liver as retinyl palmitate (ester of retinol and palmitic acid)**
Biological role: Retinal has Visual property while retinol & retinoic acid has steriod hormone activity
Hypervitaminosis : Damages lysosomal membrane

Vitamin E
"Anti sterility" vitamin
Most potent biological antioxidant: Protects biomembranes, Prevents hemolysis, Retards ageing: Decrease oxidation of LDL→ reduce the risk of atherosclerosis. Synergistic action with Se

Vitamin D
D2-Ergocalciferol- Fomed from Ergosterol in plants;
D3- Cholecalciferol – Formed from 7-dehydrocholesterol in animals
Active form of vit D – calcitriol (1, 25 DHCC); Calcitriol binds to cytoplasmic receptor
25 hydroxylation in liver- Microsomal mono oxygenase:
1 hydroxylation in kidney: Mitochondrial enz-

Other forms of Vitamin D are
D1- Molecular compound of ergocalciferol with lumisterol (1:1); D4 – Dihydrotachysterol- From D3
D5 – Sitocalciferol- from 7-dehydrocholesterol

Vitamin K
Naphthoquinone derivative
K1- Phylloquinone; K2 – Menaquinone; K3 – Menadione- Synthetic, water soluble form
Required for gamma carboxylation of glutamic acid residues (Gla) of blood clotting proteins Factors II, VII, IX and X as well as Protein C, Protein S and Protein Z (that is they also require vit K)

Water soluble vitamins: B complex, vitamin C
B complex vitamins act as coenzymes for various enzymes

Vitamin	Active form	Reactions requiring the vitamin
Thiamine	TPP	PDH, α KG DH, Transketolase, Branched chain α keto acid decarboxylase
Riboflavin	FMN, FAD	FMN: Aminoacid oxidase, NADH dehydrogenase FAD: PDH, α KG DH, Acyl CoA DH, Succinate dehydrogenase, Xanthine oxidase
Niacin	NAD, NADP	Dehydrogenases and reductases other than riboflavin linked enzymes
Pantothenic acid	ACP, CoA	Acyl transferase reactions
Pyridoxine	PLP	Transaminases, Decarboxylation of amino acids, Cystathionine synthase, Cystathionase, ALA synthase, Kynureninase, Glycogen phosphorylase
Biotin		Carboxylation
Folic acid	THF	One carbon metabolism
Cobalamin	Methyl B12 Doexy adenosyl B12	HS-Met methyl transferase Methyl malonyl CoA mutase

B COMPLEX VITAMINS WITH RING STRUCTURE

- Thiamine: Pyrimidine +Thiazole

- Riboflavin: Isoalloxazine
- Niacin: Pyridine
- Pyridoxine: Pyridine
- Biotin: Imidazole + Thiophene
- Folic acid : Pteridine + PABA
- Cobalamin: Corrin [Tetrapyrrole ring with Cobalt at its centre]

INDIVIDUAL VITAMINS, IMP ENZYMES REQUIRING VITAMIN & DEFICIENCY DISEASES

BIOTIN: Synthesised by intestinal bacteria; Anti egg white injury factor; Coenzyme for carboxylases

NADPH generating enzymes

- G6PD
- 6 phosphogluconate DH
- Cytoplasmic isocitrate DH
- Malic enzyme (malate → pyruvate)

NADPH utilising enzymes

- Enzymes of reductive biosynthetic pathways

Niacin deficiency

- Requirement: 20 mg/day: 2 / 3 is produced from Trp : 60 mg Trp → 1 mg Niacin
  - Trp → Niacin
    - Kynureninase: Require PLP as coenzyme
- Pellagra: 4 D: Diarrhoea, Dementia, Dermatitis leading to Death

Maize: Niacin present as bound unavailable form known as niacytin
Sorghum: High Leucine content inhibits QPRTase
PLP def: Kynureninase inhibited
Hartnup disease : Trp absorption defective

FMN and FAD – coenzyme forms of Riboflavin (B2)
FMN: Aminoacid oxidase, NADH dehydrogenase
FAD: Acyl CoA dehydrogenase, Succinate DH, X.O, PDH, α KG DH

B6 (Pyridoxine)
Enzymes which require PLP: Transaminase, Decarboxylation of amino acids, Cystathionine synthase, Cystathionase, ALA synthase, Kynureninase (Trp→ Niacin)

Sulphur containing B complex vitamins: Thiamine, Biotin
Pantothenic acid does not contain sulphur, but its active forms CoA and ACP contain

Pantothenic acid (B5): Present in CoA, Acyl Carrier Protein; Contains β alanine ; Deficiency: Burning foot syndrome

VITAMIN C
Only L-ascorbic acid and dehydroascorbic acid have vitamin activity; D-ascorbic acid –not active
Most animals-synthesise from glucose
Humans cannot: Absence of ***gulonolactone oxidas***e

Functions: Hydroxylation of Pro and Lys; Trp hydroxylase ; Dopamine β oxidase, Folate reductase
Antioxidant→ reduce the risk of cancer

Infantile form – Barlow's disease

Chapter 8
MINERALS

Macrominerals (Major elements): Daily requirement >100 mg
Ca, Mg, P, Na, K, Cl and S
Microminerals (Trace elements): Daily requirement <100 mg
Fe, I, Cu, Co, Mn, Mo, Se, Zn and F
Ultratrace elements: Requirement < 1 mg/day

Metalloenzymes

Iron: Cytochrome oxidase, catalase, peroxidase, xanthine oxidase
Copper: Tyrosinase, Cytochrome oxidase, SOD, lysyl oxidase
Zinc: Carbonic anhydrase, Carboxy peptidase, Alcohol dehydrogenase, ALP, LDH, Glu DH, RNA polymerase
Magnesium: Hexokinase, Enolase, PFK
Manganese: Hexokinase, Enolase
Molybdenum: Xanthine oxidase
Calcium: Lipase, Lecithinase

Calcium: Normal level: 9-11 mg/dl; 50 % ionised; 40% protein bound; 10 % complexed with anions
Alkalosis favours protein binding

Calpains

Calcium dependent Cysteine proteases that modulate cellular functions
Role in DM, Cataract, Alzheimer's disease, Muscular dystrophies
Inhibited by calpastatins

Calcium level- Reciprocal relation with phosphorus

Ionic product-constant
S.Calcium x S. Phosphorus = 40 : Children, it is 50 (Ca is more)

Zinc: 60 % in bones, 30 % in skeletal muscles
Highest concentration in (a) Hippocampus (b) Prostatic fluid
Zn and Cu competitively inhibits each other's absorption
Antioxidant: Because e.c SOD is Zn dependent
Stabilises insulin stored in pancreas

Copper:

Wilson's disease

Copper binding ATPase deficient in cells; Excretion of copper from cells defective
Copper accumulates in liver and brain cells
Low serum Cu and Ceruloplasmin; High urinary excretion of Cu
As Zn prevents Cu absorption, it can be used to reduce Cu load

Menke's kinky hair syndrome

Deficiency of intracellular Cu binding ATPase
Defective cross linking of connective tissue
X-linked recessive
Dissecting aneurysms and cardiac rupture cause death

Mineral	Deficency	Toxicity
Boron		Developmental defects, male sterility, testicular atrophy
Calcium	Osteoporosis, tetany	Renal insufficiency (milk-alkalai syndrome), nephrolithiasis, impaired iron absorption
Copper	Anemia, growth retardation, defective keratinization and pigmentation of hair, hypothermia, degenerative changes in aortic elastin, osteopenia, mental deterioration	Nausea, vomiting, diarrhea, hepatic failure, tremor, mental deterioration, hemolytic anemia, renal dysfunction
Chromium	Impaired glucose tolerance	Occupational: renal failure, dermatitis, pulmonary cancer
Fluoride	Dental caries	Dental and skeletal fluorosis, osteosclerosis
Iodine	Hypothyroidism	Acne-like eruptions
Iron	Anemia, tiredness, koilonychias etc	Gastrointestinal effects (nausea, vomiting, diarrhea, constipation), iron overload with organ damage
Manganese	Impaired growth and skeletal development, reproduction, lipid and carbohydrate metabolism; upper body rash	Neurotoxicity, Encephalitis-like syndrome, Parkinson-like syndrome, psychosis, pneumoconiosis
Molybdenum	Neurologic abnormalities	Reproductive & fetal abnormalities
Selenium	Cardiomyopathy, heart failure, striated muscle degeneration	Alopecia, peripheral neuropathy, garlic odor to breath, dermatitis Lung and nasal carcinomas, liver necrosis, pulmonary inflammation
Phoshorus	Rickets (osteomalacia), proximal muscle weakness, rhabdomyolysis, paresthesia, ataxia, seizure, confusion, heart failure, hemolysis, acidosis	Hyperphosphatemia
Zinc	Growth retardation, decresed taste sense, alopecia, dermatitis, immune dysfunction,gonadal atrophy	Reduced copper absorption, gastritis, Respiratory distress, pulmonary fibrosis

Chapter 9
ACID BASE BALANCE & pH

pH of various cellular compartments: Plasma 7.4 ± 0.02
Compatible with life 6.8-7.8
- Intracellular pH : RBC 7.2 Other cells 7.0 Skeletal muscle – as low as 6.0

Buffer = Solutions which can resist changes in pH when an acid/alkali is added
They are mixtures of weak acids and its salt with a strong base OR Mixtures of weak bases and its salt with strong acid

Henderson-Hasselbalch equation:

$$pH = pK + \log \frac{[Salt]}{[Acid]}$$

pK (Acid dissociation constant)

- Bicarbonate 6.1
- Phosphate 6.7-7.2
- Organic phosphate esters 6.5-7.6
- Protein buffers
  - His side chains 6.5-7.4
  - N-terminal amino grp 7.6-8.4
  -

Buffers of body fluids

Plasma	Intracellular Fluid	RBC
Bicarbonate Phosphate Albumin	Phosphate Protein buffer Bicarbonate	Hb buffer Phosphate Bicarbonate

Factors affecting pH of a buffer

a. pK value : The lower the pK, lower the pH
b. RATIO of salt/acid (Not the exact concentration)

Factors affecting Buffering capacity: Ability to resist changes in pH: affected by actual conc. of salt or acid
A buffer is most effective when pH=pK (salt: acid = 1:1)
Effective range of buffer is pK-1 → pK +1

Bicarbonate buffer system is the most important in plasma.
Plasma $[HCO_3]$ = 24 mmol/L $[H_2CO_3]$ = 1.2 mmol/L $PaCO_2$ = 40 mmHg
$[HCO_3]/[H_2CO_3]$ = 20 → "Alkali reserve"

Mechanisms of regulation of pH

Buffers : First line of defense
Respiratory regulation: By increasing/decreasing the rate of respiration
Renal regulation:

a. Reabsorption of bicarbonate: Occurs mainly in PCT
b. Excretion of titratable acid; Occurs in DCT . Major titratable acid present in urine is sodium acid phosphate Phosphate buffer is the most important urinary buffer
c. Excretion of ammonium ions: in DCT. Large quantity of acid can be excreted with minimum change in pH of urine. Ammonia is derived by the action of glutaminase on glutamine. Most important in acidosis because- large amounts of acids can be excreted without much change in urinary pH; Ammonia is readily availablefrom amino acids; Does not require sodium/potassium for its excretion

Normal pH of urine can vary from 4.4-6.0

ANION GAP

In plasma, total amount of cations and anions should be equal.

Measured cations are Na^+ and K^+ (95 %) ; Measured anions are HCO_3^- and Cl^- (86 %)

Unmeasured anions constitute anion gap

Anion gap = $(Na^+ + K^+) – (Cl^- + HCO_3^-)$; Normal value= 12 -16 mmol/L

Due to Protein anions, SO_4, PO_4, Organic acids: Increased in some types of metabolic acidosis

For clinical purposes, anion gap is calculated as $Na^+ – (Cl^- + HCO_3^-)$; Normal 8-12 mmol/L

Unmeasured cations- Ca, Mg, K

High anion gap

Usually due to increase in unmeasured anions (lactic acid, keto acid, non- chloride containing acids etc)

Increase in anionic albumin (increased albumin, alkalosis (charge of albumin increases)

Low anion gap

Increase in unmeasured cation

Abnormal cations added to blood (Lithium, Cationic immunoglobulins-plasma cell dyscrasia)

Hypoalbuminemia

Acidosis which decreases charge of albumin

Hyperviscosity and severe hyperlipidemia (lead to under estimation of Na and Cl levels)

Norma plasma pH: 7.4 ± 0.02

Acidosis: decrease in plasma pH Alkalosis: increase in plasma pH

"Respiratory" = Primary decrease/increase in pCO_2

"Metabolic" = Primary decrease/increase in hco_3^-

Metabolic acidosis

High anion gap:

- Renal failure,Ketoacidosis
- Lactic acidosis, Ingestion of acids
- Salicylate poisoning, Methanol
- High fever, severe exercise
- Haemorrhage, Shock, ischemia
- INH, iron toxicity

Normal anion gap (Both anions and cations lost)

- Diarrhoea
- Small intestinal fistula
- Ureterosigmoidostomy
  - ◘ These are associated with hypokalemia
- ☐ Renal Tubular Acidosis
- ☐ C.A Inhibitors

Metabolic alkalosis

Chloride responsive: (Urine $Cl^- < 10$ mmol/L)

- Intake of alkali: Sodium bicarbonate, Sodium lactate
- Loss of acid: Prolonged vomiting, gastric lavage
- Diuresis

Chloride resistant (Urine $Cl^- > 10$ mmol/L)

- Hyperaldosteronism, Cushing's syndrome

Respiratory acidosis

Any condition which produces hypoventillation / perfusion

- CNS diseases
- Drugs: Morphine, Barbiturates, Anaesthetics
- Bulbar poliomyelitis
- Pulmonary / Mediastinal tumors
- COPD, Emphysema, Pulmonary oedema, Pleurisy
- Rebreathing from a closed space

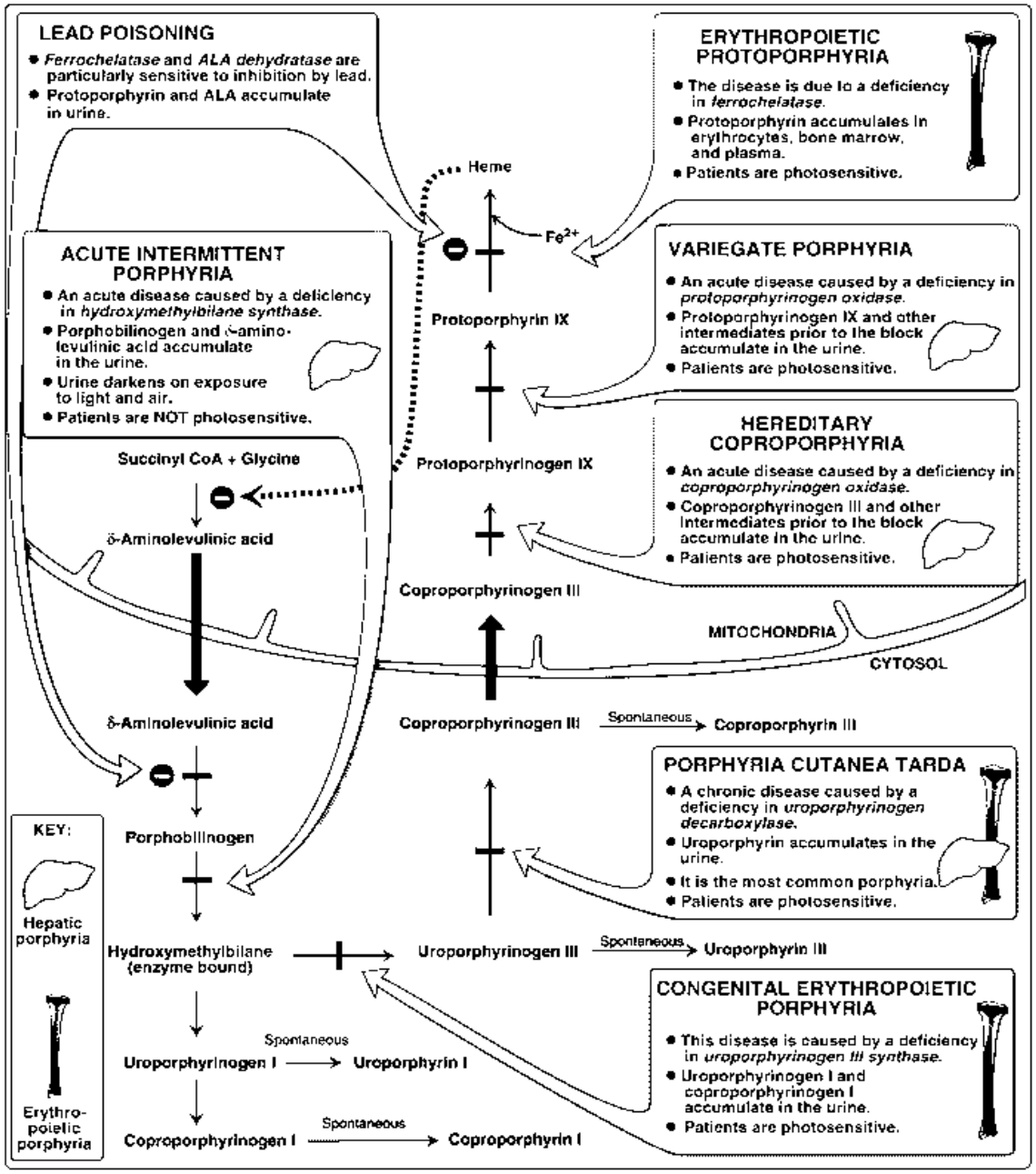
Respiratory alkalosis

Any condition leading to hyperventillation

- Anxiety, Hysteria

CNS diseases involving respiratory centre, Salicylate poisoning

Chapter 10
HEME SYNTHESIS AND DEGRADATION



Hemoproteins

They are proteins containing heme as the prosthetic group. Heme is a tetra-pyrrole with iron in centre
Examples of Hemoproteins are:

1. Hemoglobin & Myoglobin
2. Cytochromes
   - Respiratory chain: cyt b, cyt c1, cyt c, cyt aa3
   - cytochrome P_{450} (involved in xenobiotic metabolism)
   - cytochrome b_5 (a component of Methemoglobin reductase)
   - cyt b_{558} (a component of NADPH oxidase in phagocytes)
3. Enzymes like Catalase and Tryptophan Pyrrolase etc

Differential diagnosis of jaundice

Test	Hemolytic (Prehepatic)	Hepatic	Obstructive (Post hepatic)
Blood Direct Bilirubin	N	N/↑	↑
Blood indirect Bilirubin	↑	↑/N	N
Urine Bile salts	(-)	(-) / (+)	(+)
Urine bile pigments	(-)	(-) / (+)	(+)
Urine UBG	↑	N/↓	(-)
Blood ALT	Normal	↑↑	Normal
Blood AST	Normal	↑↑	Normal
Blood ALP	Normal	↑	↑↑↑↑

Hb derivatives

Oxy Hb – orange red (dark red)
Deoxy Hb – Purple
Met Hb – Dark brown
COHb – Cherry red
Sulph Hb – Green (Bluish green)

Carboxy Hb

Affinity of CO to Hb is 200 times higher than Oxygen
Occupational hazard in mines: Breathing automobile exhaust in closed space
Normal level <0.16 % Smokers-High level
CO also binds to Mb and Cyt p450
C/F when COHb > 20 %: Irritability, headache, vomiting, chest pain
At 40-60 % saturation, confusion, coma and death can occur
Treatment: Hyperbaric oxygen

Met Hb: Fe in Ferric state

Met Hb emia
Congenital: Cytochrome b5 reductase deficiency, Hemoglobin M
Acquired: Drugs: Phenacetin, Sulpha,nitrites, sodium nitroprusside

Sulph Hb

Drugs : sulphonamides, phenacetin, dapsone
Irreversible: Seen as Basophilic stippling of RBC

HbS-Sickle cell anemia

First molecular disease which supported one gene-one protein theory
Hydrophilic Glu → Hydrophobic Val: leads to stickiness
Abnormality leads to polymerization of Hb during deoxygenation → Distortion of RBC
Protection against P.Falciparum
Diagnosis
Sickling test: Blood smear kept at low oxygen tension by adding sodium dithionite: Smear shows sickled RBCs after a few hours
Solubility tests: Deoxy Hb is less soluble
Electrophoresis: slower moving than HbA
(Due to loss of Glu which is negatively charged at pH 8.6)

Antisickling agents: Urea, cyanates, aspirin

Transport of CO_2 [Isohydric transport-as bicarbonate-78%; Carbamino Hb-13%; Dissolved- 9%]
CO_2 binds to N-terminal amino acid of both chains; In beta chain it has to compete with 2,3 BPG

Hb A – $\alpha_2\beta_2$ HbA2 - $\alpha_2\delta_2$ Hb F - $\alpha_2\gamma_2$
α chain – 141 amino acids 1-Val 141-Arg
β chain – 146 amino acids 1-Val 146-His
γ chain - 146 amino acids 1-Gly 146-His
δ chain - 146 amino acids 1-Val 146-His

HbF (α 2 γ 2) has got decreased interaction with 2,3 BPG
γ chain – decreased binding with 2,3 BPG
HbF1 – 15- 20 % of HbF is acetylated at the N terminal: does not bind 2,3 BPG
Hb H= 4 beta chains (Can be considered as alpha thalassemia)

Embryonic Hb
Produced from 3rd to 8th week of gestation
Hb Gover-1 : ε2 ζ 2 (Embryonic chains only)
Hb Gover – 2 : α2 ε2
Hb Portland : γ2 δ2
By 7th week of gestation, site of erythropoiesis shifts from liver → spleen, and synthesis of HbF starts

Hb M = Proximal or Distal His → Tyr
"Proximal and Distal Histidine" (The term is In relation to the proximity of His residues of both chains to Iron atom of heme
In Alpha, Distal 58 and Proximal 87; In others Distal 63 and proximal 92

Hb M Boston: α 58 His →Tyr
HbM Iwate : α 87 His →Tyr
Hb M Saskatoon: β 63 His → Tyr
Hb M Hyde Park: β 92 His → Tyr

Hb variants with high oxygen affinity
Hb Olympia (β 20 Val → Met)
Hb Yakima (β 99 Asp →His)
Hb Kempsey (β 99 Asp →Asn)
Hb Hiroshima (β 143 His→Asp)
Hb Rainier (β 145 Tyr → His)
Hb Chesapeake (α 92 Arg → Leu)
Hb F Chesapeake (α 92 Arg → Leu, *γ2*)

Hb variants with low oxygen affinity
Hb Kansas : β 102 Asn → Thr
Hb Rothschild : β 37 Trp → Arg
Hb Hope : β 136 Gly → Asp

One Litre of plasma can carry 1.34 ml of oxygen at 38^0 C . One litre of blood (150 g Hb) can carry about 200 ml of oxygen . [87 times more efficient than plasma]
2, 3 BPG - Intermediate of glycolysis in RBC: Synthesis with the help of mutase enzyme (BPG shunt) : Normal level: 15 ±1.5 µg / g of Hb
Child>Adult Female>Male
Preferentially binds to deoxyHb: Stabilizes T state: Lowers intracellular pH
Shifts ODC to right
↑ed in anemia, cardiopulmonary insufficiency, high altitude. Decreased binding with HbF
BPG level increases after birth and reaches a peak by 3 months

Shift to right of ODC is caused by
(a) Increase in pCO2 (b) Increase in [H+] (c) Increase in 2,3 BPG (d) Increase in temperature
Haldane effect: Deoxygenation of the blood increases its ability to carry carbon dioxide
Bohr effect: Increase in CO_2 or acidity causes less affinity of Hb to Oxygen
Hill coefficient: Indicates cooperative binding
Value 1 indicates independent binding
> 1 indicates positive co operativity (Hb – 2.8 – 3)

Chapter 11
BIOENERGETICS - ELECTRON TRANSPORT CHAIN

The study of this energy transduction in our body is called **Bioenergetics**. It is also called **Biochemical thermodynamics**

Energy – capacity to do work.

1. Free energy (G) – Energy available to do work. (Useful energy or chemical potential). Δ G = Change in free energy
2. Entropy (S) = Randomness/ Disorderliness Δ S= Change in entropy
3. E = Total internal energy Δ E = Change in total internal energy

In biological systems

Standard free energy change (ΔG^0)

The free energy change (ΔG) when all reactants are at a concentration of 1 moles / L including H^+

{pH = – log $[H^+]$ = – log [1] = 0}

However in biologic system pH is 7

Therefore ΔG° in biological system is denoted by $\Delta G^{0/}$

i.e. All reactants at 1 mole/L except H^+ which is 10^{-7} mole/ L i.e. pH = 7

R = Gas constant
T = Absolute temp in ^0K
Keq = equilibrium constant

According to 2nd law of thermodynamics, for any change to occur spontaneously entropy must increase
Based on the pattern of energy transductions, biochemical reactions can be categorized

$\Delta G^{0/}$ – ve	ΔS + ve	Spontaneous	exergonic	Oxidative breakdown	catabolic
$\Delta G^{0/}$ + ve	ΔS – ve	Not spontaneous	endergonic	Reductive synthesis	anabolic
ΔG = 0	TΔS = ΔE	Equilibrium	No energy change		

Metabolism: A study of all the processes which take place in our body, whether oxidative breakdown **(catabolism)** or reductive synthesis **(anabolism).**

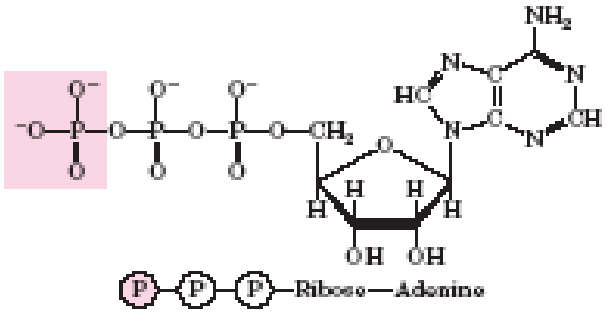
Energy currency

Endergonic reactions cannot proceed unless they are coupled with exergonic reactions. Similarly the energy liberated from exergonic reactions has to be trapped for future use. Such coupling is provided by **ATP** (the energy currency of the cell).

Advantages of ATP

1. If all high energy positive compounds are arranged in increasing or decreasing order of their $\Delta G^{0/}$ of hydrolysis ATP occupies a middle position.
2. It can easily take up $-Ⓟ$ form compounds with higher $\Delta G^{0/}$ and easily give $-Ⓟ$ to compounds with lower $\Delta G^{0/}$.

ATP → ADP + Pi	($\Delta G^{0/}$ = – 30.5 KJ/ mole or – 7.3 kcal / mole)
ATP → AMP + PPi	($\Delta G^{0/}$ = – 32.2 KJ/ mole or – 7.7 kcal / mole)
ADP → AMP + Pi	($\Delta G^{0/}$ = – 27.6 KJ/ mole or – 6.6 kcal / mole)
PPi → Pi + Pi	($\Delta G^{0/}$ = – 19.2 KJ/ mole or – 4.6 kcal / mole)
Creatine phosphate → creatine + Pi	($\Delta G^{0/}$ = – 43.1/KJ/ mole or – 10.3 Kcal/ mole)



Note: Other functions of ATP/ ADP:

1. ADP binding results in platelet activation
2. ADP – Ribosylation of proteins important in their functioning
3. Poly ADP ribosylation of nucleoproteins is important in DNA repair mechanism

Major sources of generations of ATP

1. Respiratory Chain (oxydative phosphorylation)
   Greatest quantitative source of ATP
   Requires molecular O_2

2. Glycolysis : 2 steps generate ATP (Substrate level phosphorylation)
   i. Phosphoglycerate kinase step
   ii. Pyruvate Kinase step

3. TCA cycle : only one step generates ATP (substrate level phosphorylation)
Succinate Thiokinase or succinyl CoA synthetase step

Respiratory Chain (Electron Transport Chain)

It is a series of catalysts present in the inner mitochondrial membrane in the form of four Lipid protein complexes arranged in the order of increasing redox potential.

Redox span of respiratory chain

$NAD^+/NADH \longrightarrow O2/ H_2O$ is –1.1 V

Four lipid protein complexes are:

Complex I: NADH Dehydrogenase (NADH – Q oxidoreductase)
Complex II: Succinate Dehydrogenase (Succinate – Q reductase)
Complex III: Q cytochrome c oxidoreductase
Complex IV: cytochrome c oxidase

All cytochromes except cytochrome oxidase (cyt a-a3) are anaerobic dehydrognases

Important components of respiratory chain:

1. Iron sulphur protein (FeS)
   a. Non heme iron
   b. Associated with metalloflavoproteins & cyt b (complex I, II, III)
   c. Involves single electron change
   d. Transfers electrons from flavins to Coenzyme Q and from Coenzyme Q to cyt b

2. Ubiquinone – also called coenzyme Q
   a. Mobile component (other mobile component being cyt c)
   b. Transfers electron from flavoproteins to cytochromes.
   c. Quinone (oxidized form) in aerobic condition
   d. Quinol (Reduced form) in anaerobic condition
   e. Isoprenoid (polyprenoid) resembling vitamin E & vitamin K.
   f. In large stoichiometric excess as compared to other components
   g. Constituent of mitochondrial membrane lipids (other lipids being phospholipid cardiolipin)

3. Cytochromes
   a. Hemoproteins (other Hemoproteins Hb, Mb, Catalase, Tryptophan Pyrrolase)
   b. Iron oscillates from Fe^{3+} to Fe^{2+}

Cytochromes of respiratory chain

Cytochrome b: Only cytochrome containing FeS protein.
Cytochrome c_1
Cytochrome c: Only soluble cytochrome.
Cytochrome oxidase (aa_3):
- Terminal component of respiratory chain
- Only irreversible reactions of respiratory chain
- Main driving force of respiratory chain which gives direction to the flow of reducing equivalents. Functions maximally till virtual depletion of O_2.
- Directs ATP production coupled with flow of reducing equivalents.
- Has very high affinity for O_2.
- Only cytochrome with 2 subunits i.e. a & a3.
- Each subunit contains heme with iron & Cu.
- Only cytochrome containing Cu in addition to iron

Oxidative Phosphorylation

Oxidation (i.e. movement of reducing equivalents or electrons through respiratory chain) is coupled with phosphorylation of ADP to form ATP.

How the 2 processes are coupled is explained by chemiosmotic theory proposed by Peter Mitchell in 1961.

Passage of reducing equivalents (electrons) across respiratory chain leads to translocation of H^+ (protons) from inside (matrix) to outside (inter membranous space). This generates
- Chemical gradient
- Osmotic force

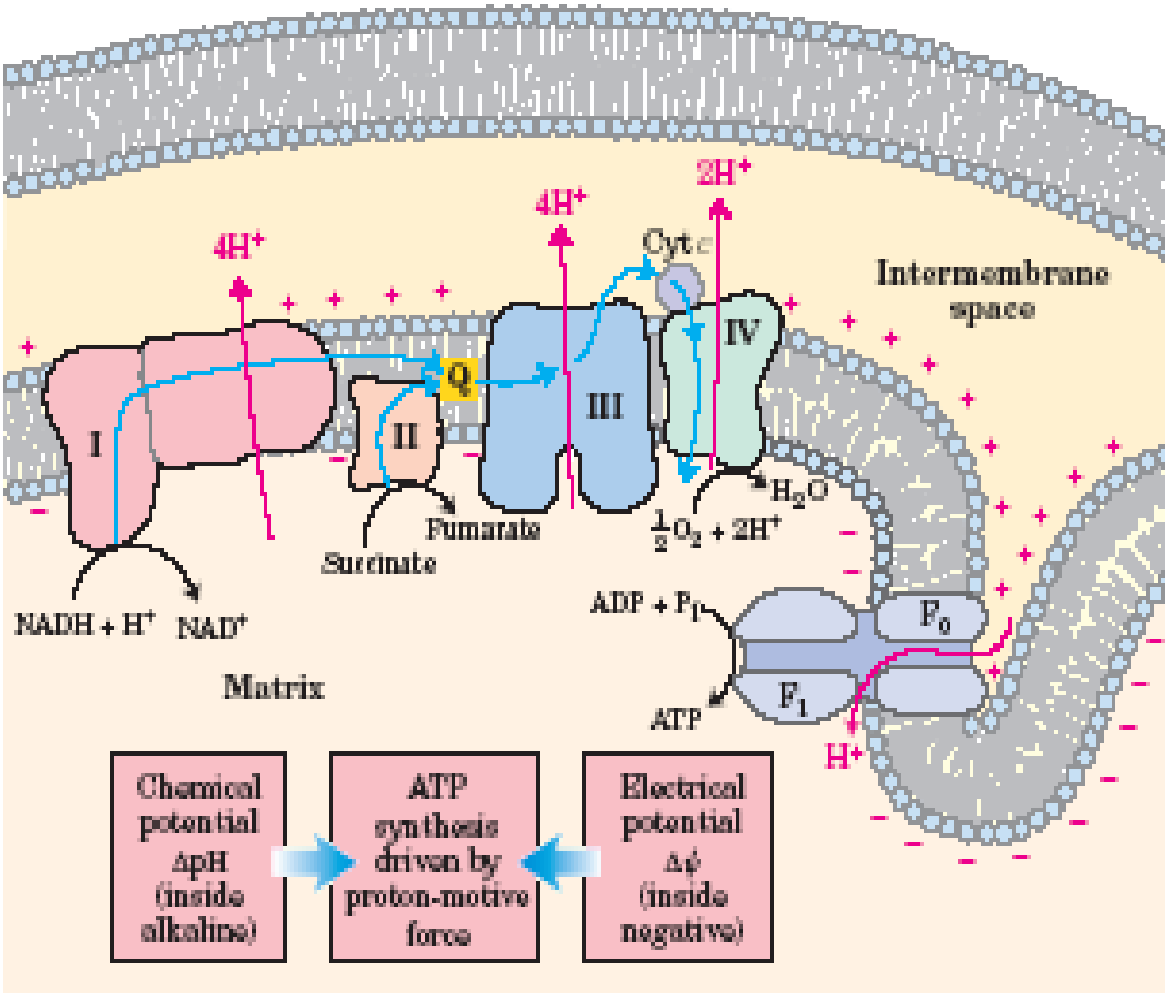
Inner mitochondrial membrane is impermeable to H^+, which comes inside through ATP synthase complex having two subunits:

F_0 subunit
- Trans-membranous
- Forms a passage
- Connected to F_1 subunit through stalk

F_1 subunit
- Extends into matrix.
- Has ATP synthase enzyme activity

$$ADP + Pi \rightarrow ATP$$

P/O ratio – No. of inorganic phosphates incorporated into ATP for every atom (1/2 mole) if O_2 consumed.
4 H^+ are equivalent to one ATP. Only complex I (4 H^+**)**, III (4 H^+**)** & IV (2 H^+**)** act as proton pump (Not complex II).
1 mole NADH produces 2.5 moles ATP / ½ mole O_2 consumed (P/ **O** ratio = 2.5)
1 mole $FADH_2$ produces 1.5 moles ATP / ½ moles O_2 consumed (P/ **O** ratio = 1.5)



In skeletal muscle, cytoplasmic NADH are transported to mitochondria as $FADH_2$ through glycerol-3-PO_4 shuttle. So only 1.5 ATPs produced
In other tissues, it is by malate shuttle – generates 2.5 ATP

Inhibitors of respiratory chain

There are 2 types of inhibitors of respiratory chain

I. Inhibitors of respiratory chain proper
   1. complex I (inhibit electron transfer from FeS to Q): Piericidin, Amobarbital & Rotenone
   2. Complex II (inhibit electron transfer from FeS to Q): Carboxin, TTFA
      Malonate is inhibitor of succinate dehydrogenase
   3. Complex III (inhibit electron transfer from cytochrome b to c):BAL & antimycin
   4. cytochrome oxidase: H_2S, CO and Cyanide

II. Inhibitor of oxidative phosphorylation: oligomycin (Block Fo)

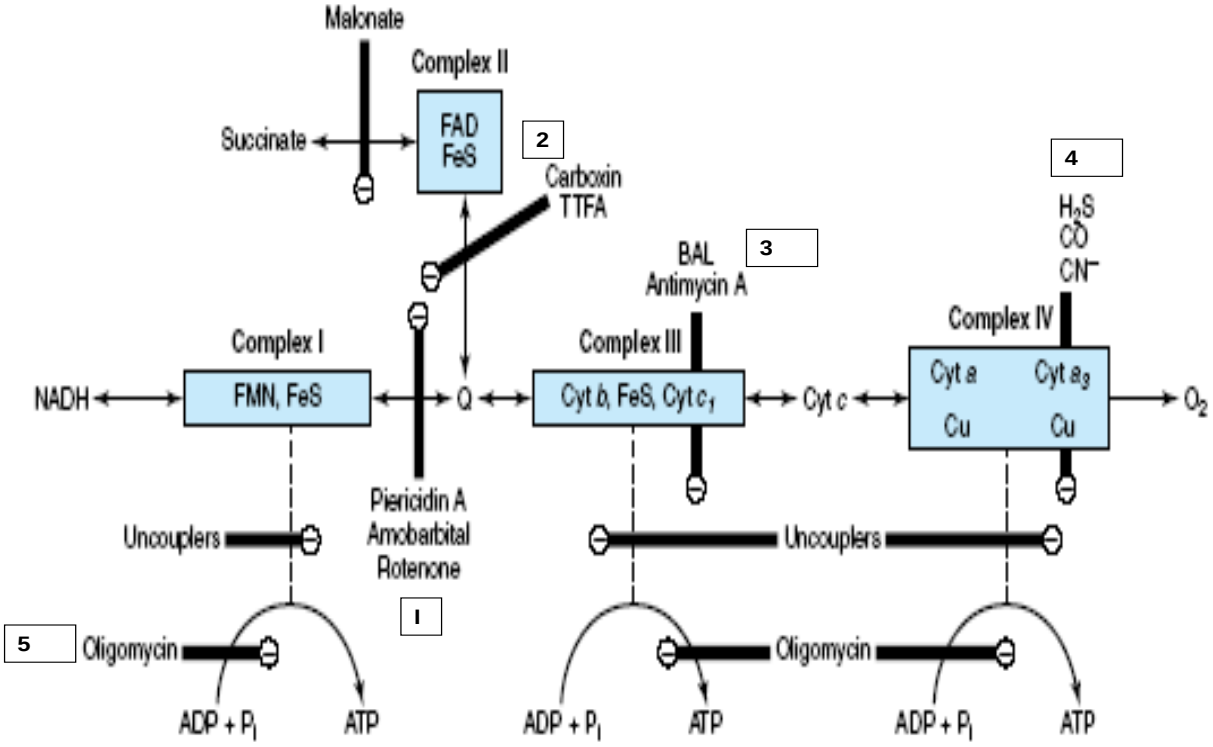
III. Adenine nucleotide transport inhibitor: atractyloside

IV. Uncouplers:
   - Amphipathic molecules.
   - Form channels
   - Allow H^+ to pass through
   - Oxidation takes place without ATP production (uncoupled)
   - Heat is produced instead of ATP

Examples:

- 2, 4 dinitrophenol
- Dinitrocresol
- Pentachlorophenol
- CCCP (chloro carbonyl cyanide phenylhydrazone)

Thermogenin – physiological Uncoupler present in brown adipose tissue and functions to generate heat in newborns

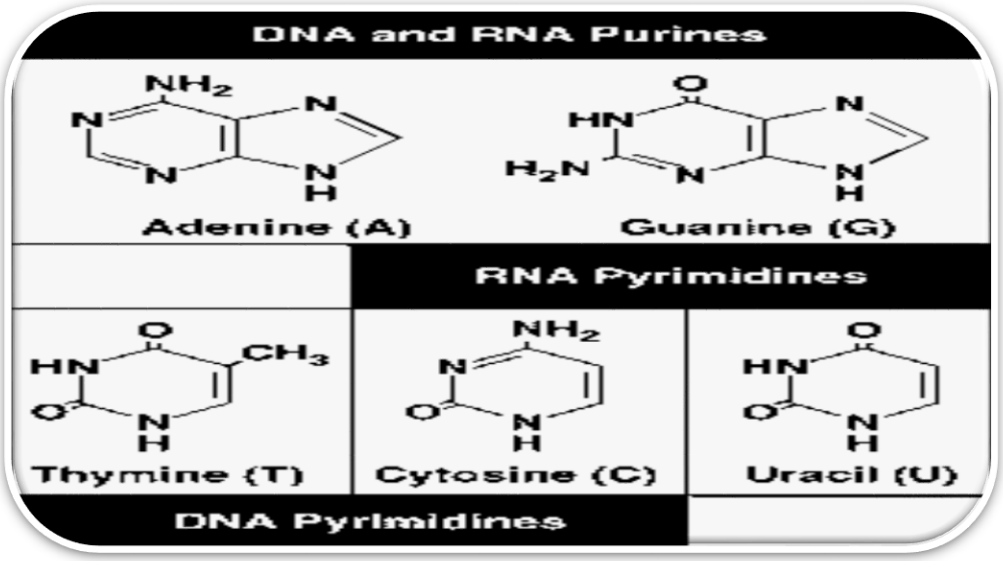


Chapter 12
NUCLEIC ACID – CHEMISTRY AND METABOLISM

Nucleic acids contain apart from A, T, G, C and U Certain unusual or minor bases like 5 Methyl cytosine, N^6 methyladenine, N^6N^6 dimethyl adenine
1, 3-dimethyl derivative of xanthine is called Theophylline present in tea) and 1,3,7 trimethyl derivative of xanthine is Caffeine)
The nitrogen atom of purine or pyrimidine to which the sugar is attached to form a nucleoside are N_9 and N_1 respectively. The carbon atom of sugar is always C_1.
In a Nucleotide the phosphate group is generally attached to carbon at 3' or 5' of the sugar
Nucleotide or NA (DNA or RNA) at physiological pH has Negative charge (due to phosphate group)

Nitrogenous bases

Purine Bases
Adenine- 6 amino purine
Guanine – 2 amino 6 oxo purine
Hypoxanthine – 6 Oxo purine
Xanthine -2,6 dioxo purine
Uric acid – 2,6,8 tri oxo purine
Pyrimidine Bases
Uracil - 2,4 Dioxo Pyrimidine :
Cytosine – 2 Oxo 4 amino Pyrimidine
Thymine – 5 Methyl Uracil



Nitrogen base	Its corresponding nucleoside	Corresponding Nucleotide
Purines		
Adenine	Adenosine	Adenosine monophosphate or adenylate
Guanine	Guanosine	Guanosine mono phosphate or guanylate
Hypoxanthine	Inosine	Inosine mono phosphate IMP
Pyrimidines		
Cytosine	Cytidine	Cytidine monophosphate or cytidylate
Thymine	Thymidine	Thymidine monophosphate or thymidylate
Uracil	Uridine	Uridine monophosphate or uridylate

Functions of Nucleotides

I. Adenosine Derivatives

1. ATP: - Energy currency of the cell
2. Cyclic AMP (Adenosine 3′ 5′ mono phosphate)
    a. Formed by the action of adenylate cyclase
    b. 2nd messenger to many hormones
        i. Adr & Noradrenalin (α2 & β receptors)
        ii. CRH, ADH
        iii. ACTH, FSH, LH, TSH
        iv. Calcitonin, PTH
        v. Glucagon
        vi. hCG, Lipotrophin H (LPH), MSH, Angiotensin II, Somatostatin.
    c. Acts through cAMP dependent protein kinases.(Protein kinase A)
    d. Hydrolyzed to 5′ AMP by phosphodiesterase.(PDE)
3. Adenosine 3′ phosphate 5′ phosphosulfate (PAPS) or active sulphate.
    a. Sulfate donor for sulphated proteoglycans.
    b. Sulfate donor for conjugation of drugs.
4. S- Adenosyl Methionine (Active Methionine):
    a. Donor of methyl groups in methylation reactions.
    b. Source of propylamine in synthesis of polyamines.
5. Coenzyme A - Carrier of Acyl radicals e.g. Acyl CoA, Acetyl CoA, Propionyl CoA etc.
6. NAD/ NADP: coenzymes in redox reactions
7. FAD (FMN is a nucleotide but does not contain adenine): both are coenzymes in redox reactions
8. Amino Acid Adenylate: COOH group of Amino Acid is attached to Adenosine of CCA of tRNA

II. Guanosine Derivative

1. GTP:
    a. Provides energy in various reactions.
    b. Energy source for protein synthesis
    c. Required for activation of adenylate cyclase through G proteins
    d. Allosteric regulator
2. cGMP (Guanosine 3′ 5′ Mono phosphate):
    a. Formed by the action of Guanylate cyclase.
    b. 2nd messenger to
        i. ANF (Atrial Natriuretic Factor)
    c. NO (Nitric Oxide)
    d. Acts via cGMP dependent protein kinase
    e. Hydrolyzed by phosphodiesterase to 5′ GMP
3. GDP Mannose: for glycoprotein synthesis
4. GDP Fucose: for glycoprotein synthesis

III. **Uridine Derivatives**

1. UDP- glucose
    a. Glycogen synthesis
    b. Galactose metabolism
    c. Lactose synthesis
    d. Glycoprotein, Glycolipid & proteoglycan synthesis
2. UDP- Galactose
    a. Galactose metabolism
    b. Lactose synthesis
    c. Glycoprotein, Glycolipid & proteoglycans synthesis.
3. UDP- glucuronic acid
    a. Proteoglycan synthesis
    b. Billirubin conjugation

c. Conjugation of steroids & xenobiotics

4. UDP – Xylose for Glycoprotein synthesis
5. UDP – N acetyl Glucosamine for Glycoprotein synthesis
6. UDP – N acetyl Galactosamine for Glycoprotein synthesis

IV. **Cytidine derivatives**

1. CMP – neuraminic acid for glycoprotein synthesis
2. CDP-diacylglycerol: in synthesis of cardiolipin & phosphatidyl inositol.
3. CDP-choline: in synthesis of PAF, Lecithin, Phosphatidyl ethanolamine, Phosphatidyl serine.
4. CDP-ethanolamine- in synthesis of plasmalogens.

Coenzymes as Nucleotide derivatives

1. S- Adenosyl Methionine (Active methionine).
2. Adenosine 3′phosphate 5′phosphosulphate (3′–phosphoadenosyl – 5′–phosphosulfate) (PAPS).
3. NAD^+ & $NADP^+$
4. FAD & FMN
5. Coenzyme A (CoASH)

"Central dogma" of genetics

- Genetic information in a DNA is copied and transferred to daughter cells (replication)
- The informations are selectively transferred to mRNA (Transcription)
- Protein is synthesised as per the information provided by the mRNA (Translation)

DNA in different organism

Eukaryotic DNA (e.g. Human)	Prokaryotic DNA (e.g. Bacterial)
Chromosomal DNA: - 23 pairs of long stretches of DNA - Present in nucleus - Stabilized by histones	Chromosomal DNA - Single circular DNA - Nucleoid - Stabilized by Histone like proteins & RNA
Extra chromosomal DNA: - Human mitochondrial (similar to prokaryotic DNA) - plants Chloroplasts	Extra chromosomal DNA: Plasmids; - Circular DNA different from bacterial chromosomal DNA - Found in gm – ve bacteria - Replicated independently - Carries genes for antibiotic resistance & conjugation

STRUCTURE OF DNA

DNA is a polymer of deoxyribonucleoside monophosphates covalently linked by 3'→5' phosphodiester bonds. Most are double stranded (ds): some viruses are (ss)

Two strands wind around each other, forming a double helix. Phosphodiester bonds join the 3' OH group of the deoxypentose of one nucleotide to the 5' OH of the deoxypentose of an adjacent nucleotide through a phosphate group. The resulting long chain has a polarity with a 5' end (the end with free phosphate) and a 3' end (the end with free OH group)

Watson and Crick Model

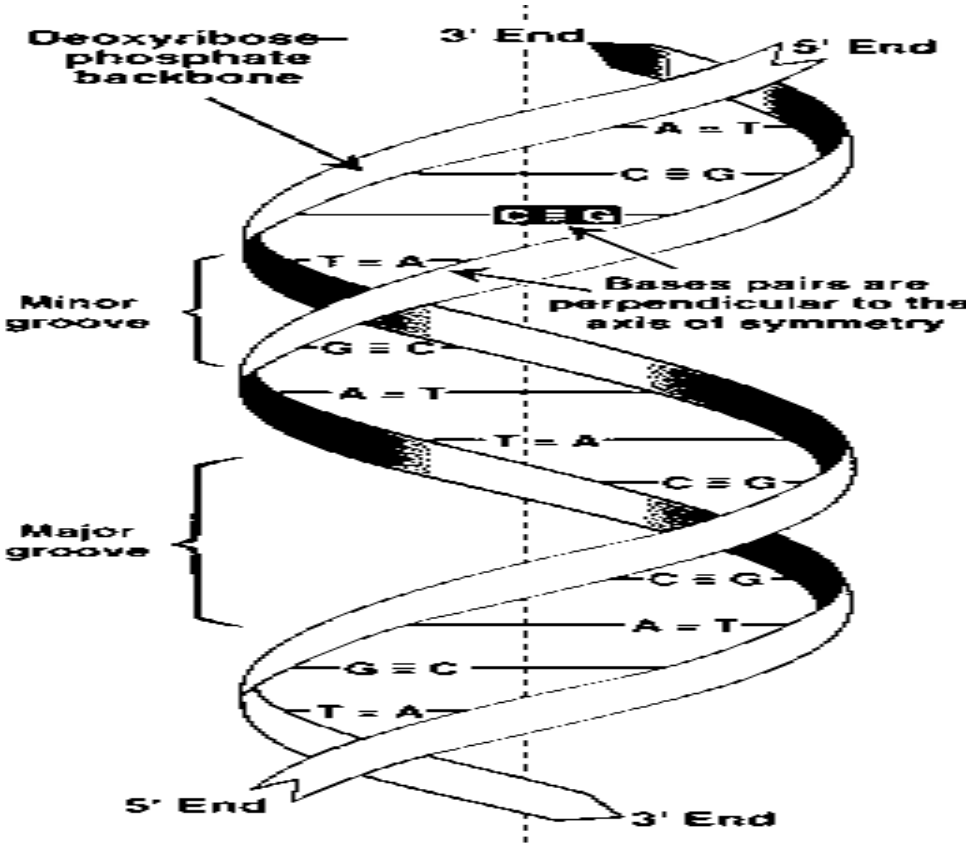
Deduced the double helical structure of DNA. The two chains are coiled around a common axis called the axis of symmetry The chains are paired in an antiparallel manner. ie 5' end of one strand is paired with 3' end of other strand

Deoxyribose-phosphate forms the backbone of each chain: it is hydrophilic. The purine and pyrimidine bases are hydrophobic and stacked inside, perpendicular to the axis of the helix. Overall structure resembles a twisted ladder. The spatial relationship between the 2 strands in the helix creates a major groove and a minor groove. These grooves provide access for the binding of regulatory proteins

Base pairing : Adenine pairs with Thymine; Guanine pairs with Cytosine. The bases obey Chargaff's rule (Base pairing rule) :- In dsDNA, the amount of adenine equals the amount of Thymine: the amount of

Different forms of DNA : (A, B, C, D, E and Z)

Under physiological conditions, B form is usually seen



B DNA : Under physiological conditions, B form is usually seen

Right handed double helix : Each turn of helix contains 10 base pairs

One turn spans a distance of 3.4 nm : (Adjacent bases are separated by 0.34 nm)

The width (Helical diameter) is 2 nm

A DNA

Produced by moderately dehydrating B DNA: Right handed double helix

11 base pairs (bp) per turn: Planes of base pairs are tilted 20^0

DNA-RNA hybrids or RNA-RNA double stranded regions contain similar forms

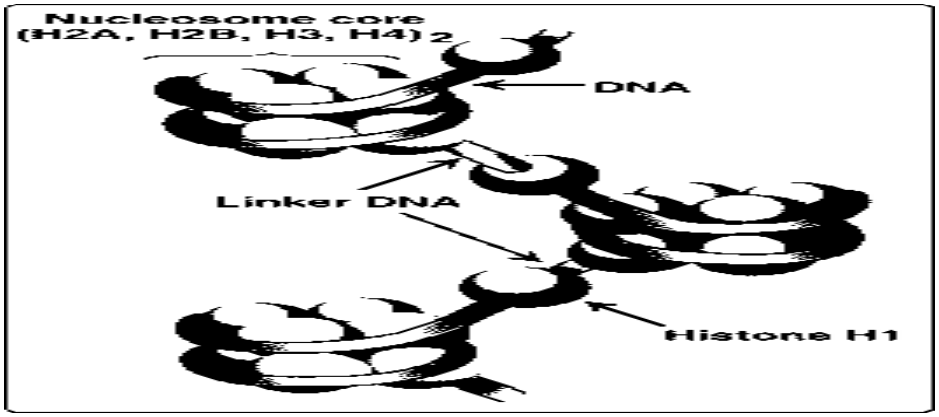
Z DNA

- Left handed double helix: dRibose-PO_4 backbone "zigzags"
- The alternating purine – pyrimidine sequence is important in Z DNA
- Longer and thinner than B DNA
- **Z DNA has 12 bp per turn (B DNA 10 bp)**
- More likely to be found in 5' end of genes
- Methylation favors Z DNA: B to Z and vice versa is possible by methylation

Circular DNA
Mitochondria of eukaryotes
Chloroplasts
Prokaryotes contain one double-stranded, supercoiled, circular chromosome
Many bacteria contain circular, extrachromosmal DNA called plasmids
Denaturation of DNA
Denaturation of DNA is called melting of DNA. The strands of a given molecule of DNA is separated .The temperature at which half of DNA strands is separated is known as melting temperature(Tm)
Tm is greater for G≡C pairs than for A=T pairs
Primary structure is intact on denaturation. Breaks the hydrogen bonds and becomes single stranded. Causes stacking of the bases and becomes a rigid rod like structure and looses viscosity. This causes an increase in optical absorbance (260 nm)which is known as hyperchromicity of denaturation
Used to analyze DNA structure. Can be done by increasing the temperature or decreasing the salt concentration. In recombinant DNA tech, Formamide is used to lower Tm, as it destabilizes H bonds

Organization of Eukaryotic DNA

DNA is associated with tightly bound basic proteins called HISTONES
DNA + Histones form nucleosomes
Histones
Histones are a small family of closely related basic proteins
Most abundant of chromatin proteins
They are positively charged at physiologcal pH due to the high content of Arginine and Lysine.
H2A and H2B are Lys rich: H3 and H4 are Arg rich.
The aminoterminal region is rich in basic amino acids
There are 5 classes of Histones - H1, H2A, H2B, H3 and H4
Because of the +ve charge, Histones form ionic bonds with –vely charged DNA. Along with Mg2+ Histones help neutralize the –vely charged DNA phosphate groups
2 molecules of H2A, H2B, H3 and H4 form an octamer. They are known as "core" histones as DNA is wound around this octamer.
A segment of DNA is wound nearly twice (1.75 turns) around a Histone octamer, forming a negatively supertwisted helix
Neighbouring nucleosomes are joined by "linker" DNA (approx 30 - 50 bp)
H1 binds to the linker DNA between the nucleosomes. H1 is the most tissue specific and species specific of the Histones It facilitates packing of nucleosomes into the more compact structures



Core histones are subject to covalent modifications
H1 is bound less tightly and can be separated by salt solution
SUMO= Small Ubiquitin-related Modifier

Modified Histones

Modification	Role

Acetylation (H3 and 4)	Regulation of transcription
Acetylation	Chromosomal assembly
Phosphorylation (H1)	Chromosomal condensation
ADP ribosylation	DNA repair
Methylation	Regulation of transcription
Mono ubiquitylation	Gene activation, repression
Sumoylation	Transcription repression

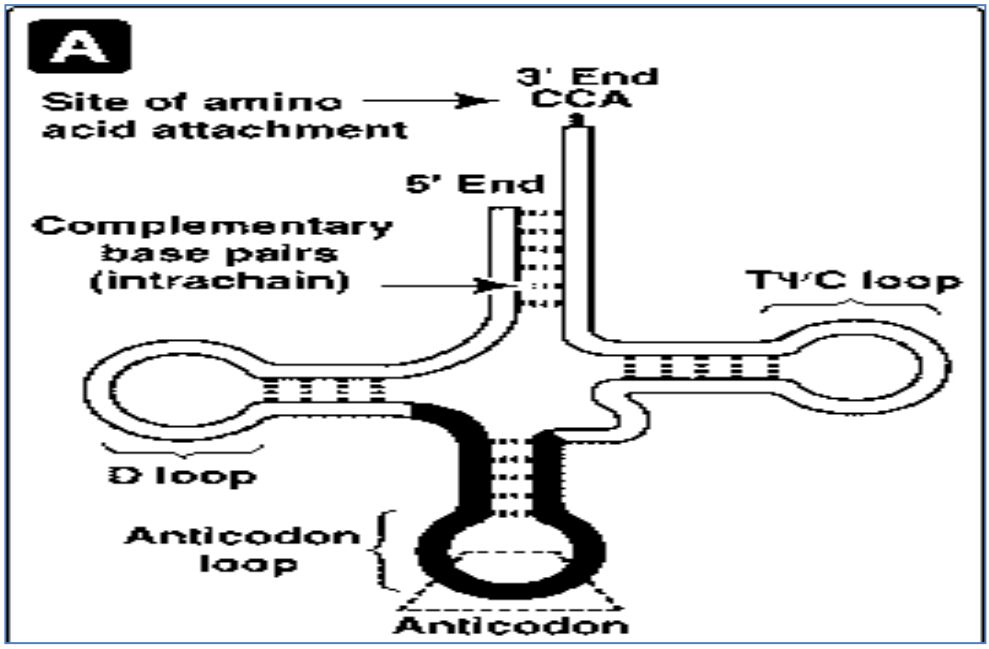
RNA

mRNA transfer genetic information from DNA to protein synthesizing machinery
tRNA – for translation of RNA information into specific amino acids
rRNA – role in protein synthesizing machinery by synthesizing ribosomes
snRNA- role in RNA processing

Transfer RNA

tRNA are the smallest (4S) of the three major species of RNA molecules:
Also known as "soluble RNA (sRNA)"
Transfer amino acids from cytoplasm to ribosomal assembly for protein synthesis
Eukaryotic RNA's

RNA	Types	Abundance	Stability
Ribosomal (rRNA)	28 S, 18 S, 5.8 S, 5 S	80% of total	High (very stable)
Messenger (mRNA)	About 10^5 different species	4-5% of total	Unstable to stable (vary)
Transfer (tRNA)	About 60 different species	15% of total	High
Small nuclear (snRNA)	About 30 different species	≤ 1% of total	High



METABOLISM OF NUCLEOTIDES:

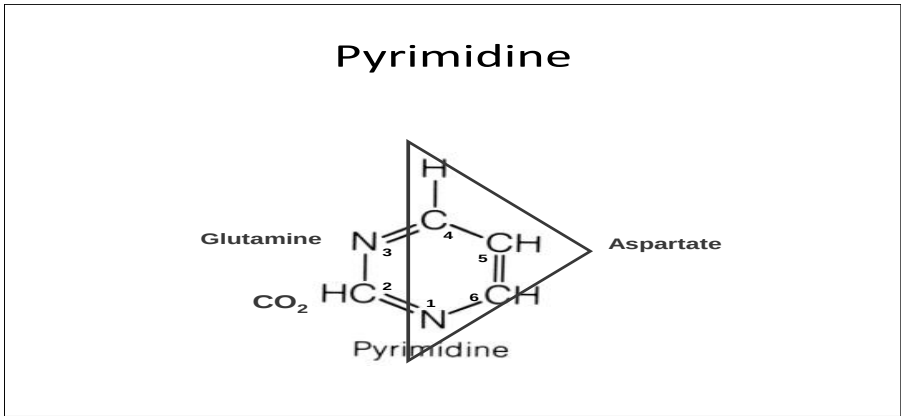
Nucleotides are synthesized by 2 pathways; Salvage and De-novo

Salvage is single step and uses preformed adenine, guanine and hypoxanthine nitrogenous base.
The enzymes are APRTase and HGPRTase
Brain and RBC are dependent on salvage pathway and do not have De-novo pathway.

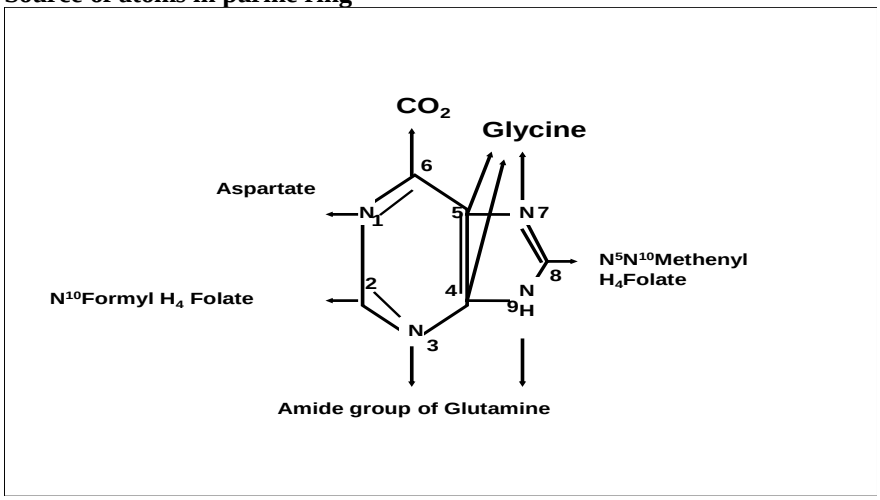
DeNovo pathway of pyrimidines and purines:

There are six enzymes involved in synthesis of Pyrimidines, activities of which are located in three gene products.
CPS II, Aspatate transcarbamoylase, Dihydro orotase : in cytoplasm
Dihydro orotate dehydrogenase : in mitochondria.
OPRTase, Orotate decarboxylase : in cytoplasm

Source of atoms in pyrimidine ring



Source of atoms in purine ring



Catabolism of purines and pyrimidines

Purine catabolism

End product of purine catabolism – Uric acid (2,6,8 trioxopurine)

Rate limiting enzyme – Xanthine oxidase

The solubility of uric acid is less. At physiological pH of 7.4 uric acid is Mono Na urate, which is present in plasma.
In urinary tract:

a. Up to DCT & CD (pH 7.4) – Mono Na urate (less risk of stones)
b. After DCT & CD (pH falls to 6.0) – Uric acid (more risk of stones)
c. Mono Na urate is much more soluble than uric acid
    i. Uric acid stones are far more common than mono sodium Urate
    ii. Uric acid stones are far more likely in distal nephron than proximal nephron.
    iii. Alkalization of urine reduces stone formation.
    iv. Lactic acidosis (alcoholism) increases Uric Acid form

Pyrimidine catabolism

Uracil and Cytosine → Beta alanine , NH3 and CO2

Thymine→ Beta amino isobutyric acid→Methyl malonate semialdehyde→ Succinate, NH3 and CO2

Note: Pyrimidine catabolism: forms highly water soluble β – Alanine & β – Amino isobutyrate. Therefore no problem like uric acid

Disorders of Pyrimidine Metabolism:

1. Orotic Aciduria
2. Reye′s Syndrome- Severely damaged mitochondria → ↑ Carbamoyl phosphate (Underutilized) → ↑Orotate production → Orotic Aciduria
3. Deficiency of Ornithine transcarbamoylase (OTC) of urea cycle also has same effects.
4. Allopurinol –Competitive inhibitor of orotidylate decrboxylase → Orotic Aciduria & Orotidinuria

Disorders of purine catabolism:

1. **ADA deficiency:**
   a. Deficiency leads to accumulation adenosine, AMP, ADP, ATP and their deoxy forms
   b. dATP inhibits Ribonucleotide Reductase
   c. This leads to deficiency of dGDP, dGTP, dCTP, TMP, TDP and TTP
   d. Deficiency of deoxy ribonucleotides affects DNA synthesis, replication & cell division.
   e. ADA deficiency leads to deficient Lymphocyte proliferation (both T cells & B cells are affected) This prevents proliferation especially of T and B lymphocytes
   f. This leads to deficient humoral as well as cellular immunity ultimately leading to severe combined immuno deficiency (SCID)
2. **Hyperuricemia**
   a. There is increased deposition of uric acid crystals & stones in urinary tract mainly as uric acid after DCT & CD.
   b. Deposition of Na urate crystals in soft tissues & joints leading to their inflammation called gout
   c. Na Urate crystals are negatively Birefringent i.e. in polarizing microscope (plane polarized light) they appear yellow when their long axis is parallel (y e **l l** o w) & blue when perpendicular to the plane of light (**D/D pseudo gout in which calcium pyrrophosphate is deposited)**
   d. Na Urate crystals are needle shaped (Rhomboid in pseudogout)

Inhbitors of purine synthesis

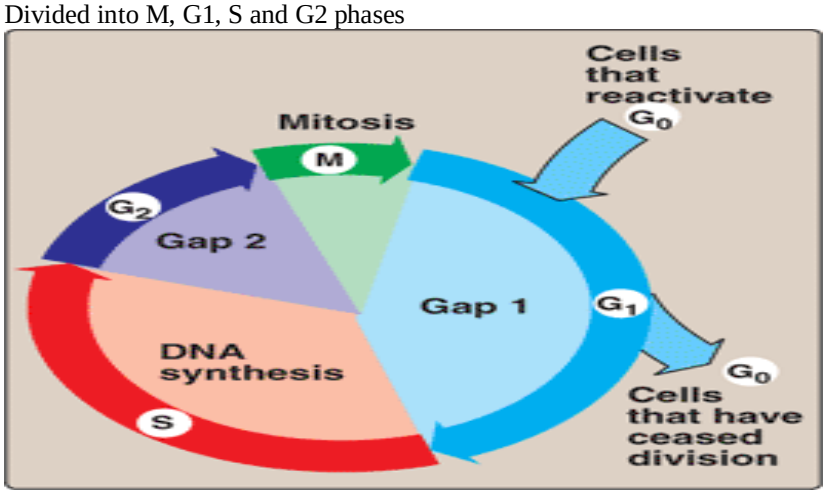
Azaserine	Formyl glycinamide R5P synthetase
Diazanorleucine	PRPP-Gln amidotransferase
6-mercaptopurine	Adenylosuccinase
	IMP DH
Mycophenolic acid	IMP DH

Azathioprine

Suppress CMI. Used to prevent graft rejection
Converted to 6-mercaptopurine in the body
Azathioprine and 6-mercaptopurine are metabolized by Xathine oxidase
Therefore increase uric acid level
Inhibited by allopurinol

CELL CYCLE

Events occurring during the period between 2 mitotic divisions
Takes about 20-22 hours in mammalian cells
Divided into M, G1, S and G2 phases



M phase (Mitosis)
Shortest phase (1 hr). Cell division takes place in M phase: After cell division, the cells can enter a Go phase-dormant: Cells can also re-enter the cycle for growth and repair
G_0 phase: Dormant phase; In a normal cell population, most of the cells are in Go phase
G1 phase (gap-1): 12 hours. Protein and RNA contents increase
S (Synthetic phase); 6-8 hrs; DNA replication occurs during this phase
G2 (Gap-2) phase: 4-5 hrs. Cytoplasmic enlargement; DNA repair; Production of Histones
G1, S and G2 phases are together called interphase (the phase between 2 mitosis)
Regulation of Cell Cycle
Cell cycle is controlled by
4 types of cyclins (A, B, D, E) and 5 cyclin dependent kinases (CDK 1,2,4,5,6)
Cyclins activate CDK; CDK phosphorylate regulatory proteins
The most important check points occur in G1 and G2 phase entry points
Growth factors trigger the cells from Go → G1
CDK2-cyclin E complex - G1→S
CDK2-cyclin A completes S phase - (S→G2)
CDK2- cyclins A&B completes G2 - (G2→M)
MPF (M phase promoting factor) Promotes mitosis
Cyclins are synthesised throughout cell cycle, and are destroyed during mitosis

Chapter 13

GENETICS

REPLICATION OF DNA

New DNA synthesis during cell division
Semiconservative : demonstrated by messelson-Stahl experiment

- The strands separate
- Each strand acts as a template for the synthesis of a new strand
- Bases in the new strand will be complementary to those in the template strand, obeying Chargaff's rule
- The synthesis of new strand is with the help of DNA polymerase, occurs in 5' → 3' direction (or: the template is read in 3'→5' direction)
- New strand is joined to the template strand by H bonds between base pairs
- Thus 2 double stranded (duplex) DNA are produced: Each goes to one daughter cell

Initiation of replication
DNA polymerase can use only ssDNA as a template: So, DNA strands should be separated first
In prokaryotes, replication begins at a single site called the ORIGIN OF REPLICATION. : AT rich sequence (called consensus sequence)
In eukaryotes, replication begins at multiple sites- AUTONOMUS REPLICATNG SEQUENCES
Formation of the replication fork
As the 2 strands separate, they form a "V" shaped area: REPLICATION FORK
It moves along the DNA molecule as synthesis occurs
Replication of dsDNA is bidirectional, ie, replication forks move in both directions from the origin
(But the synthesis in each new strand occurs in 5'→3' direction only)
Proteins required for strand separation:
ori binding proteins (dna A proteins in prokaryotes). It recognizes the origin site, rich in AT base pairs and separate the strands forming local single stranded DNA. Progressive unwinding is brought by DNA helicases (known as dnaB protein in prokaryotes). SSB proteins stabalise the complex by keeping two starand separated. SSB (single strand binding proteins) prevent premature reannealing
Thus dnaA protein, DNA helicases cause strand separation: Both utilize ATP.
Positive supercoiling during replication
When 2 strands are separated, positive supercoils ("Supertwists") appear in the region of DNA ahead of the replication fork. These interfere with further unwinding of DNA: solved by enzymes called topoisomerases
Topoisomerases : Produce nicks in DNA and reseal it
There are 2 types of topoisomerases

Topoisomerase I	Topoisomerase II
Cuts single strand	Cuts both strands
Does not insert super coiling	Inserts super coiling
ATP not required	ATP is required

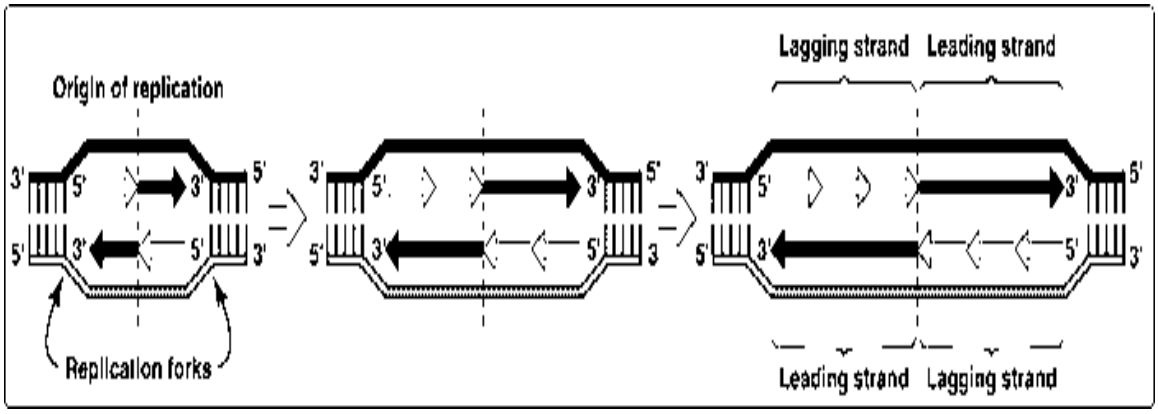
Note: DNA Gyrase is a type of Topoisomerase II in E. coli which produces negative super coils using ATP before replication

RNA primer: First an RNA primer is synthesized by RNA primase (dna G in EColi is primase). Once the RNA primer is formed, DNA polymerase starts adding nuleotides to the 3' end of primer. The added nucleotides will have bases complementary to the DNA strand

Synthesis can occur continuously in 5'→3 direction: (Leading strand). The other strand is synthesized as small fragments (okazaki fragments: (Lagging strand)

Primosome: Complex of dnaA, SSB proteins, Helicase (dnaB) & Primase (dnaG), formed in the lagging strand. It moves backwards i.e. 3′ to 5′ (opposite to the direction of synthesis). Forms RNA primers at several steps in the lagging strand

Primosome is followed by DNA Polymerase III which synthesizes both leading and lagging strands.



After replication, DNAP III proof reads it
RNA primers are removed by DNAP I, gaps filled by DNAP I, and joined by DNA ligases
Telomere: 5' end of the new strand is not synthesized by this ssDNA area is called telomere: Corrected by telomerase which acts like reverse transcriptase and adds $(TTAGGG)_n$ to telomere.
This prevents shortening of chromosome with each cell division

Inhibitors of DNA replication

Affects Bacterial DNA Gyrase - (antibacterials):	Ciprofloxacin, Norfloxacin, Naidixic acid, Novobiocin
Affects Human topoisomerase – (Anticancer):	Etoposide, Doxorubicin, Daunorubicin

Prokaryotic DNA polymerase

Pol I – removal of RNA primers and Gap filling (also called Kornberg's enzyme)
Pol II – DNA proof reading and repair
Pol III – Synthesis of leading strand and okazaki fragments, Proof reading

Eukaryotic DNAP

Alpha- RNA primase activity (+) Initiates DNA synthesis, Gap filling
Beta- Repair
Gamma- Mitochondrila DNA synthesis
Delta- Synthesis of leading strand and Okazaki fragments
Epsilon- Repair

End replication problem in eukaryotes:

Since the eukaryotic DNA is in the form of long segments, a small portion at the end of lagging strand after removal of RNA primer remains unreplicated due to lack of free 3′ OH group to initiate replication. Primer gaps are formed at the ends of both new DNA strands. This phenomenon occurs at the ends of chromosomes i.e. telomeres.

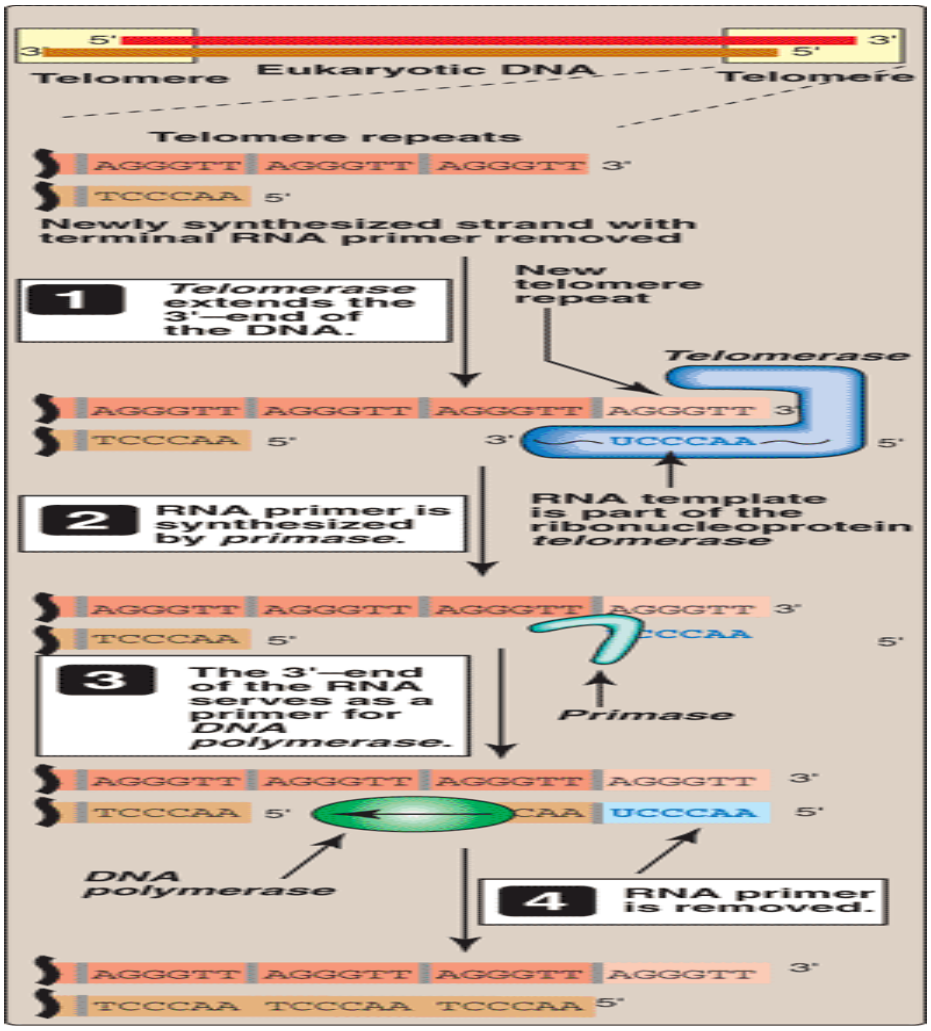
Telomeres:

- Several thousand tandem repeats of a 5–8 bp sequence e.g. AGGGTTAGGGTTAGGGTT
- Few hundred nucleotide long segment of ssDNA folds back on itself and stabilized by proteins

- In a normal cell, telomeres get shortened in each replication cycle. This is inevitable.

After the telomeres get shortened by a critical length, the cell stops dividing and enters G_O phase (senescence).

However germ cells, cancer cells & stem cells have telomerase activity which prevents telomere shortening



A specific enzyme Telomerase fills these gaps

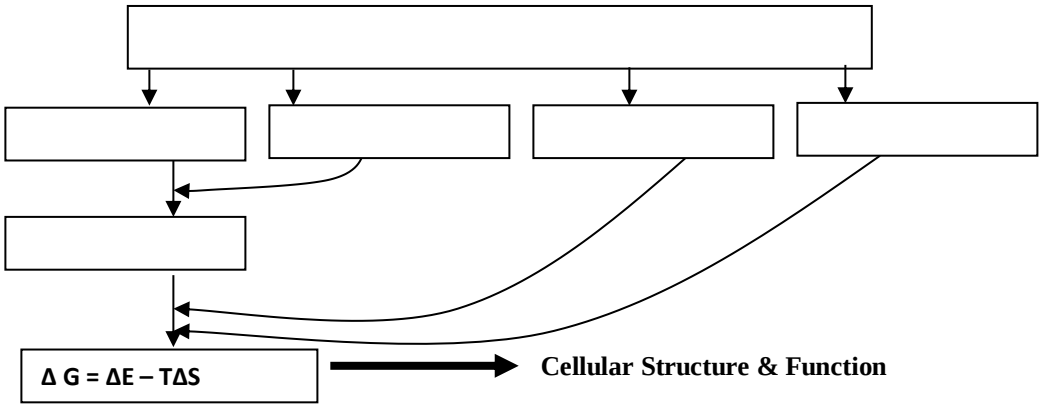
Telomerase:

- A Ribonucleoprotein
- Has reverse transcriptase activity (RNA dependent DNA polymerase)
- 9 – 30 nucleotide long region of RNA with

  5′ – AACCCUAACCCU – 3′ sequence
- It adds 3′ – TTGGGATTGGGA – 5′ sequence to 3′ end of parent strand
- Now fresh DNA can be synthesized on this template

TRANSCRIPTION

Synthesis of RNA from DNA template is called transcription.



Main feature of Transcription is that it is highly selective i.e. RNA polymerase recognizes the signals on DNA where to start and where to end the transcription.
Main enzyme for transcription is RNA polymerase.
Bacterial RNA polymerase is a multi-subunit enzyme.

Prokaryotic RNA polymerase

- In prokaryotes all types of RNA except RNA primers for replication are synthesized by the same type of RNA polymerase: Multisubunit enzyme: 2 identical α subunits, similar but non-identical β and β' subunits and 1 ω subunit constitute the core complex (E)
- The core enzyme (E) associates with a protein factor called sigma (σ) factor to form the holoenzyme (E σ): σ factor helps RNAP to recognize the "promoter" on the DNA
- 2α, 1β, and 1β', are responsible for the 5′→3′ RNA polymerase activity
- Function of ω subunit is not clear
- Inhibited by the Rifampicin, which binds to the β subunit

Eukaryotic RNA Polymerase

3 different types of RNAP: They have 2 large subunits and many small subunits- the number of this varies in different types

Type	Inhibition by amanitin	Products
I	Insensitive	5.8, 18, 28SrRNA
II	Highly sensitive	mRNA, miRNA, snRNA
III	Intermediate sensitivity	tRNA, 5S rRNA

Mitochondrial RNA polymerase is similar to prokaryotic RNA polymerase.
Differences between DNA polymerase & RNA polymerase:

1. RNA Polymerase doesn′t require primer.
2. RNA polymerase does not have proof reading ability.
3. RNA Polymerase does not require Helicase. It has its own helices activity.

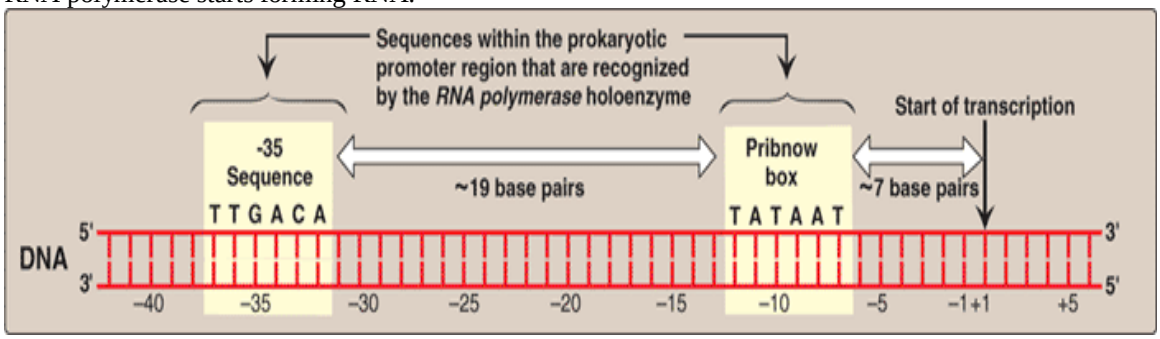
Similarities between DNA & RNA polymerases:

1. Both are 5′ → 3′ polymerases
2. Both require Nucleoside Tri phosphates (NTPs) as precursors

Transcription in prokaryotes

I. Initiation

σ Protein recognizes & binds to promoter region in prokaryotic DNA. After initiation, the factor dissociates and RNA polymerase starts forming RNA.

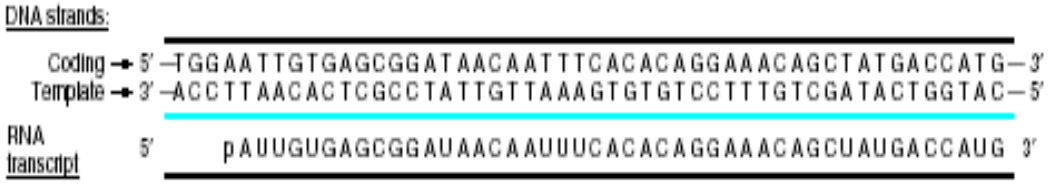


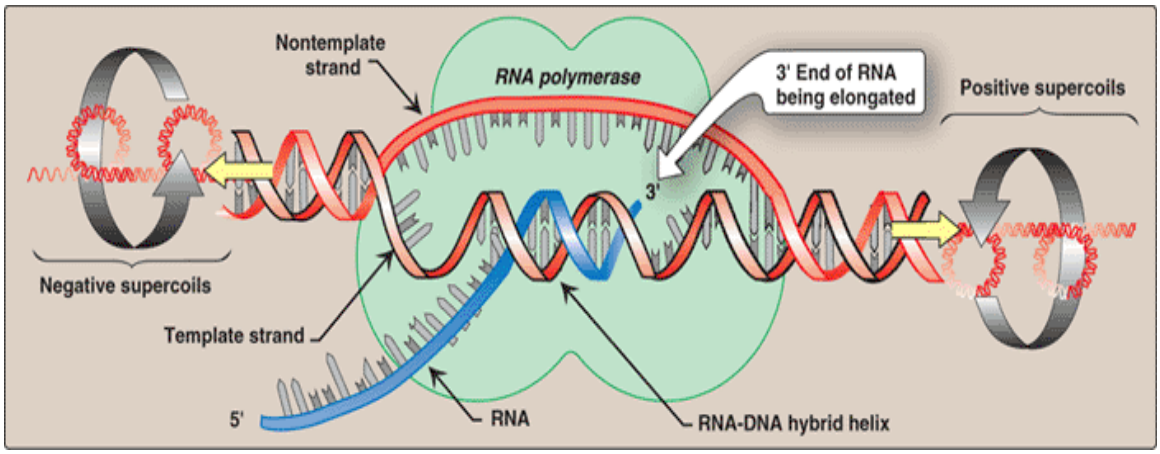
II. Elongation-

Core RNA polymerase synthesizes RNA similar to DNA polymerase. It has its own helicase activity and super coils are taken care of by Topoisomerases.

Main features of Elongation:

- RNA synthesis always takes place from 5′→ 3′ direction.
- Ribonucleoside tri phosphates are used to form phosphodiester linkage.
- PPi released is broken to 2Pi pulling the reaction to completion.
- Sequence of RNA transcript is same as coding strand **(sense strand)** except U in place of A.
- The strand complimentary to coding strand **(anti-coding or anti-sense strand)** serves as a template.
- Transcription is a continuous process.
- No Endo or Exonuclease activity.
- No proof reading.
- No repair of mistakes.





III. Termination

There are two mechanisms for termination of transcription.

1) **ρ Dependent termination**

- ρ factor binds C rich ρ recognition site near 3′ end of RNA transcript
- Gradually it moves to RNA polymerase paused at the termination site
- ρ factor has RNA dependent ATPase activity
- It also has intrinsic ATP dependent RNA DNA helicase activity which helps release the primary RNA transcript.

2) **ρ independent termination**

- Formation of a stable hairpin of RNA transcript due to DNA Palindromes.
- Formation of poly Us after hairpin.
- A–U bonding is weak, which helps RNA to detach from DNA.

Note: Palindromes –A sequence of double stranded DNA, where two strands have the same sequence from 5′→ 3′.

Transcription in Eukaryotes

Formation of pre-initiation complex (PIC): at least 50 different factors are involved in formation of PIC ensuring the fidelity of transcription

Elongation: same as prokaryotes

III. Termination: poorly understood

Inhibitors of RNA synthesis

Actinomycin D → Inserts phenoxazone ring between 2 G-C bp of DNA
Rifampicin → Binds to β subunit of RNA polymerase and inactivates it
Alpha amanitin → Inactivates RNA polymerase II
3'-deoxy adenosine→ incorrect entry into chain causing chain termination

Usually transcription is by DNA dependent RNA polymerase
Reverse transcriptase is RNA dependent DNA polymerase
Present in some RNA viruses-retroviruses
Produce a dsDNA copy of their RNA genome

PROMOTERS OF TRANSCRIPTION

- Sequences in DNA which directs RNAP to start transcription: they are on coding strand

- Eukaryotes:
  - 25 bp upstream – HG box (TATAAA)
  - 70bp upstream - CAAT box GGCCAATCT
- Prokaryotes :
  - 10 bp upstream - Pribnow box/ TATA box (TATAAT)

35 bp upstream -5'TGTTGACA3
Sigma factor recognizes promoter site
Rho factor is involved in termination of transcription

Post-transcriptional processing:

mRNA is transcribed as a primary transcript, which is long and contains exons & introns. Undergoes processing to get mRNA. Includes endonuclease cleavage, Poly-A tailing, 5' capping, Methylation, Removal of introns, Splicing of exons.

5' capping: 7 methyl GTP cap. Helps in recognition of mRNA by translation machinery
Prevents attack by 5' exonuclease
Poly A tail: 20-250 adenylate residues. Prevents attack by 3' exonuclease

A few mRNAs esp those for some histones do not have poly A tail

Areas that are transcriptionally active are less densly stained and are called euchromatin.
Areas that are transcriptionally inactive are densly packed at interphase and appear as densly stained areas under electron microscope- called heterochromatin.
Some areas are always condensed and transcriptionally inactive- Constitutive heterochromatin
Certain areas of heterochromatin decondenses at times and become transcriptionally active Facultative heterochromatin

Features of genetic code

Triplet codons : Each codon consist of 3 bases
Non-overlapping; Non punctuated
Degeneracy- One amino acid has more than one codon
Unambiguous – one codon stands only for 1 amino acid
Universal ("from E.Coli→Elephant): In all organisms, the one codon codes for the same amino acid
Wobbling- reduced stringency between 3rd base of codon and complementary base in anticodon
Initiator-AUG
Terminator- UGA,UAG, UAA
Tota 64 codons. 3 are stop codons. Remaining 61 code for 20 amino acid. Some amino acids have more than one codon
Single Codon: AUG - Met, UGG - Trp
2 Codons: Asp, Glu, Asn, Gln, Phe, Tyr, Lys, Cys, His.
3 Codons: Ile.
4 Codons: Gly, Ala, Val, Pro, Thr.
6 Codons: Ser, Leu, Arg.
Stop Codons -3

Mitochondrial DNA (mtDNA)- Closed circular dsDNA

Majority of proteins in mitochondria are coded by nuclear genes. Others are by mitochondrial DNA
Encodes 13 protein subunits of respiratory chain

- 7 subunits of NADH dehydrogenase
- Cyt b
- 3 subunits of cyt oxidase
- 2 subunits of ATP synthase

Genetic code differs slightly from that of mtDNA

- UGA → Trp
- AGA and AGG → stop codons

Special features of mitochondrial DNA

Mitochondrial DNA (mtDNA) is somewhat similar to prokaryotic DNA:-

i) It is a circular double stranded helically coiled DNA.
ii) There are 2 – 10 copies of circular DNA per mitochondrion. Certain cells in embryo which are undergoing differentiation may contain up to 100 copies of circular duplex DNA
iii) In embryo all the mitochondria are derived from ovum and hence in an individual mDNA is maternally acquired
iv) mDNA codes for mitochondrial tRNAs, rRNAs and a few mitochondrial proteins. More than 95% mitochondrial proteins are encoded by nuclear DNA
v) Mitochondria also divide along with cell division. The mDNA is replicated before and during cell division
vi) mtDNA genes have a much higher mutation rate than nuclear DNA genes.
vii) Mitochondria undergo replicative segregation at cell division.
viii) Somatic mDNA mutations accumulate in post-mitotic tissues with age, reducing the ATP generating capacity.

SnRNAs: (Snurps or (short nuclear ribonucleoproteins):

Small nuclear RNAs. **SnRNAs** combine with proteins to form small nuclear Ribonucleoproteins **(snurps)**

Involved in splicing of exons
Size: 90 -300 nucleotides.About 1 million copies of Sn RNAs/cell.
6 different SnRNAs- uracil rich: U1,U2, U4, U5,U6 and U7 .

U1- Cuts the 5'end: U5 - attacks the 3' end of the intron
U2 – is the main splicing enzyme.
U7- Specific for Histones
All of them are located in nucleus.

TRANSLATION

It has following steps:

- Activation of amino acids
- Initiation
- Elongation
- Termination
- For many proteins, this is followed by post translational modifications

Activation of amino acid

- With the help of aminoacyl tRNA synthetases
- Amino acid + tRNA + ATP →Aminoacyl tRNA + AMP
- DHU arm of tRNA is important in the recognition of tRNA by this enzyme

Initiation of translation

- A mRNA is selected by ribosome for translation
- This involves the action of many eukaryotic initiation factors (eIF)
- Can be divided into 4 steps
  - Ribosomal dissociation
  - Formation of Pre-initiation complex
  - Formation of initiation complex
  - Ribosoml assembly

Ribosomal dissociation

- 80 s ribosome splits into 40s and 60s subunits
- eIF-3 and eIF-1A bind to the 40s subunit
- This delays its reassociation with 60s subunits
- It allows other initiation factors to associate with 40s

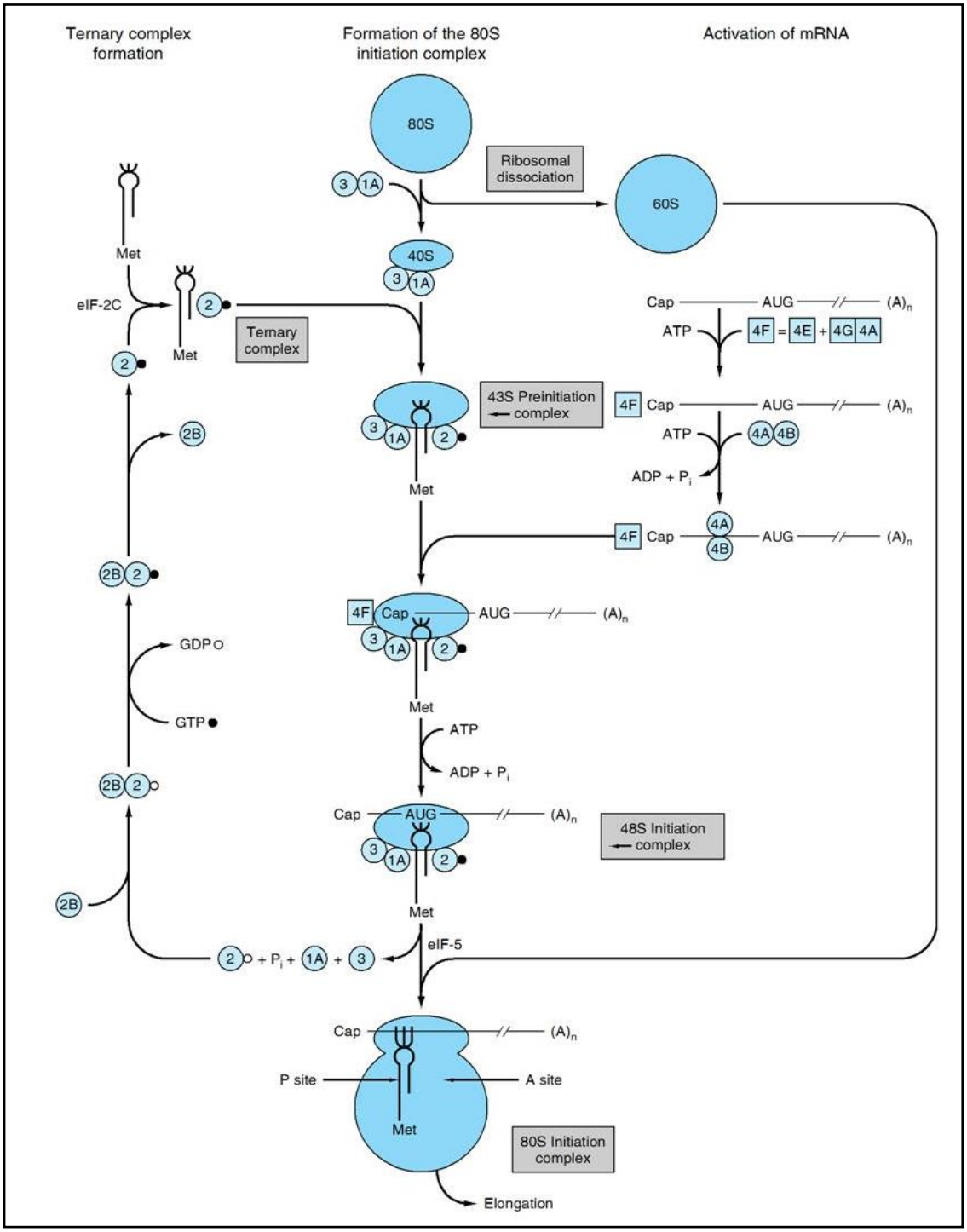
Formation of 43s pre-initiation complex

- GTP binds with eIF-2
- This binds to Met-tRNA
- This binds to 40s to form 43s pre initiation complex
- eIF-3 and 1A stabilizes it

Formation of 48s initiation complex

- mRNA cap facilitates binding of mRNA to 43s pre initiation complex
- A protein complex called eIF-4F binds to the cap. eIF-4A and 4B reduces the complex secondary structure of 5' end of mRNA through ATP dependent helicase activities
- Now this complex scans mRNA for a suitable initiator codon
- First AUG after the marker sequence is taken as initiator codon
- In prokaryotes, the marker sequence is a purine rich sequence (eg:- UAAGGAGG) known as Shine-Dalgarno sequence, located 6-10 bases upstream of the initiator codon. It helps in binding of 30 S ribosomal subunit to mRNA

In eukaryotes, the marker is known as "Kozak" sequence



Formation of 80s initiation complex (Ribosomal assembly)

- 60 s ribosomal subunit binds to 48s initiation complex, using GTP
- Initiation factors bound to 48s are released
- 40s and 60s assembles to form 80s ribosomal assembly
- The initiating AUG is recognized by a Met-tRNA. This is facilitated by eIF2 and GTP
- (in prokaryotes and in mitochondria this tRNA carries N-formylated Met)
- The charged Met-tRNA enters the P site of ribosomal assembly
- **This Met or fMet is cleaved even before completion of termination**

Elongation

- Involves
  - Binding of new aminoacyl tRNA
  - Peptide bond formation
  - Translocation

Binding of new amino acyl – tRNA to 'A' site

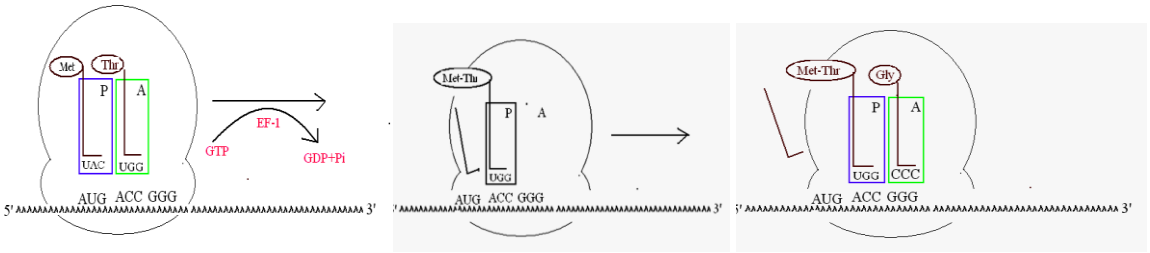
- The codon after the initiator codon decides which aminoacyl-tRNA is to be added next.
- The new aminoacyl-tRNA comes to the 'A' site: This requires elongation factor EF-1 and GTP
- GTP hydrolysed; EF-1 released

Peptide bond formation

- The amino group of the new aminoacid forms a peptide bond with the COOH of the previous amino acid, catalysed by "peptidyl transferase" (a ribozyme)
- As the amino acid is already activated this does not require energy
- Now the previous tRNA, which is on the 'P' site does not carry amino acid

Translocation

- Ribosome moves in 5' → 3' direction of the mRNA, to te next codon
- Now the empty tRNA reaches the 'E' site and is expelled from ribosome
- tRNA which carries the growing peptide reaches the 'P' site
- New incoming aminoacyl tRNA as decided by the next codon comes to the 'A' site
- This requires EF-2 and GTP



Energy requirements

- For each peptide bond formed, a total of 4 high energy bonds are used
- 2 for activation of amino acid + 1 for EF-1 step (binding of tRNA to A site) + 1 for EF-2 step (translocation)
- Step of formation of peptide bond does not require energy
- In addition, Energy is required for initiation complex formation and termination

TERMINATION

- When the A site of ribosomal assembly reaches a terminator codon sequence in mRNA, translation stops. There is no tRNA with anticodon for these
- eRF recognises terminator codon (in prokaruotes, it is by RF-1 and RF-2)
- It induces peptidyltransferase to hydrolyze the polypeptide from tRNA
- eRF-3 releases the releasing factors, utilizing GTP (in prokaryotes, RF-3)
- 80S ribosomal assembly dissocates into 60S and 40S subunits

Example of chain terminating codons are UAA (Ochre), UGA (opal) and UAG (amber)

INHIBITORS

Ciprofloxacin, Nalidixic acid and Novobiocin inhibit Bacterial DNA gyrase
Etoposide, Adriamycin and doxorubicin inhibit Human topoisomerase
6-mercaptopurine inhibits DNA polymerase
5-Fluoro uracil inhibits Thymidylate synthase

PROTEIN TARGETING

Most proteins are synthesized in cytoplasm; But many of them functions in specific cellular organelles.
So, after translation, these should reach the correct destination
This is with the help of specific amno acid sequences signals in the amino terminal region
In addition to this the carboxy terminal region also contains sequences which carry an "address" for its destination
Co-translational glycosylation also has a role in proein targetting
Defective protein targeting can lead to diseases like Zellweger's syndrome, Primary hyperoxaluria etc

Zellweger's syndrome: Due to defective protein targetting, the enzymes required for oxidation of very long chain fatty acids do not reach peroxisomes. Accumulation of VLCFA in nervous tissue leads to neurological impairment and early death

Primary hyperoxaluria: Defective targeting of alanine glyoxalate aminotrasferase
Normally it is present in peroxisomes ; Due to targeting defect it is seen in mitochondria
Leads to increased pool size of glyoxalate → excess production of oxalate

REGULATION OF GENE EXPRESSION

"Gene expression" means the production of a functional gene product (Protein or RNA) from the information in a gene. This is mainly regulated at the level of transcription. In eukaryotes, regulation also occurs at the level of post-transcriptional and post-translational modifications.
All genes are not regulated. **Constitutive genes (House keeping genes)** encode products required for basic cellular functions. They are continuously expressed.
Regulated genes are expressed only under certain conditions
Eg: Insulin gene is expressed only in β cells of pancreas
Some proteins are produced only when needed (Lactose metabolising enzymes in E.Coli)
Regulatory sequences in DNA (cis-acting elements) interact with regulatory proteins (trans-acting elements) to regulate transcription. Binding of regulatory proteins to DNA is through structural motifs like zinc finger, leucine zipper or helix-turn-helix
Examples of DNA binding regulatory proteins with binding motifs

Regulatory proteins binding to DNA	**Motif**
Lac repressor and CAP of E coli	Helix turn helix
Homeo box proteins in mammals	Helix turn helix
Gene 32 protein of E coli	Zinc finger
Steroid receptor family in mammals	Zinc finger
CRE binding protein in mammals	Leucine zipper

Induction and repression

- Induction and repression acts as important mechanisms of regulation
- Induction is the increased synthesis of a protein/enzyme in response to a signal. (the gene is "on")
- Repression is decreased synthesis (the gene "off")

OPERON CONCEPT OF GENE REGULATION

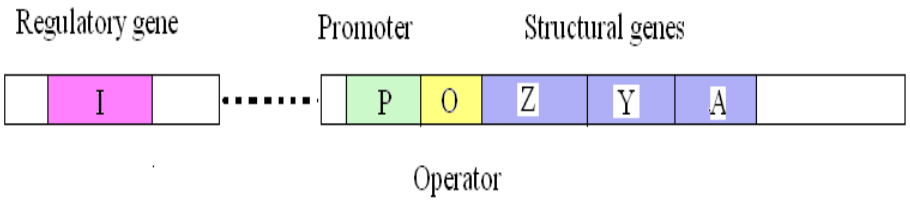
Put forward by Jacob and Monod
This was based on studies in E.Coli . E.Coli grown in glucose medium do not produce lactose metabolizing enzymes. But when they are grown in lactose,(and no glucose) lactase enzyme(beta galactosidase) is produced . The mechanism suggested is as follows:

- In bacteria, the structural genes that code for proteins in a particular pathway are sequentially grouped on the DNA along with cis-acting elements. The product is a polycistronic mRNA. The genes for this thus act as a unit which can be cocordinately controlled. This entire package is called "OPERON"
- Operon is a unit of gene expression; It includes

Structural genes: control elements: regulator/inhibitor gene: Promoter and operator areas

LAC OPERON

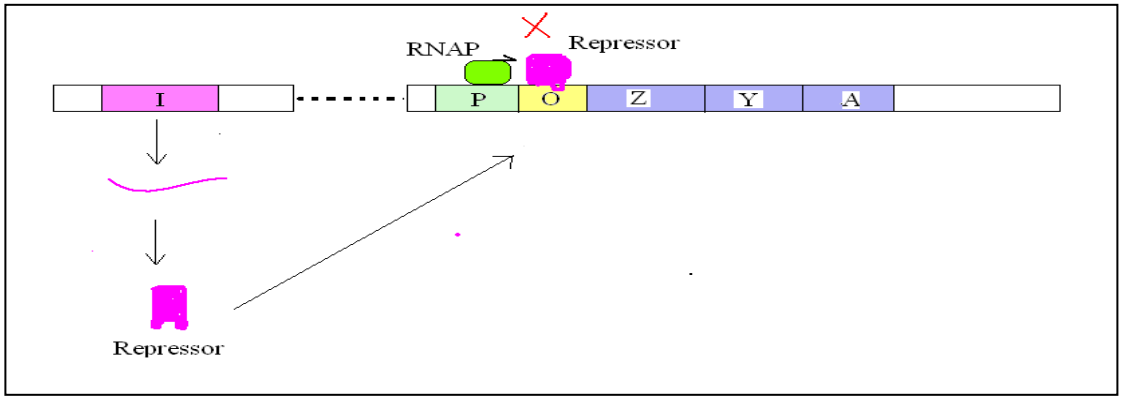
- This is the operon related to lactose metabolism
- Includes **3 structural genes** (Z,Yand A) which code for 3 proteins, a **regulator(inhibitor)** gene, a **promoter** area and an **operator**
- Z gene codes for β galactosidase, Y gene codes for permease (which transports lactose and galactose into cell) and A gene codes for thiogalactoside transacetylase



Transcription of these 3 genes start from a common promoter located close to Z gene. RNAP binds to the promoter and transcribes these 3 genes as a single mRNA.

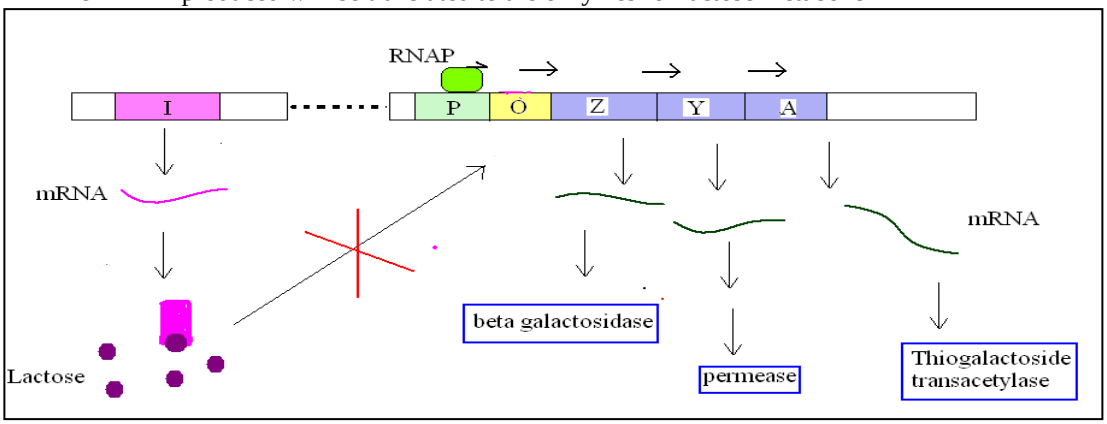
When glucose is available and lactose is absent, transcription is repressed

- Regulator (Inhibitor) gene produces a repressor molecue (150kD)
- Repressor has strong affinity to the operator site and binds tightly
- RNAP finds the promoter and moves forwards towards the structural genes, but its movement beyond operator is prevented by repressor protein bound to the operator
- So transcription of structural genes does not occur; lactose metabolising enzymes not produced



When lactose is present and glucose is absent, transcription is induced or "derepressed"

- Lactose combines with repressor protein
- This Lactose-repressor complex cannot bind to the operator site
- RNAP can move forward and transcribe the structural genes
- The mRNA produced will be translated to the enzymes for lactose metabolism



This shows that the synthesis of these proteins are normally repressed
Lactose is an inducer; When lactose is present, the genes are de-repressed or induced

Role of **Catabolite Activator Protein**

- If both glucose & lactose present, E.Coli utilize Glucose. Lactose metabolizing enzymes are produced only if glucose is absent
- This is because RNAP can attach only when CAP –cAMP complex is available
- cAMP accumulates only when the bacteria starves (when no glucose) ; then CAP binds with cAMP to produce the complex. This helps binding of RNAP to promoter site.

MUTATIONS

"Spontaneous abrupt origin of a new character"
Due to an alteration in the genetic material
One or a few nucleotides may be altered, or a large area may be altered
Mutagenicity of a substance is detected by Ame's test
Mutation can occur due to
Point mutations (base substitution) OR Frameshift mutations (base addition or deletion)
Point mutations
Transitions (Pyrimidine → Pyrimidine or Purine → Purine)
Transversions (Purine → Pyrimidine / Pyrimidine → Purine)
Results in: Silent mutations
Missense mutation (Acceptable, Partially acceptable, Unacceptable)
Nonsense mutation

DNA REPAIR MECHANISMS

Repair mechanism	Defect	Rectification
Mismatch repair	Single base or 2-5 base unpaired loops	Methyl directed strand cutting, Exonuclease digestion and replacement
Base excision repair	Damage to a single base (Spontaneous, chemical or radiation induced)	Base removal by N-glycosylase, abasic sugar removal, replacement
Nucleotide excision repair	Damage to a DNA segment	Removal of approx 30 nucleotide oligomer, and replacement
Double-strand break repair	Damage to strands (radiation, chemotherapy etc)	Ku protein, DNA-PK acts Unwinding, alignment and ligation

Xeroderma pigmentosum
Autosomal recessive disorder
Exposure to UV light causes dimerisation of Thymine bases
Not corrected because Nucleotide excision repair is defective
This requires excision endonuclease which is defective in Xeroderma Pigmentosa.
Disease characterized by marked sensitivity to UV light leading to cancer.

Diseases associated with DNA repair mechanisms

Disease	Defect	Features
Xeroderma Pigmentosum	Nucleotide excision repair defective	Sensitivity to UV light, Skin malignancy
Fanconi's anemia	DNA cross link repair	AR; Anemia, increased incidence of cancer
Ataxia telangectasia	ATM gene	Sensitivity to UV light, Lymphoreticular neoplasms
Bloom' syndrome	DNA ligase / helicase	Lymphoreticular neoplasms
Hereditary polyposis colon cancer (Lynch syndrome)	Mismatch repair	Ca Colon
Cockayne syndrome	NER defective	A.R. Progeria like features No increased tendency for cancer

ONCOGENIC VIRUSES

Infection by some viruses can produce cancer
This is because the viral DNA is incorporated into the human DNA
Viral DNA multiplication will not be controlled by human mechanisms

Leads to uncontrolled multiplication of the cells

Virus	Cancer produced
Epstein-Barr virus (EBV)	Burkitt's lymphoma Nasopharyngeal carcinoma
Human papilloma virus (HPV)	Cancer of uterine cervix
Hepatitis B(HBV)	Hepatoma

Antimutagens

Substances which interefere with development of cancer

Eg; Vitamin A, Carotenoids, Vitamin E, Vitamin C, Dietary fibre

ONCOGENES

They are certain genes, normally present in cells, which are capable of causing cancer. Also called proto-oncogenes ; First demonstrated in Rous sarcoma virus

This virus can produce sarcoma in birds; But, if the virus is deficient in a particular gene, it cannot do; This gene is named sarcoma gene (src)

Same sequences are present in normal avian cells- they are called C-src (cellular). Viral gene is called V-src

Proto oncogenes are regulatory genes, and they are expressed only when required

Oncogene	Location in human chromosome	Virus carrying the gene	Product of the gene
abl	9	Abelson leukemia virus in mouse	Tyrosine kinase
erb-A	17	Erythroblastosis virus in chicken	Receptor for epidermal growth factor
myc	8	Myelocytoma virus in chicken	DNA-binding protein
sis	22	Simian sarcoma virus in monkey	Platelet Derived Growth Factor (PDGF)
src	20	Rous sarcoma in chicken	Tyrosine kinase
ras	12	Rat sarcoma	GTPase

- Activation of oncogenes by carcinogens or due to point mutations may result in malignancy

Oncosuppressor genes (Anti oncogenes)

- These are genes which protect the cell from developing cancer
- When these are deleted or mutated, it results in cancer
- Eg:- Retinoblastoma oncosuppressor gene
  P53 oncosuppressor gene

P53 Oncosuppressor gene

Present in short arm of chromosome 17

Producese a phosphoprotein with m:wt 53 kD

This prevents development of cancer by 3 means

- If DNA of a cell is damaged, it blocks cell division untill the damage is repaired. (It downregulates PCNA gene transcription and causes arrest in G1 phase)
- If the damage is irreparable, it directs the cell for apoptosis
- It can also prevent the activity of oncogenic viruses

Many cancer cells show an absence of p53 gene or a mutated non functional p53

Retinoblastoma gene

Located in chromosome 13; Encodes a protein called p105 (m:wt 105 kD)

Called so because, firs isolated from retinoblastoma patients

It inactivates transcription factor E2F

Inhibits cell cycle at G1 phase

In normal cells, this arrest is prevented by the action of cyclin D (Separates Rb from E2F)

If the genome is deleted of this gene, retinoblastoma results

Certain tumor antigens from oncogenic viruses combine with Rb→ then cannot inhibit cell cycle → cancer

ONCOFETAL ANTIGENS

These are products of certain genes which are normally expressed only in fetal life after which the gene is repressed. But when malignant transformation occurs in some cells, the gene is de repressed and these proteins are produced. They are clinically used as tumor markers

Eg:-

Alpha fetoprotein – in hepatoma

Carcino embryonic antigen – Colon cancer

TUMOR MARKERS

Enzymes

- ALP: Bone
- Regan isoenzyme: Ca of Liver, Lung and Gut
- Nagao isoenzyme: Pleural malignancy
- ACP, PSA : Ca prostate
- Neuron specific enolase: tumors involving nervous system

Hormones

- Beta hCG- Choriocarcinoma
- Calcitonin- Medullary Ca thyroid
- Vasoactive Intestinal polypeptide- Apudomas
- VMA – Phaeochromocytoma and neuroblastoma
- 5 HIAA- Carcinoid syndrome

Oncofetal products

- Alpha fetoprotein: Hepatoma, germ cell tumors
- CEA: Colorectal, GI and Lung cancers

Carbohydrate antigens

- CA – 125: Ovarian cancer

Tissue catabolic product

- Hydroxy proline- Bone metastasis

Other proteins

- Immunoglobulins- Multiple myeloma, macroglobulinemia
- Bence-Jones proteins- Multiple myeloma

RECOMBINANT DNA TECHNONLOGY:

Following are the Steps:

Preparation of specific human gene
Preparation of chimeric DNA
Transfection / Transformation of vector into the host
Selection of colony having the required gene

Preparation of specific human gene

- The specific mRNA is first selected from the tissue
- A cDNA (complementary copy DNA) is prepared using reverse transcriptase: (This is done because isolation of a specific gene from human DNA is a difficult process)

Preparation of chimeric DNA

- A vector carrying a foreign DNA is called Chimeric DNA (Hybrid DNA) (Recombinant DNA)
- A vector DNA (Eg: Plasmid) is cut with a specific R.E. This may produce either sticky ends or blunt ends
- Human DNA is also cut with the same R.E: Same sequences are generated at the ends of the cut pieces
- Now the sticky ends of both vector and human DNA have complementary sequences.When these are incubated together, annealing takes place. The fragments are joined together by DNA ligase - a circular chmeric DNA is formed

Following are the different vectors with the size

Vector	DNA insert size
Plasmid pBR322	0.01-10 kb
Lambda phage	10-20 kb
Cosmids	35-50 kb
BAC	50-250 kb
YAC	500-3000 kb

Transfection/Transformation

- The process of introducing vector into a cell is called transformation for bacteria and transfection for eukaryotes
- The host E.Coli cells and vectors are incubated in hypertonic medium with calcium. Opens up calcium channels and vector enters the cell
- The bacteria are cultured
- As the host cell multiplies, it forms a clone in which every bacterium carries copies of the same inserted DNA fragment, hence the name "cloning."

Selection of colonies having the required gene

- The colonies which contain the desired foreign DNA are selected using properties like antibiotic resistance. The cloned DNA is released from its vector by cleavage (using the appropriate restriction endonuclease) and is isolated. By this mechanism, many identical copies of the DNA of interest can be produced.
    - pBR322 has Apr, Tcr and Cmr genes
    - EcoRI cuts in the middle of Cmr gene
    - PstI cuts wthin Apr gene

Expression vectors: If a vector carrying foreign gene is translated to the foreign protein, it is called 'expression vector".

Homopolymer tailing

- Ends of sticky ends may get sponatneously reconneted to produce tandem hetrogenous inserts
- To prevent this, blunt ends produced first and then homopolymer tailing is given using terminal transferase

GENE THERAPY

- Therapeutic delivery of genes into a cell: Applied to patient with a defective gene which produces disease. The normal, cloned DNA for a gene is introduced into the somatic cells of the patient. The DNA become permanently integrated into the patient's chromosomes→ expressed to produce the correct protein
- Vectors used for gene delivery are
    - Retroviruses
    - Adenoviruses
    - Plasmid-liposome complex: (can carry human genome of big size

Gene gun method: Tungsten particles coated with plasmid are accelerated to the tissues by helium pressure discharge

Diseases treated with gene therapy

SCID (Severe Combined Immuno Deficiency): ADA deficiency: First successful gene therapy
Cystic fibrosis; Hemophilia; Familial hypercholesterolemia ; Duchenne muscular dystrophy

TRANSGENESIS

- A form of germ cell gene therapy: The hybrid DNA is inroduced into the fertilized ova
- Embryos develop in the uterus of another animal; produce transgenic animal
  → incresed milk producton in cows
  → to correct genetically determined hypogonadism in mice

FUSION PROTEINS: Product of a fusion gene

- Fusion gene: produced by removing a stop codon from a gene and appending DNA sequence coding for another protein

Useful in tumor cell targeted immunotherapy

Important Terms /Tools In Genetics

Antisense Therapy

- mRNA contains a "sense" or message that is to be translated
- If an olignucleotide with a complementary sequence is made, it is called "antisense"
- If antisense RNA is introduced into a cell, it can trap the normal mRNA and can inhibit the protein synthesis
- This is being tried on AIDS and Cancer
- Antisense molecules can be introduced into cells by liposome encapsulation

Balanced Polymorphism

When a gene is mutated, the abnormal gene will be eliminated from the next generation in population . So the ratio of normal & abnormal gene is maintained in all generation (Hardy-Weinberg law). But an abnormal gene need not always be eliminated from population, especially if it has got an advantage.
Eg: Sickle cell trait and malaria
The lethal nature of the gene in homozygous state is counterbalanced by the advantage in heterozygous state. This is called balanced polymorphism

Apoptosis: Programmed Cell Death

- Removal of aged or partially damaged cells is done by apoptosis
- The changes include Chromatin condensation, Nuclear shrinkage, membrane blebbing and removal of these bodies by macrophages: In electrphoresis- Step ladder pattern of DNA
- Apoptosis mediating genes- suicidal genes
- Apoptosis is finally effected by activation of caspases : (Cysteinyl aspartate specific proteases)

Restriction Endnucleases

Presence in a given bacterium restrict the growth of bacteriophages
Cut DNA in a sequence-specific manner
Protects the host bacterial DNA from DNA of foreign organisms
Always exist in pair with site-specific DNA methylases
Site - specific DNA methylases protect host DNA from digestion by Restriction endonucleases

Regulation by repression in humans

- ALA synthase (rate limiting enzyme in heme synthesis) is repressed by heme
- An aporepressor is produced by a regulatory gene. The aporepressor binds with heme (co-repressor) to form the holo-repressor
- Holorepressor binds to the operator site and prevents the movement of RNAP
- Thus transcription of ALA synthase gene is repressed
- When heme is not available, holo repressor is not formed; So de-repression occurs and ALA synthase is produced

Hormone Response Elements (HRE) in regulation of gene expression

- Hormones or their second messengers act as inducers in higher organisms
- Steroid hormones bind to cytoplasmic receptors and the complex goes to nucleus. Exert their effect by attaching to HRE in DNA
- The receptor binds at the enhancer region → activates promoter → accelerates transcription

Gene Switching

- Sometimes one gene is switched off while a closely related gene takes up its function
- Eg:- In fetal life, genes for alpha & gamma chains of Hb are actve, After birth, it gradually switch over to genes of alpha and beta chains

Gene Amplification

- Amplification of a few genes by repeated initiation of DNA synthesis
- Eg: drug resistance to methotrexate can be produced by amplification of Folate reductase gene by cancer cells

Gene Silencing:

It is switching OFF a gene by mechanisms other than genetic modifications

- Transcriptional gene silencing- by histone modifications

- Post-transcriptional gene silencing – by mRNA of a particular gene being destroyed or blocked Eg: RNAinterference (by miRNA and siRNA)

mi RNA	si RNA
Form imperfect duplexes with 3' untranslated regions of mRNA and arrest translation	Form perfect hybrids anywhere with mRNA. - These hybrids are degraded in P bodies - Leads to arrest of translation

DNA Finger Printing

- Tandem repeats are short sequences of DNA at scattered locations in the genome, repeated in tandem (one after another)
- Probes are used to hybridize to these tandem repeats
- The number of these units varies from person to person, but is unique for any given individual

Therefore, it serves as a molecular fingerprint.

Applications

- Disputed paternity / parenthood
- To identify the culprit of a crime
  - DNA could be isolated form blood /seminal stains even after several years

RFLP (Restriction Fragment Length Polymorphism)

Genome variations are differences in the sequence of DNA among individuals; include both polymorphisms and mutations

A polymorphism is a clinically harmless DNA variation that does not affect the phenotype; occur in the intervening sequences that do not code for proteins.When variation occurs in more than 1% of population, it is called polymorphism

Two types of DNA variation commonly result in RFLPs:

- single-base changes
- tandem repeats

- single-base changes at restriction sites or a change at other sites may result in fragments of lengths differing from the normal
- Tandem repeats: short sequences of DNA at scattered locations in the genome, repeated in tandem (one after another)

RFLP can be found out by cleaving the DNA into fragments (restriction fragments) with a restriction endonuclease . The existence of 2 or more types of restriction fragment patterns in population is called RFLP

Nucleic Acid Hybridization Techniques: In Situ Hybridization

- DNA / mRNA can be viewed in the context of tissue morphology
- When the probe is tagged with fluorescin, it is called FISH
- CISH is chromogenic in situ hybridization

DNA Microarray

DNA probes of about 500-5000 genes are prepared and added to small wells in polystyrene plates
cDNA from patient's cell is added to the wells: Hybridized and fluorescence assessed

Used to identify expression of various genes
Blot techniques: Sothern,Northern,Western,South-western

CHROMOSOME JUMPING

- A technique for physical mapping of genome
- Used to bypass regions difficult to clone eg: repetitive DNA sequences
- DNA of interest is identified, cut into fragments with REand circularised
- From a known sequence a primer is designed to sequence across the circularised junction
- This primer is used to jump 100 kb-300 kb intervals: a sequence 100 kb away would have come near the known sequence on circularisation
- Thus, sequences not reachable by chromosome walking can be sequenced

JUMPING GENES- "TRANSPOSONS"

- Discovered by Barabara McClintock
- Sequences of DNA that can move or **transpose** themselves to new positions within the genome of a single cell [either "copy and paste" or "cut and paste"]
- Cause mutations and can alter genome size
- Transposons are considered "junk" DNA
- Used in biotechnology to alter DNA in a living organism

POLYMERASE CHAIN REACTION

In vitro amplification of a segment of DNA, whose flanking sequences are used as primers.
Enzyme used is Taq polymerase

Reverse transcriptase PCR

- Polymerase from Thermus thermophilus ; Has RTase and Polymerase activity
- Produce cDNA from mRNA present in the cell, it is amplified
- ie , it detects mRNA; Means it is from a live organism

Nested PCR

- Use 2 different sets of primers in 2 successive cycles
- This is to avoid contamination in products due to unexpected primer binding sites

RACE PCR: Rapid Amplification of cDNA Ends

- Unknown sequence at the ends of mRNA is amplified by a RT PCR, using known information from the centre of the transcript
- Can be used to generate 5' or 3' end of mRNA
- Also called one sided PCR or anchored PCR

TRIPLEX DNA (TRIPLE STRANDED DNA)

3 oligonucleotides wind around each other and form a triple helix. In this structure, one strand binds to a B-form DNA double helix through Hoogsteen or reversed Hoogsteen hydrogen bonds
A **Hoogsteen base pair** is a variation of base-pairing in nucleic acids such as the A•T pair. In this manner, two bases on each strand can be held together by hydrogen bonds in the major groove. A Hoogsteen base pair applies the N7 position of the purine (as a hydrogen bond acceptor) and C6 amino group (as a donor), which bind the Watson-Crick (N3–N4) face of the pyrimidine base.

Fluorescence In Situ Hybridization (FISH)

FISH (Fluorescent in situ hybridization) is a cytogenetic technique which can be used to detect and localize the presence or absence of specific DNA sequences on chromosomes. It uses fluorescent probes which bind only to those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out where the fluorescent probe bound to the chromosome. FISH is often used for finding specific features in DNA. These features can be used in genetic counseling, medicine, and species identification. FISH uses fluorescent molecules to vividly paint genes or chromosomes. This technique is particularly useful for gene mapping and for identifying chromosomal abnormalities.

How does FISH work?

FISH involves the preparation of short sequences of single-stranded DNA, called probes, which are complementary to the DNA sequences the researchers wish to paint and examine. These probes hybridize, or bind, to the complementary DNA and, because they are labeled with fluorescent tags, allow researchers to see the location of those sequences of DNA. Unlike most other techniques used to study chromosomes, which require that the cells be actively dividing, FISH can also be performed on nondividing cells, making it a highly versatile procedure.

What is FISH used for?

Scientists use three different types of FISH probes, each of which has a different application:

Locus specific probes hybridize to a particular region of a chromosome. This type of probe is useful when scientists have isolated a small portion of a gene and wish to determine which chromosome that gene is located on. They prepare a probe from the piece of the gene and observe which chromosome the probe hybridizes to.

Alphoid or centromeric repeat probes are generated from repetitive sequences found at the centromeres of chromosomes. Because each chromosome can be painted in a different color, researchers use this technique to determine whether an individual has the correct number of chromosomes or, for example, whether a person has an extra copy of a chromosome.

Whole chromosome probes are actually collections of smaller probes, each of which hybridizes to a different sequence along the length of the same chromosome. Using these libraries of probes, scientists are able to paint an entire chromosome and generate a spectral karyotype. This full color image of the chromosomes allows scientists to distinguish between the chromosomes based on their colors, rather than based on their dark and light banding patterns, viewed in black and white through traditional karyotyping. Whole chromosome probes are particularly useful for examining chromosomal abnormalities, for example, when a piece of one chromosome is attached to the end of another chromosome.

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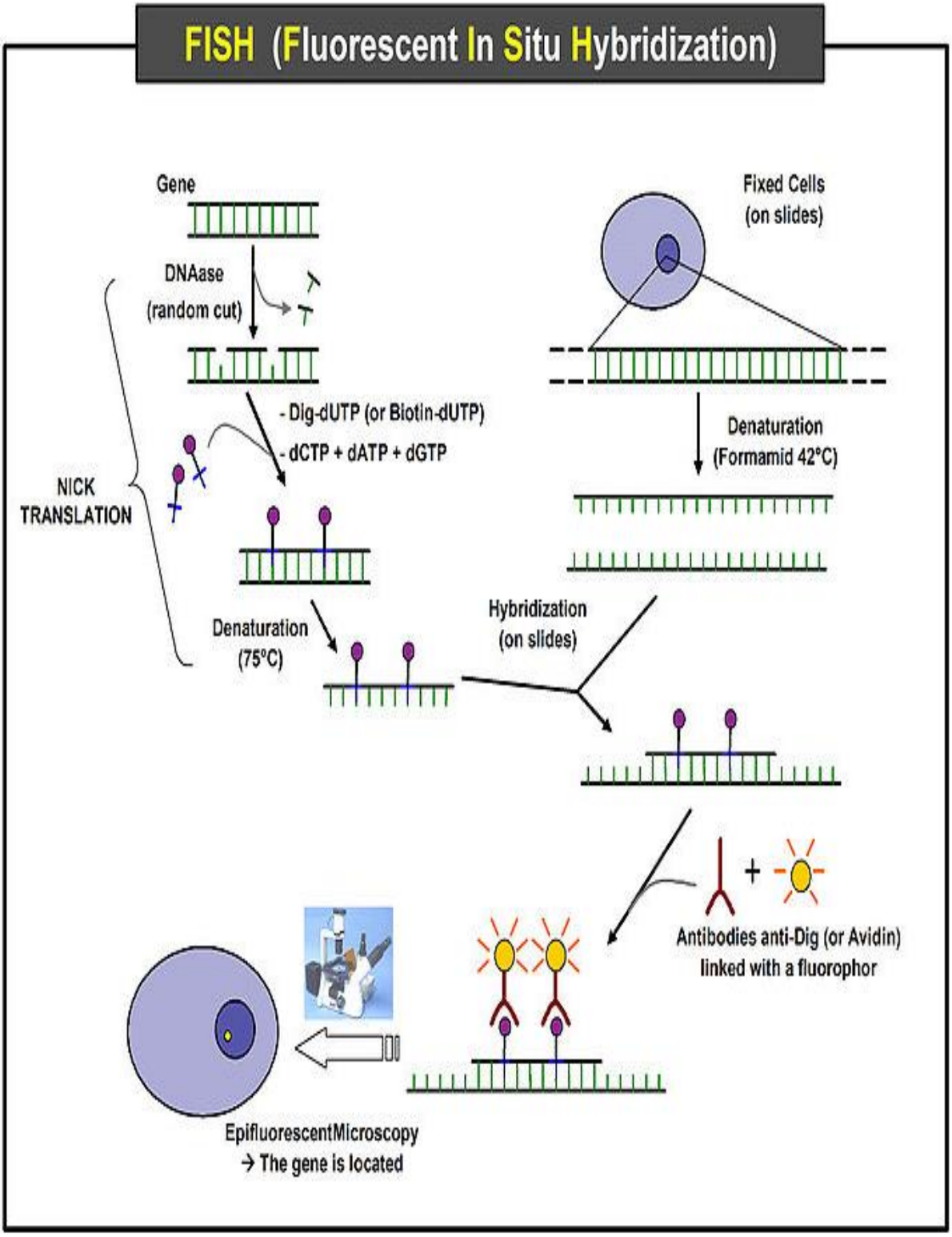

FISH (Fluorescent In Situ Hybridization)
Gene
DNAase
(random cut)
- Dig-dUTP (or Biotin-dUTP)
- dCTP + dATP + dGTP
NICK
TRANSLATION
Denaturation
(75°C)
Fixed Cells
(on slides)
Denaturation
(Formamid 42°C)
Hybridization
(on slides)
Antibodies anti-Dig (or Avidin)
linked with a fluorophor
EpifluorescentMicroscopy
→ The gene is located

SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis is a molecular biology technique in which a mutation is created at a defined site in a DNA molecule, usually a circular molecule known as a plasmid. In general, site-directed mutagenesis requires that the wild type gene sequence be known.

This technique is also known as **site-specific mutagenesis or oligonucleotide-directed mutagenesis**. For our purposes, we will define site-directed mutagenesis as any technique for introducing specific base-pair changes at specific locations within a target DNA sequence such as a gene. In general, changes are limited in extent - most often just one or two base pairs - with the intention of changing, for example, a restriction site, a codon, or the sequence of some regulatory element. Occasionally, more extensive changes can be made such as, for example, introducing six histidine codons so that a His-tagged protein my be expressed and isolated.

Site-directed mutagenesis can also be achieved by using PCR

This approach to mutagenesis was pioneered by the late Michael Smith at the Univerity of British Columbia. He shared the 1993 Nobel Prize in Chemistry for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies. Kary Mullis was the co-recipient. He received the prize for his invention of the polymerase chain reaction (PCR) method

In this strategy, a piece of DNA is first cloned in a suitable vector. A ssDNA copy of the recombinant plasmid is then prepared to which is annealed an oligonucleotide containing the desired sequenced changes. (Sometimes additional oligonucleotides are used as suggested in this figure.) The annealed DNA now serves as a template for DNA polymerase and the resulting heteroduplex DNA is then transformed into E. coli where the mutated clones will be identified.

In theory, if all goes well then 50% of the clones will contain the original sequence and 50% will contain the mutated sequence. In practice, the bias lies greatly in favour of the original sequence. This is due to inefficiencies in synthesising the complementary DNA strand, primer annealing improperly to other sequences in template DNA, and to the host cell repair systems which recognise the heteroduplex and remove the mutation that you are attempting to introduce**.**

Site-directed mutagenesis has been widely used in the study of protein functions. There are many approaches. An oligonucleotide-based method is illustrated in Figure below

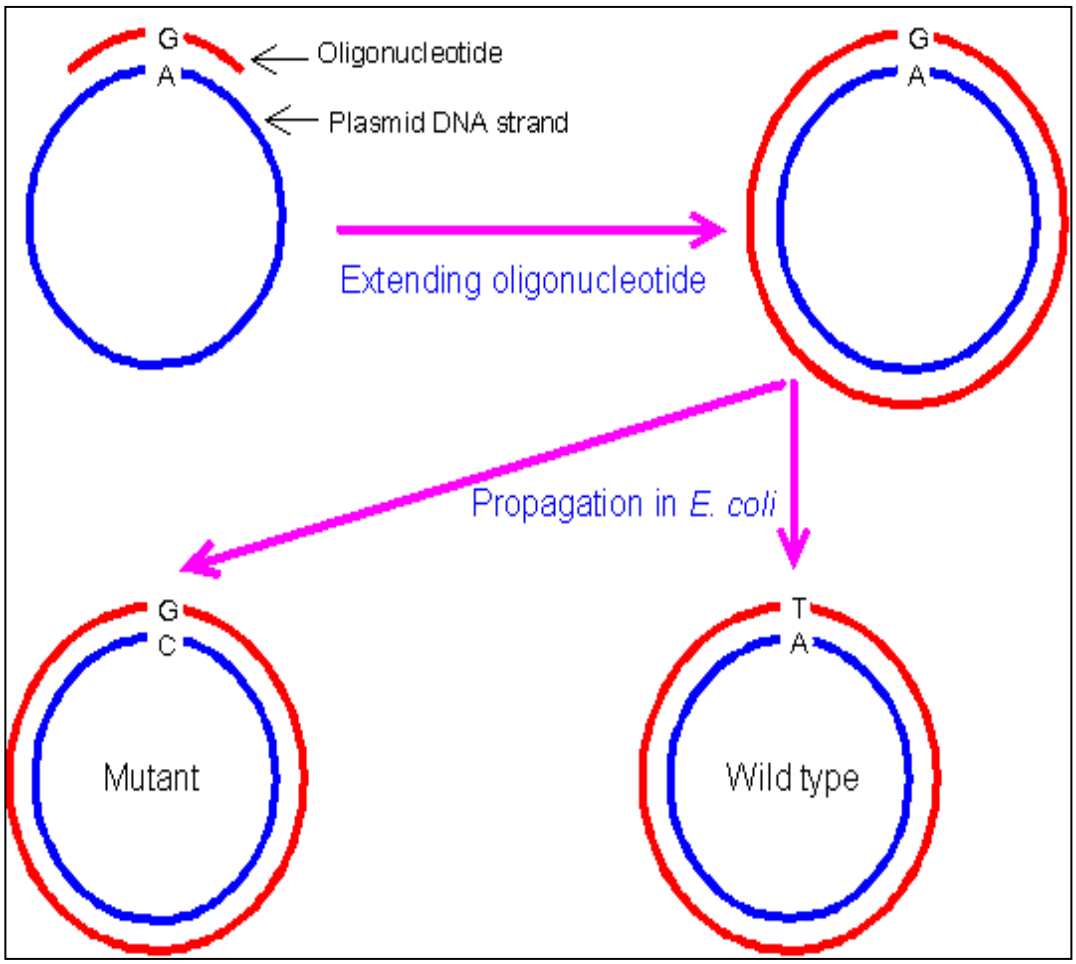


Figure is a illustration of the basic steps in a site-directed mutagenesis method.

(1) Cloning the DNA of interest into a plasmid vector.

(2) The plasmid DNA is denatured to produce single strands.

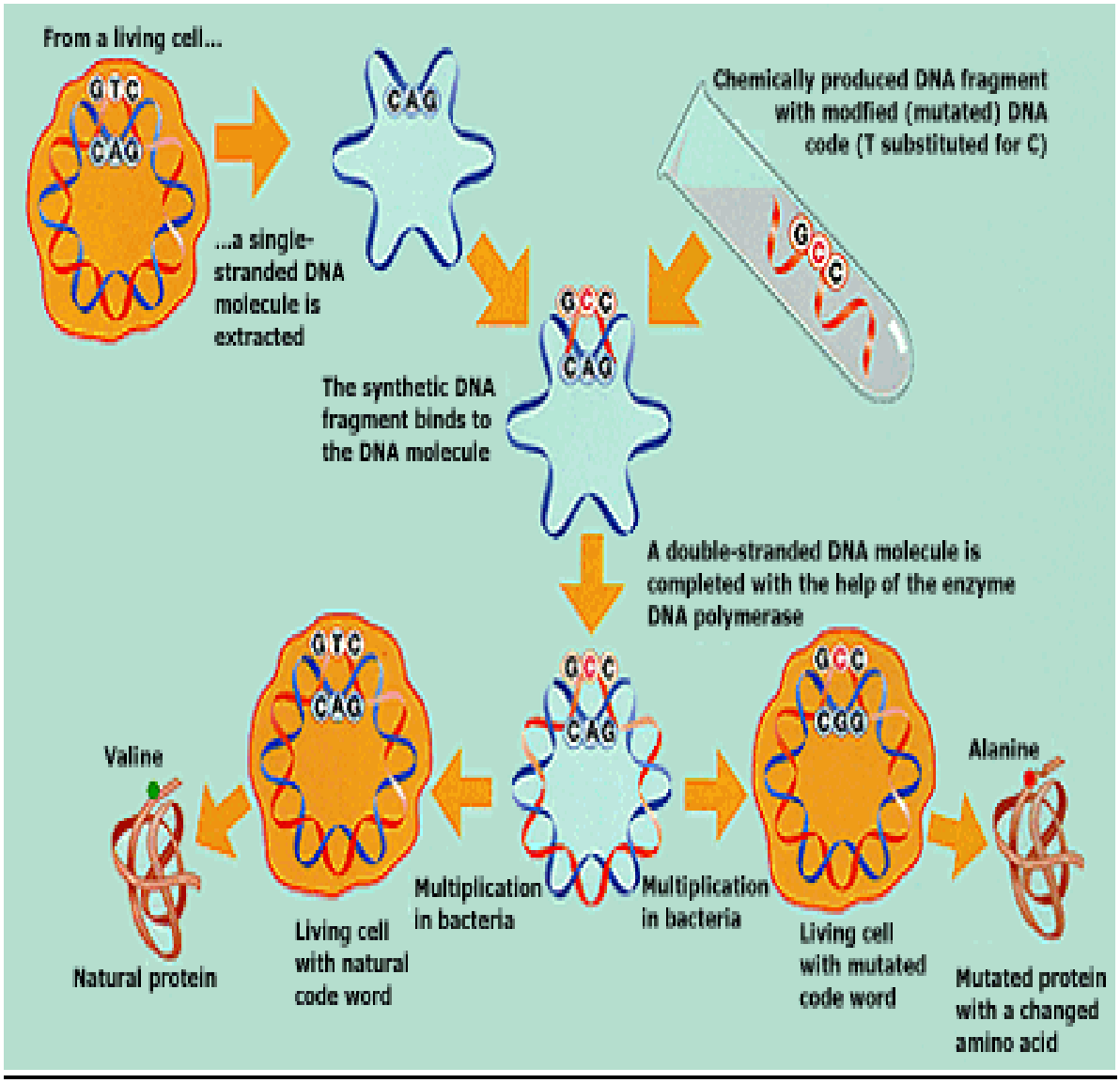
(3) A synthetic oligonucleotide with desired mutation (point mutation, deletion, or insertion) is annealed to the target region. In this figure, the T to G point mutation is used as an example.

(4) Extending the mutant oligonucleotide using a plasmid DNA strand as the template.

(5) The heteroduplex is propagated by transformation in *E. coli*.

After propagation, in theory, about 50% of the produced heteroduplexes will be mutants and the other 50% will be the "wild type" (no mutation). In commercial mutagenesis kits, some selection and enrichment methods have been used to favor the production of mutants.

Same site directed mutagenesis can be demonstrated by figure below



STUDYING GENE FUNCTION: CREATING KNOCKOUT MICE

Construction a knockout mouse

For decades researchers have tried to create tools that allowed for precise control over a specific gene in order to study its function. **In the early 1980′s a breakthrough technology known as transgenics or gene transfer was developed**. This new technology involved the process of pronuclear microinjection, a method involving the injection of genetic material into the nuclei of fertilized eggs (*Genetic material is injected directly into the fertilized egg which is then implanted back into a mouse and allowed to come to term*). Following injection, DNA would incorporate into the genome of the cell. The transformed fertilized eggs were then injected back into pregnant females and brought to term. **A major downfall of this technique is that researchers could neither predict nor control where in the genome the foreign genetic material would be inserted**. Since a gene's location in the genome is important for its expression pattern, mouse lines carrying the same transgene could display wildly varying phenotypes.

An innovative solution that resolved this conflict was originated by a team of scientists led by Martin Evans, Oliver Smithies and Mario Capecchi who created what is known as a genetic "knockout". By creating a knockout they proved that it was possible to aim the inserted gene at a precise location in the mouse genome. This gave scientists the ability to replace, or knock out, a specific gene with an inactive or mutated allele. Therefore, knockout animals are considered an investigative technique that allows for a particular gene of interest to be removed in an attempt to define what effect that gene has in the life of the organism. The gene knockout is created by selectively disabling a specific target gene in a particular type of cells called embryonic stem cells. The three scientists received the Lasker Award in 2001 for this achievement. The technique has been used to make several thousand different knockout mice. Knockout mice have become one of the most useful scientific tools in helping to understand the human genome and its roles in disease.

The process of gene targeting involves two processes. First a vector must be constructed. Once this has been done, the vector is put into embryonic stem cells and a new mouse is made from these cells. The vector consists of two regions of homology at either end, the disrupted gene of interest, and two additional genes to allow for quality control in the process. The regions of homology are located at either end of the vector and are complementary to specific sites on the genome surrounding the gene of interest. The vector binds to the genome at these two points of homology. This technology then takes advantage of the process of recombination to swap the disrupted gene for the real one. Here the two additional genes are used. In order to make sure that the vector has been inserted into the genome, the neomycin resistance gene is included. If the vector has not been inserted then all cells will die when the cells are grown in neomycin. It is also possible that the vector has been inserted into the genome in the wrong place. To make sure this has not happened, the thymidine kinase (tk) gene is also added into the construct outside the region of homology. If recombination has taken place then the tk gene will not be in the genome and so cells will be insensitive to gancyclovir. However if the vector has been inserted into the genome completely then the cell will die when grown in gancyclovir. Thus through positive and negative selection, cells with the correct insert can be isolated.

Embryonic stem cells are used as the target cells for the insertion of these vectors because they are pluripotent, meaning that they can give rise to every different type of cell in the adult body. Scientists interested in examining a specific gene will remove or "knock out" the gene in an embryonic stem cell as described above, then put the cell into a recently fertilized embryo. The manipulated cell then divides and eventually develops into many different tissues. The result is a mouse in which some of the cells have developed from the knocked-out embryonic stem cells. Often, embryonic stem cells which give rise to mice with a brown coat colour are injected into blastocysts which give rise to mice with a white coat colour. This allows the researcher to see which mice have contributions from both the injected cells and the blastocyst: if a mouse is completely white, then no injected cells grafted with the blastocyst. On the other hand if the mouse has both brown and white markings on its coat, then the injection of knockout embryonic stem cells was successful. After finding mice which have contributions from both cell types, the mice are bred to propagate the knockout gene to a certain percentage of the progeny, a process known as germ-line transmission. Once germ-line transmission is achieved, it is possible to breed mice where some are missing the knocked-out gene while others are not. In this case the latter group can act as a control group to see if the experiment has worked.

Uses of knockout mice

Knockout mice are used in a variety of ways. They allow to test the specific functions of particular genes and to observe the processes that these particular genes could regulate. What actions does this gene turn off and on? By examining what is happening in an *in vivo* (inside the body) model, we are able to determine the effects a particular gene may have. These effects would be impossible to observe in a culture dish. However, to completely establish and assign an action to a particular gene is very challenging and requires an incredible amount of work. One of the most exciting applications of knockout technology is in biomedical research. Scientists are using these models to study the progress of thousands of genetically based diseases at the molecular level. The hope is that by better understanding how a certain gene contributes to a particular disease, researchers can then take the knowledge a step further and look for drugs that act on that gene. Another essential application of knockout mice is in drug development. The knockout technology may lead to the discovery of the next generation of blockbuster therapies for curing numerous diseases based on novel targets from the human genome.

Although knockout technology is highly advantageous for both biomedical research and drug development, it also contains a number of limitations. For example, because of developmental defects, many knockout mice die while they are still embryos before the researcher has a chance to use the model for experimentation. Even if a mouse survives, several mouse models have somewhat different physical and physiological (or phenotypic) traits than their human counterparts. An example of this phenomenon is the p53 knockout. Gene p53 has been implicated in as many as half of all human cancers. However p53 knockout mice develop a completely different range of tumors than do humans. In particular, mice develop lymphomas and sarcomas, whereas humans tend to develop epithelial cell-derived cancers [2]. Because such differences exist it cannot be assumed that a particular gene will exhibit identical function in both mouse and human, and thus limits the utility of knockout mice as models of human disease.

Disadvantages of knockout technology

Although knockout mice are extremely useful in studying gene function, producing custom knockout mice is very expensive costing between $3,000 to $30,000. There are hundreds of knockout mice commercially available and can be purchased from a variety of companies. It is also important to consider the cost of care for genetically altered animals, as they require special transgenic core facilities where staff can assist researchers with housing and taking proper measures to keep these knockout animals at optimal conditions for research purposes. The cost of equipping and maintaining such a facility is usually very high and may be a limiting factor.

Thus knockout mice are a wonderful tool used to study the function of specific genes in a system. Despite numerous concerns and limitations applying this technique, it provides tremendous insight into understanding the disease process. The development of a gene knockout mouse has been a massive advance to the biomedical and pharmaceutical field presenting researchers with a very powerful tool for analyzing gene function during development, as well as in disease. In short, gene knockout technologies have become invaluable experimental tools for modeling genetic disorders, assigning functions to genes, evaluating drugs and toxins, and for helping to answer fundamental questions in basic and applied research.

MICROSATELLITES AND DISEASE

Microsatellite sequences are repeating DNA sequences. For example, in one part of chromosome number 4, CAG nucleotides are repeated many times over. They look like this: CAGCAGCAGCAG..... If the trinucleotides are repeated too many times this would cause the person to get Huntington's disease in adult life. Other diseases that involve repeats of three nucleotides are also known to cause neurological diseases (see below). At this time 14 neurological disor ders have been shown to result from the expansion of trinucleotide repeats, establishing an expanding class of diseases. Trinucleotide repeat diseases can be categorised into two subclasses based on the location of the trinucleotide repeats: diseases involving noncoding repeats (untranslated sequences) and diseases involving coding sequences (exonic). In general trinucleotide repeat disorders are either dominantly inherited or X-linked, the one exception being Friedrich's ataxia, which is autosomal recessive (Goldstein and Schlotterer, 1999; Cummings and Zoghbi, 2000).

Not all diseases are caused by a mistake in one gene. Sometimes many genes may be involved in a disease, for example, in schizophrenia. For these diseases microsatellite sequences have been used as a marker for locating the diseased region of the chromosome. This method is called positional cloning. Microsatellite markers close to the disease gene correlate with the heredity of the disease, and by analysis of these markers within families scientists can predict how the disease will be inherited (Risch, 2000).

EXAMPLES OF DISEASES INVOLVING TRINUCLEOTIDE REPEATS-

Huntington's disease
Symptoms: Late onset dementia and loss of motor control, resulting in full-blown chorea after 10-20 years. Motor disorder is often preceded or accompanied by memory deficits, cognitive decline or changes in personality. Juvenile onset is rare and patients show rigidity, bradykinesia, epilepsy, severe dementia and an accelerated disease course.
Involvement of microsatellites: CAG coding repeat in the first exon of the HD gene. Normal gene contains between 6 and 35 repeats and the affected gene from 36 to 121 repeats. Adult onset typically occurs when the repeat contains 40 - 50 units, whereas alleles containing more than 70 repeats typically result in the more severe juvenile form. The microsatellite adds a string of glutamine amino acids to the huntingtin protein
Chromosome location: #4

Fragile X
Symptoms: Mental retardation, long and prominent ears and jaws, high-pitched speech, hyperactivity, poor eye contact, and stereotypic hand movements (e.g. hand-flapping and hand-biting). 1 in 4000 males are affected, and fewer females are affected, depending on the ratio of cells with normal X chromosome active to abnormal X active.
Involvement of microsatellites: The repeat is CGG in a non-coding region of the FMR2 gene, and normal is 6 - 53 repeats. The disease occurs if the repeat is between 60 - 200.
Chromosome location: X

Myotonic dystrophy
Symptoms: Congenital DM is the most severe form of this disease involving hypotonia, respiratory distress at birth and developmental abnormalities. Adult onset includes variable loss of mental function, myotonia, muscle weakness and progressive muscle wasting. Other features may include facial dysmorphology, presenile cataracts, testicular atrophy, premature balding in males, kidney failure, hyperinsulin secretion and cardiac conduction abnormalities.
Involvement of microsatellites: CTG repeats in a non-coding region of the DMPK gene. Normal is between 5 and 37, the disease may involve from 50 - 1000s of repeats.
Chromosome location: #19

Spinalbulbar muscular atrophy
Symptoms: Neurological degeneration leading to difficulties in speech, articulation and swallowing, muscle weakness and atrophy. Signs of mild androgen insensitivity are typically seen at adolescence.
Involvement of microsatellites: CAG repeat in first coding exon of the androgen receptor (AR) gene. Between 9 and 36 repeats is normal, and people with 38-62 repeats develop the disease.
Chromosome location: X, recessive.

Friedrich's ataxia
Symptoms: Ataxia, diminished tendon reflexes, loss of position and vibratory senses, dysarthria (slurred speech), cardiomyopathy, diabetes mellitus, optical atrophy, scoliosis and skeletal abnormalities. Age of onset is typically early childhood.
Involvement of microsatellites: GAA repeat in non-coding region of gene X25. Normal gene contains between 7 and 34 repeats. The disease gene has 34 to 80 repeats.
Chromosome location: #9, recessive

EXAMPLES OF DISEASES FOUND BY POSITIONAL CLONING

Schizophrenia and Bipolar Disorder
Symptoms: Schizophrenia is characterized by auditory and visual hallucinations and delusions; symptoms of bipolar disorder involve severe mood swings, between mania and depression.
Involvement of microsatellites: Using 388 microsatellite markers within 8 families the researchers (Bailer et al., 2002) found a susceptibility locus for both schizophrenia and bipolar disorder on chromosome 3.
Chromosome location: #3

Congenital generalized hypertrichosis
Symptoms: Rare hair growth disorder, characterized by excessive hair growth on the face and upper body; sometimes called "werewolf" syndrome.
Involvement of microsatellites: Using hypervariable microsatellite markers the researchers (Figurea et al., 1995) localized the gene to a part of the X chromosome.
Chromosome location: X, dominant.

Asthma and Bronchial Hyperresponsiveness
Symptoms: Common respiratory disorder characterized by recurrent episodes of coughing, wheezing and breathlessness.
Involvement of microsatellites: A putative asthma susceptibility gene was identified on the ADAM33 region of chromosome 20 (Van Eerdewgh et al., 2002).
Chromosome location: #20

Detecting Cancer
The rate of microsatellite expansion (that is, increase in the number of repeats) or contraction (decrease in number of repeats) in cells is increased in some types of cancers, due to defects in enzymes that correct copying mistakes in DNA. Early clinical detection of some types of colon and bladder cancers using changes in microsatellite repeats have been successful (Yonekura et al., 2002; Moxon and Willis, 1999).

Chapter 14
BIOTECHNOLOGY

RESTRICTION ENDONUCLEASES

Cleave double-stranded (ds) DNA into smaller fragments. Each enzyme cleaves DNA at a specific nucleotide sequence-used experimentally to obtain precisely defined DNA segments called restriction fragments.

-Named after the bacterium from which they are isolated

- EcoRI From E Coli
- BamHI from Bacillus amyloliquifaciens

RE recognize short stretches of DNA (4-6 base pairs) that contain specific palindrome nucleotide sequences; this site is called restriction site

Produce "sticky or blunt" ends

Using the enzyme DNA ligase sticky ends of a DNA fragment can be covalently joined with other DNA fragments that have sticky ends produced by cleavage with the same RE. Another ligase, encoded by bacteriophage T4, can covalently join blunt-ended fragments.

DNA CLONING

- Introduction of a foreign DNA molecule into a replicating cell permits the amplification (production of many copies) of the DNA.
- To clone a nucleotide sequence of interest, the total cellular DNA is first cleaved with a specific restriction enzyme, creating millions of fragments.
- Each of the resulting DNA fragments is joined to a DNA vector molecule to form a recombinant molecule.
- This molecule conveys its inserted DNA fragment into a single host cell, (eg: a bacterium), where it is replicated (or "amplified").
- The process of introducing foreign DNA into a cell is called transformation for bacteria and transfection for eukaryotes.
- As the host cell multiplies, it forms a clone in which every bacterium carries copies of the same inserted DNA fragment, hence the name "cloning."
- The cloned DNA is released from its vector by cleavage (using the appropriate restriction endonuclease) and is isolated.
- By this mechanism, many identical copies of the DNA of interest can be produced.

VECTORS

A vector is a molecule of DNA to which the fragment of DNA to be cloned is joined

- Prokaryotic plasmids: Plasmids may carry genes that convey antibiotic resistance to the host bacterium, and may facilitate the transfer of genetic information from one bacterium to another. They can be readily isolated from bacterial cells, their circular DNA cleaved at specific sites by RE and foreign DNA inserted. The hybrid plasmid can be reintroduced into a bacterium, and large numbers of copies of the plasmid containing the foreign DNA produced

Other vectors:

- viruses that infect bacteria (bacteriophage λ) or mammalian cells (eg: retroviruses)
- artificial constructs such as cosmids and bacterial or yeast artificial chromosomes (BACs or YACs, respectively)

Recombinant DNA techbology

DNA LIBRARIES

A DNA library is a collection of cloned restriction fragments of the DNA of an organism.

- Genomic libraries: contain a copy of every dsDNA nucleotide sequence in the genome.
- cDNA libraries contain those DNA sequences that appear as mRNA molecules, and these differ from one cell type to another.
- Cloned cDNA lack introns and the control regions of the genes, whereas these are present in genomic libraries.

PROBES

Cleavage of large DNA molecules by restriction endonucleases produces an array of fragments. To pick a specific gene or DNA sequence of interest from the mixture of millions of DNA fragments, probes are used. Probe is a short, single-stranded piece of DNA, labeled with a radioisotope, or with a nonradioactive probe, such as biotin. The nucleotide sequence of a probe is complementary to the target DNA

BLOT TECHNIQUES

Southern blot detects DNA
Northern blot – RNA
Western blot - Protein
Southern Blotting

- Southern blotting is a technique that can detect mutations in DNA. It combines the use of restriction enzymes, electrophoresis, and DNA probes.
- Invented by Edward M Southern
- DNA is extracted from cells
- DNA is cleaved into many fragments using a restriction enzyme
- The fragments are separated by electrophoresis.
- The DNA fragments in the gel are denatured and blotted to a nitrocellulose membrane for analysis.
- A probe is used to identify the DNA fragments of interest.
- The patterns observed on Southern blot analysis depend both on the specific restriction endonuclease and on the probe used to visualize the restriction fragments

RESTRICTION FRAGMENT LENGTH POLYMORPHISM

- Genome variations are differences in the sequence of DNA among individuals. It has been estimated that the genomes of nonrelated people differ at about 0.1% of the genome.
- These genome variations include both polymorphisms and mutations. A polymorphism is a clinically harmless DNA variation that does not affect the phenotype. In contrast, mutation refers to an infrequent, but potentially harmful, genome variation that is associated with a specific human disease.. Polymorphisms often occur in the intervening sequences that do not code for proteins.
- A restriction fragment length polymorphism (RFLP) is a genetic variant that can be examined by cleaving the DNA into fragments (restriction fragments) with a restriction enzyme. The length of the restriction fragments is altered if the genetic variant alters the DNA so as to create or abolish a restriction site.
- RFLP can be used to detect human genetic variations

Two types of DNA variation commonly result in RFLPs:

- ✓ Single-base changes in the nucleotide sequence
- ✓ Tandem repeats of DNA sequences.

POLYMERASE CHAIN REACTION

- Invented by Karry Mullis 1989 (Nobel prize 1993)
- In vitro DNA amplification: Millions of copies of a sequence of DNA produced: no cloning needed
- DNAP is Taq polymerase

Advantages of PCR
The major advantages of PCR over cloning are sensitivity and speed.
Applications

- Comparison of a normal cloned gene with an uncloned mutant form of the gene:
- Detection of low-abundance nucleic acid sequences: eg: viruses during window period
- Forensic analysis of DNA samples: DNA fingerprinting by means of PCR has revolutionized the analysis of evidence from crime scenes.
- Prenatal diagnosis and carrier detection of diseases like cystic fibrosis:
- Detection of malignancy
- Fossil studies

ANALYSIS OF GENE EXPRESSION

Can be done by

- Determination of mRNA levels

- Analysis of proteins

Determination of mRNA levels can be by
Northern blot
DNA microarray

NORTHERN BLOTS:

Very similar to Southern blots except that the original sample contains a mixture of mRNA molecules that are separated by electrophoresis, then transferred to a membrane and hybridized to a radioactive probe. The bands obtained by autoradiography give a measure of the amount and size of particular mRNA molecules in the sample

- Microarrays: DNA microarrays contain thousands of immobilized DNA sequences organized in an area no larger than a microscope slide.
- used to analyze a sample for the presence of gene variations or mutations (genotyping)
- used to determine the patterns of mRNA production (gene expression analysis), analyzing thousands of genes at the same time.
- For genotyping analysis, the cellular sample is genomic DNA.
- For expression analysis, mRNA molecules from a particular cell type is converted to cDNA and labeled with a fluorescent tag
- This mixture is then exposed to a gene chip, which is a glass slide or membrane containing thousands of tiny spots of DNA, each corresponding to a different gene.
- The amount of fluorescence bound to each spot indicates the amount of that particular mRNA in the sample.
- DNA microarrays are often used to determine the differing patterns of gene expression in two different types of cell—for example, normal and cancer cells

ANALYSIS OF PROTEINS

Can be done by
ELISA
Western blot

GENE THERAPY

- The goal of gene therapy is to insert the normal, cloned DNA for a gene into the somatic cells of a patient who is defective in that gene.
- The DNA must become permanently integrated into the patient's chromosomes in such a way as to be properly expressed to produce the correct protein.

TRANSGENIC ANIMALS

- Transgenic animals can be produced by injecting a cloned gene into the fertilized egg.
- If the gene becomes successfully integrated into a chromosome, it will be present in the germline of the resulting animal, and can be passed along from generation to generation.
- Transgenic goats and cows can be designed that produce human proteins, such as blood clotting factors, in their milk.

Chapter 15
SEPARATION TECHNIQUES

CHROMATOGRAPHY

Chromatography is a technique by which the components in a mixture of similar substances are separated by a continuous redistribution between 2 phases, one stationary and the other mobile. The differences in the attractive forces between the stationary phase and the substances to be separated leads to selective retardation of these substances, relative to the mobile phase.

The technique was first applied by **Tswett** to a mixture of plant pigments. (Hence the name).

Depending on the type of stationary and mobile phases, chromatography can be of different types.

- Liquid-solid chromatography → mobile phase liquid; stationary phase solid
- Liquid-liquid chromatography → mobile phase liquid; stationary phase also liquid
- Gas-solid chromatography → mobile phase gas; stationary phase solid
- Gas-liquid chromatography → mobile phase gas; stationary phase liquid

When the stationary phase is liquid, it is supported on a solid like paper, silica etc.

Reverse phase chromatography

In liquid-liquid chromatography, the stationary phase is polar and mobile phase is less polar. But in some cases, we make the stationary phase non-polar and mobile phase polar. This is called reverse phase chromatography.

Depending on the mechanism of selective retardation of the particles, chromatography can be of different types like;

- Partition chromatography
- Adsorption chromatography
- Ion exchange chromatography
- Affinity chromatography
- Gel filtration chromatography (Molecular sieve chromatography)

Partition chromatography

Most commonly done method. Various components of the mixture are separated depending on their partition coefficient.

There are mainly 3 types of partition chromatography.

1. Paper chromatography
2. Thin layer chromatography
3. Gas-liquid chromatography

Paper chromatography

This is a *liquid-liquid partition* chromatography. The *stationary* phase is *water*, held on a solid support of filter paper. Mobile phase is a mixture of solvents like butanol and acetic acid or phenol and ammonia.

If the mobile phase is applied from the bottom, it is called ***ascending chromatography*** and if it is applied from the top, ***descending chromatography*****.**

The mixture to be separated is applied as a small spot near a corner of the paper and this paper is mounted on the solvent in a tank. The solvent ascends in the filter paper by capillary action and the substances are separated by the difference in partition coefficient.

Thin layer chromatography (TLC)

Silica gel is spread as a thin layer on a glass slide. The sample is applied on one corner. The plate is then placed in a tank containing the solvent. Water is held as the stationary phase and the non-polar solvent acts as the mobile phase (so it is also a liquid-liquid chromatography). It is less time consuming than paper chromatography (takes only 3-4 hours whereas paper chromatography takes 12-16 hours).

In paper as well as thin layer chromatography, once the procedure is completed it is necessary to visualize the spots formed by various substances. For this, substances called ***location reagents*** are sprayed to the paper or glass slide, which will react with the substances to give coloured spots.

Examples of location reagents:

- Ninhydrin for amino acids
- Diphenylamine for sugars

Identification of the spots

After locating the spots, the next aim is to identify the substances. For this we have to calculate the ***R_f values (Ratio of fronts)*** of the spots and compare them with R_f values of known standards.

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

R_f value of a particular substance is a constant for a particular solvent system at a given temperature.

In paper chromatography, the solvent used is butanol-acetic acid-water mixture. Water trapped in between cellulose fibres act as the stationary phase. So hydrophilic amino acids move less than hydrophobic amino acids In a paper chromatography, Rf value (distance travelled by the solute/ distance travelled by the solvent) is calculated. The more the Rf. The more it moves
Highest Rf is for Leucine.
Leucine > Isoleucine > Phenyl alaine > valine > Tyrosine ……. …….
…… …… Arg > His > Asparagine > Lysine > Cystine

2 dimensional chromatography: When the R_f values of the substances are very close to one another, the separation may not be satisfactory. Then we do a second chromatography in a direction perpendicular to the first one, using a different solvent system.

Gas liquid chromatography

A type of partition chromatography.
Stationary phase is liquid supported by inert materials like silica kept as a long narrow column. This method is suitable for those *substances which can resist high temperature*. The mixture is made volatile at one end of the column, mixed with an inert gas and passed through the column. A detecting device is used for detecting the various fractions which emerge at the other end of the column.

Adsorption chromatography

Based on the differences in adsorption of the substances to a solid medium. Silica gel, alumina etc are made into columns and used as the stationary media. The mixture is applied in a solvent on the top of the column. Due to the difference in adsorption of each substance, they move down with different speeds and get separated. Each fraction can be separated using suitable solvents.

Ion-exchange chromatography

In this, charged biological molecules are separated based on their electrostatic attraction with substances called ***ion-exchange resins.***
Ion-exchange resins are cross-linked polymers with ionic groups as part of their structure. These are 2 types: *anion exchange resins* (contain a quarternary N^+) and *cation exchange resins* (contain sulphonic or carboxylic groups).
Column chromatography is done using the resin as stationary medium. The substances bind to the resins depending on their charges. Then these are eluted by using buffers of increasing pH gradient.

Affinity chromatography

Based on the high affinity of some proteins to specific substances. These specific substances are used in columns to isolate or purify the proteins.
Eg:-

- Coenzymes are used for isolating enzymes.
- Amino phenyl boronic acid is used for isolating glycated Hb
- Antibodies are used for purification of antigens.

Gel filtration chromatography: also known as (Molecular sieve chromatography)

Here the mixture to be separated is passed through a column of porous hydrophilic gel (eg:Agarose, Acryl amide). This column acts as a molecular sieve, which allows small molecules
to enter into the gel particles, and prevent large molecules from entering. So large molecules bypass the gel particles and move down at a faster rate than the smaller particles. This technique is mainly used for separation, purification or molecular weight determination of proteins.

HPLC (High performance liquid chromatography)

It is a type of column chromatography in which the basic mechanisms are the same as adsorption, partition or ion-exchange chromatography. But the mobile phase is passed through the column under high pressure. This leads to a **high resolution**, and it requires **only a few minutes**.

ELECTROPHORESIS

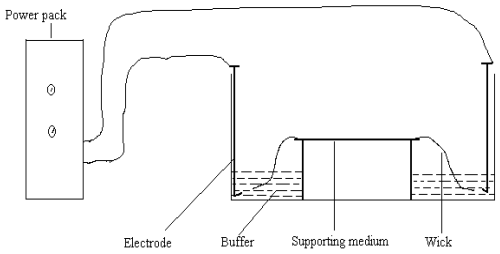
Electrophoresis is the movement of charged particles through an electrolyte when subjected to an electric field.

Factors affecting electrophoresis

The rate of movement of the particles during electrophoresis depends on:

1. Net charge on the particles
2. Strength of the electric field
3. Mass and shape of the particles
4. pH of the medium
5. Properties of the supporting medium
6. Temperature

Electrophoresis apparatus



The electrophoresis apparatus consists of a power pack for a stable continuous supply of electricity, a tank to hold the buffer, electrodes and the support medium for electrophoresis, which is connected to the buffer by wicks.

Common supporting media in use are:

1. Filter paper- requires 16-18 hours
2. Cellulose acetate membrane strips- takes only <1 hour; expensive
3. Agar gel- takes 1½ -2 hours; less expensive; reasonably good separation.
4. Poly acryl amide gel-it has high molecular sieving effect; so ensures good separation.

Different types of buffer can be used depending on the substances to be separated.

After the electrophoretic run is completed, the substances are fixed to the supporting medium using a fixative. The mixture will be separated into different bands. Amount of each component in the mixture can be calculated by 2 methods:

- Elution
- Scanning using densitometer.

Serum protein separation using agar gel electrophoresis

The buffer used is barbitone buffer at the pH of 8.6. At this pH all the serum proteins have a net negative charge and will move towards the anode.

Agar gel is prepared in barbitone buffer. This gel is liquid when hot and is spread on a glass slide. This solidifies on cooling. A few microlitres of sample is applied by cutting into the gel using a sharp object. Then it is subjected to electrophoresis for about 90 minutes.

The slide is taken out, dried. The various fractions are fixed using methanol. Then the bands are stained by using a dye (Amidoschwarz). Excess stain is removed by dil. acetic acid.

Albumin moves most rapidly, so is far away from the point of application. This is followed by ***α_1, α_2, β and γ globulins***. Gamma globulins have the smallest negative charge, so move only slightly- or it may slightly move to the opposite side due to electroendosmosis. If plasma is used, fibrinogen is found as a narrow band between β and γ globulins.

Variations in the patterns can occur depending on the blood level of various fractions. In ***multiple myeloma*** we get a sharp spike of γ globulin band which is called ***myeloma band (M band).*** Similar bands may be seen in *Waldenstorm's macroglobulinemia and heavy chain disease.*

PAGE (Poly Acrylamide Gel Electrophoresis)

Poly acryl amide has a high molecular sieving effect and so gives a better separation. Serum proteins are separated into more than 20 different bands.

In SDS-PAGE, proteins are boiled for 1-2 minutes with a denaturing agent, ***sodium dodecyl sulphate (SDS)***. The negative charge of SDS will cover the protein molecules, making them strongly electronegative: so the separation is then based on their **molecular size**. This method is mainly used for molecular weight determination of proteins.

Isoelectric focusing

A pH gradient is generated from anode to cathode. Each protein gets concentrated at the point where the local pH is the same as the isoelectric point. This method is useful for separation of isoenzymes, Hb variants etc.

Immunoelectrophoresis

Agarose gel electrophoresis is done first with the serum sample applied in wells cut into the gel. To visualize the different fractions, a specific antibody is applied to a trough cut into the gel and incubated. Precipitation arcs are formed when the antigen and antibody molecules are at 1:1 ratio. It is a highly sensitive method to study proteins.

High voltage electrophoresis

- 400 – 2000 V is used; Takes < half an hour; Used for separation of nucleotides

Capillary electrophoresis

Very low quantity of sample is required

- Gel taken in capillary tube
- High voltage 25,000 V
- Complete in a few minutes

Pulsed field gel electrophoresis

Direction of current is altered at regular intervals (electrical field is cycled between electrodes)

- Used to separate DNA fragments of > 50 kBp length

2 dimensional electrophoresis

- First electrophoresis based on charge in one direction; Second, based on m:wt
- Identify mutant DNA, abnormal proteins in genetic disorders