25B.Sc (HONOURS) MICROBIOLOGY (CBCS STRUCTURE) CC-3: BIOCHEMISTRY (THEORY) SEMESTER – 2 MCB-A-CC-2-3-TH TOTAL HOURS: 50 CREDITS: 4 Unit 1 Bioenergetics No. of Hours: 6 First and second laws of Thermodynamics. Definitions of Gibb's Free Energy, enthalpy,and Entropy and mathematical relationship among them, Standard free energy change and equilibrium constant Coupled reactions and additive nature of standard free energy change, Energy rich compounds: Phosphoenolpyruvate, 1,3- Bisphosphoglycerate, Thioesters, ATP

Unit 2 Carbohydrates No. of Hours: 10 Families of monosaccharides: aldoses and ketoses, trioses, tetroses, pentoses, and hexoses. Stereo isomerism of monosaccharides, epimers, Mutarotation and anomers of glucose.Furanose and pyranose forms of glucose and fructose, Haworth projection formulae for glucose; chair and boat forms of glucose, Sugar derivatives, glucosamine, galactosamine, muramic acid, N- acetyl neuraminic acid, Disaccharides; concept of reducing and non-reducing sugars, occurrence and Haworth projections of maltose, lactose, and sucrose, Polysaccharides, storage polysaccharides, starch and glycogen. Structural Polysaccharides, cellulose, peptidoglycan and chitin

Unit 3 Lipids No. of Hours: 10 Definition and major classes of storage and structural lipids.Storage lipids.Fatty acids structure and functions.Essential fatty acids.Triacylglycerols structure, functions and properties. Saponification Structural lipids.Phosphoglycerides: Building blocks, General structure, functions and properties. Structure of phosphatidylethanolamine and phosphatidylcholine, Sphingolipids: building blocks, structure of sphingosine, ceramide. Special mention of sphingomyelins, cerebrosides and gangliosides Lipid functions: cell signals, cofactors, prostaglandins, Introduction of lipid micelles, monolayers, bilayers

Unit 4 Proteins No. of Hours: 10 Functions of proteins, Primary structures of proteins: Amino acids, the building blocks of proteins. General formula of amino acid and concept of zwitterion. Titration curve of amino acid and its Significance, Classification, biochemical structure and notation of standard protein amino acids Ninhydrinreaction.Natural modifications of amino acids in proteins hydrolysine, cystine and hydroxyproline, Non protein amino acids: Gramicidin, beta-alanine, D-alanine and D- glutamic acid Oligopeptides: Structure and functions of naturally occurring glutathione and insulin and synthetic aspartame, Secondary structure of proteins: Peptide unit and its salient features. The alpha helix, the beta pleated sheet and their occurrence in proteins, Tertiary and quaternary structures of proteins. Forces holding the polypeptide together. Human haemoglobin structure, Quaternary structures of proteins

Unit 5. Enzymes Structure of enzyme: Apoenzyme and cofactors, prosthetic group-TPP, coenzyme No. of Hours: 10 NAD,metal cofactors, Classification of enzymes, Mechanism of action of enzymes: active site, transition state complex and activation energy. Lock and key hypothesis, and Induced Fit hypothesis. Significance of hyperbolic, double reciprocal plots of enzyme activity, Km, and allosteric mechanism Definitions of terms – enzyme unit, specific activity and turnover number, Multienzymecomplex : pyruvate dehydrogenase; isozyme: lactate dehydrogenase, Effect of pH and temperature on enzyme activity. Enzyme inhibition: competitive- sulfa drugs; non-competitive-heavy metal salts

Unit 6. Vitamins Classification and characteristics with suitable examples, sources and importance

Name " Enzyme " Introduced by Frederick W. Kuhne.

Classification of Enzymes

The 7 major classes of enzymes with some important examples from some subclasses are described below :

1. Oxidoreductases
2. Transferases
3. Hydrolases
4. Lyases or Desmolases
5. Isomerases
6. Ligases or Synthetases
7. Translocases

1. Oxidoreductases EC1

- This class comprises the enzymes which were earlier called dehydrogenases, oxidases, peroxidases, hydroxylases, oxygenases etc
- The group, in fact, includes those enzymes which bring about oxidation-reduction reactions between two substrates

$$A_{red} + B_{ox} \rightleftharpoons A_{ox} + B_{red}$$

2. Transferases EC2

- Catalyze the transfer or exchange of certain groups among some substrates
- In these are included the enzymes catalyzing the transfer of one-carbon groups, aldehydic or ketonic residues and acyl, glycosyl, alkyl, phosphorus or sulfur-containing groups
- Choline acetyltransferase, Phosphorylase, Hexokinase

$$\text{ATP} + \text{D-hexose} \longrightarrow \text{ADP} + \text{D-hexose-6-phosphate}$$

$$\text{A-B} + \text{C} \longrightarrow \text{A} + \text{B-C}$$

3. Hydrolases EC3

- Accelerate the hydrolysis of substrates
- These catalyze the hydrolysis of their substrates by adding constituents of
- water across the bond they split
- The substrates include ester, glycosyl, ether, peptide, acid-anhydride, C—C, halide and P—N bonds
- Lipase, Beta-galactosidase, Arginase, Trypsin. Pepsin, plasmin,

$$\text{L-arginine} + H_2O \longrightarrow \text{L-ornithine} + \text{urea}$$

$$\text{A-B} + H_2O \longrightarrow \text{A-H} + \text{B-OH}$$

5. Isomerases EC5

- Facilitate the conversion of isomers, geometric isomers or optical isomers
- Alanine racemase, Cis-trans isomerases. Retinine isomerase, Glucosephosphate isomerase

D-glucose-6-phosphate ⟶ D-fructose-6-phosphate

All *trans*-retinene ⟶ 11-*cis*-retinene

A-B-C ⇌ A-C-B

4. Lyases EC4

- Promote the removal of a group from the substrate to leave a double bond reaction or catalyze its reverse reaction
- In these are included the enzymes acting on C—C, C—O, C—N, C—S and C—halide bonds
- Aldolase, Fumarase, Histidase

L-malate ⟶ Fumarate + H_2O

A-B ⇌ A + B

(reverse reaction: synthase)

6. Ligases EC6

- Catalyze the synthesis of two molecular substrates into one molecular compound with the release energy
- These are the enzymes catalyzing the linking together of two compounds utilizing the energy made available due to simultaneous breaking of a pyrophosphate bond in ATP or a similar compound
- This category includes enzymes catalyzing reactions forming C—O, C—S, C—N and C—C bonds
- Acetyl-CoA synthetase, Glutamine synthetase, Acetyl-CoA carboxylase

$$\text{ATP} + \text{acetate} + \text{CoA} \longrightarrow \text{AMP} + \text{pyrophosphate} + \text{acetyl-CoA}$$

$$\text{A} + \text{B} + \text{ATP} \longrightarrow \text{A-B} + \text{ADP} + \text{P}_\text{i}$$

7. Translocase EC7

- Catalyze the movement of ions or molecules across membranes or their separation within membranes
- he reaction is designated as a transfer from “side 1” to “side 2”
- Translocases are the most common secretion system in Gram positive bacteria
- Translocase of the outer membrane (TOM) can work in conjunction with translocase of the inner membrane (TIM) to transport proteins into the mitochondrion

Active Site

As the substrate molecules are comparatively much smaller than the enzyme molecules, there should be some specific regions or sites on the enzyme for binding with the substrate. Such sites of attachment are variously called as **'active sites'** or **'catalytic sites'** or **'substrate sites'.**

Properties of Active Site

1. The active site occupies a relatively small portion of the enzyme molecule
2. The active site is neither a point nor a line or even a plane but is a 3- dimensional entity. It is made up of groups that come from different parts of the linear amino acid sequence. For example lysozyme has 6 subsites in the active site. The amino acid residues located at the active site are 35, 52, 59, 62, 63 and 107
3. Usually the arrangement of atoms in the active site is well defined, resulting in a marked specificity of the enzymes. Although cases are known where the active site changes its configuration in order to bind a substance which is only slightly different in structure from its own substrate

Properties of Active Site

4. The active site binds the substrate molecule by relatively weak forces
5. The active sites in the enzyme molecules are grooves or crevices from which water is largely excluded. It contains amino acids such as aspartic acid, glutamic acid, lysine serine etc. The side chain groups like -- COOH, --NH2, --CH2OH etc., serve as catalytic groups in the active site. Besides, the crevice creates a micro-environment in which certain polar residues acquire special properties which are essential for catalysis

Nomenclature

- The first three numbers represent the class, subclass and sub-subclass to which an enzyme belongs, and the fourth digit is a serial number to identify the particular enzyme within a sub-subclass.
- The class, subclass and sub-subclass provide additional information about the reaction classified. For example, in the case of EC 1.2.3.4, the digits indicate that the enzyme is an oxidoreductase (class 1), that it acts on the aldehyde or oxo group of donors (subclass 2), that oxygen is an acceptor (sub-subclass 3) and that it was the fourth enzyme classified in this sub-subclass (serial number 4).
- The last printed list of enzymes appeared in the year 1992. Since then it has been updated and maintained online.

Holo enzyme = Apo enzyme / Apo protein + Cofactor

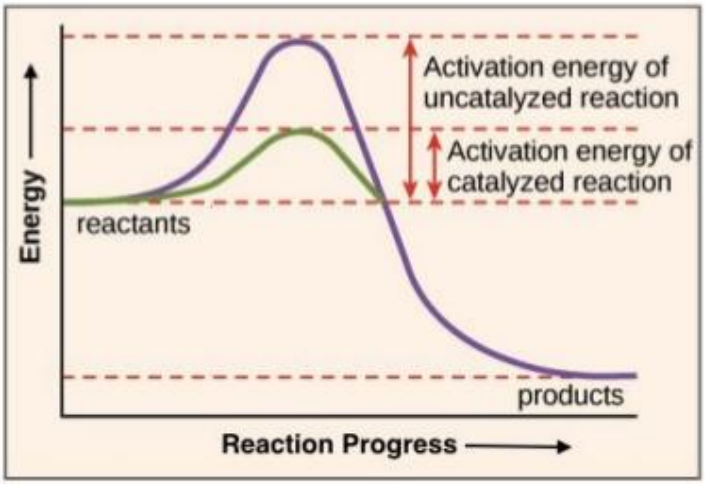
TABLE 8.2 Enzyme cofactors

Cofactor	Enzyme
Coenzyme	
Thiamine pyrophosphate	Pyruvate dehydrogenase
Flavin adenine nucleotide	Monoamine oxidase
Nicotinamide adenine dinucleotide	Lactate dehydrogenase
Pyridoxal phosphate	Glycogen phosphorylase
Coenzyme A (CoA)	Acetyl CoA carboxylase
Biotin	Pyruvate carboxylase
5′-Deoxyadenosyl cobalamin	Methylmalonyl mutase
Tetrahydrofolate	Thymidylate synthase
Metal	
Zn^{2+}	Carbonic anhydrase
Zn^{2+}	Carboxypeptidase
Mg^{2+}	*Eco*RV
Mg^{2+}	Hexokinase
Ni^{2+}	Urease
Mo	Nitrate reductase
Se	Glutathione peroxidase
Mn^{2+}	Superoxide dismutase
K^{+}	Propionyl CoA carboxylase

Enzyme affect reaction rates, not equilibria

E + S = ES= EP= E + P

Activation Energy



Standard free energy change for the reaction is negetive means the free energy of the ground state of Product is lower than that of substrate. So Δ G is negetive for the equilibrium which favors the formation of product.

Transition State: A molecular moment in which events such as bond breakage , bond formation, charge development have proceeded to the precise point at which decomposition to either substrate or product is equally likely.

Activation Energy: The difference between the energy level of the ground state and the transition state is called activation energy.

Catalysis enhances the reaction rate by lowering the activation energy.

Properties of Enzymes

1. Colloidal Nature

- On account of their large size, the enzyme molecules possess extremely low rates of diffusion and form colloidal systems in water
- Being colloidal in nature, the enzymes are nondialyzable although some contain dialyzable or dissociable component in the form of coenzyme.

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1. Colloidal Nature

- On account of their large size, the enzyme molecules possess extremely low rates of diffusion and form colloidal systems in water
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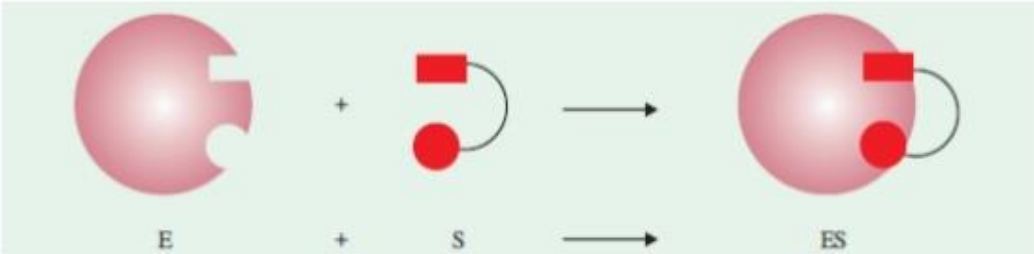
Fischer's Lock and Key Model

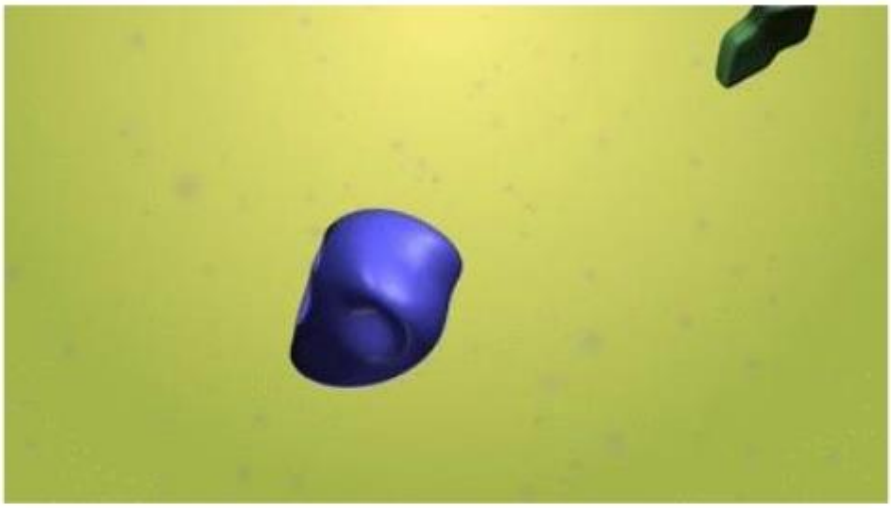
- also known as template model - proposed by Emil Fischer in 1898
- the union between the substrate and the enzyme takes place at the active site more or less in a manner in which a key fits a lock and results in the formation of an enzyme substrate complex



Fischer's Lock and Key Model

- In fact, the enzyme-substrate union depends on a reciprocal fit between the molecular structure of the enzyme and the substrate
- And as the two molecules (that of the substrate and the enzyme) are involved, this hypothesis is also known as **the concept of intermolecular fit**
- The enzyme-substrate complex is highly unstable and almost immediately this complex decomposes to produce the end products of the reaction and to regenerate the free enzyme.
- The enzyme-substrate union results in the release of energy. It is this energy which, in fact, raises the energy level of the substrate molecule, thus inducing the activated state
- In this activated state, certain bonds of the substrate molecule become more susceptible to cleavage.

Fischer's Lock and Key Model



Koshland's Induced Fit Model

- unfortunate feature of Fischer's model is the rigidity of the active site
- Koshland presumed that the enzyme molecule does not retain its original shape and structure. But the contact of the substrate induces
- some configurational or geometrical changes in the active site of the enzyme molecule.
- Consequently, the enzyme molecule is made to fit completely the configuration and active centres of the substrate
- At the same time, other amino acid residues may become buried in the interior of the molecule

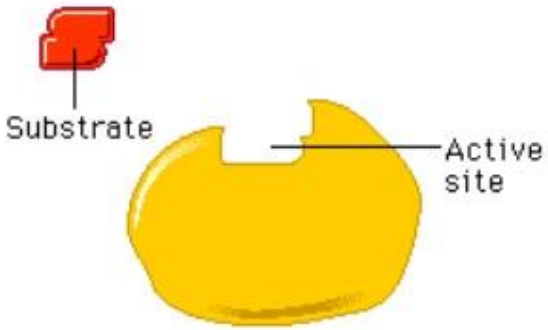
Koshland's Induced Fit Model

- As to the sequence of events during the conformational changes, 3 possibilities exist
  1. The enzyme may first undergo a conformational change, then bind substrate
  2. An alternative pathway is that the substrate may first be bound and then a conformational change may occur
  3. Both the processes may occur simultaneously with further isomerization to the final conformation

- Koshland's model has now gained much experimental support. Conformational changes during substrate binding and catalysis have been demonstrated for various enzymes such as phosphoglucomutase, creatine kinase, carboxypeptidase

Enzyme is complementary to transition state.

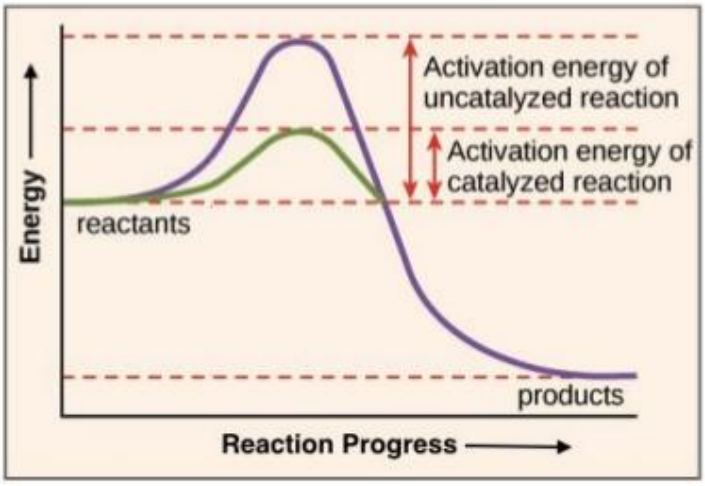
Koshland's Induced Fit Model



Activation Energy

- All the chemical reactions in a biological system have an energy barrier which prevents reactions from proceeding in an uncontrolled and spontaneous manner. The input of energy required to break this energy barrier or to start a reaction is called the **activation energy**
- Enzymes lower the activation energy of a reaction - that is the required amount of energy needed for a reaction to occur. They do this by binding to a substrate and holding it in a way that allows the reaction to happen more efficiently.

Activation Energy



Activation Energy

- It is important to remember that enzymes do not change the reaction's ΔG
- In other words, they do not change whether a reaction is exergonic (spontaneous) or endergonic
- This is because they do not change the reactants' or products' free energy
- They only reduce the activation energy required to reach the transition state

Michaelis Menten Hypothesis

Leonor Michaelis and **Maud L. Menten** (1913), while studying the hydrolysis of sucrose catalyzed by the enzyme invertase, proposed this theory. Their theory is, however, based on the following assumptions :

1. Only a single substrate and a single product are involved
2. The process proceeds essentially to completion
3. The concentration of the substrate is much greater than that of the enzyme in the system
4. An intermediate enzyme-substrate complex is formed
5. The rate of decomposition of the substrate is proportional to the concentration of the enzyme substrate complex

E+S=ES K1. K-1, ES= EP K2, K-2, EP = E+ P K3

Michaelis Menten Hypothesis

- The theory postulates that the enzyme (E) forms a weakly-bonded complex (ES) with the substrate (S)
- This enyzme-substrate complex, on hydrolysis, decomposes to yield the reaction product (P) and the free enzyme (E)

$$\mathbf{E + S \Leftrightarrow ES \rightarrow E + P}$$

$$V = \frac{V_{max} \times S}{K_m + S}$$

Michaelis-Menten equation

$$\frac{V}{V_{max}} = \frac{S}{K_m + S} \quad \text{or} \quad K_m = S\left[\frac{V_{max}}{V} - 1\right]$$

1/v = Km + S / V max xS

1/V = Km/ Vmax x S + 1/Vmax

The Lineweave r–Burk plot puts 1/[S] on the x-axis and 1/V on the y-axis.

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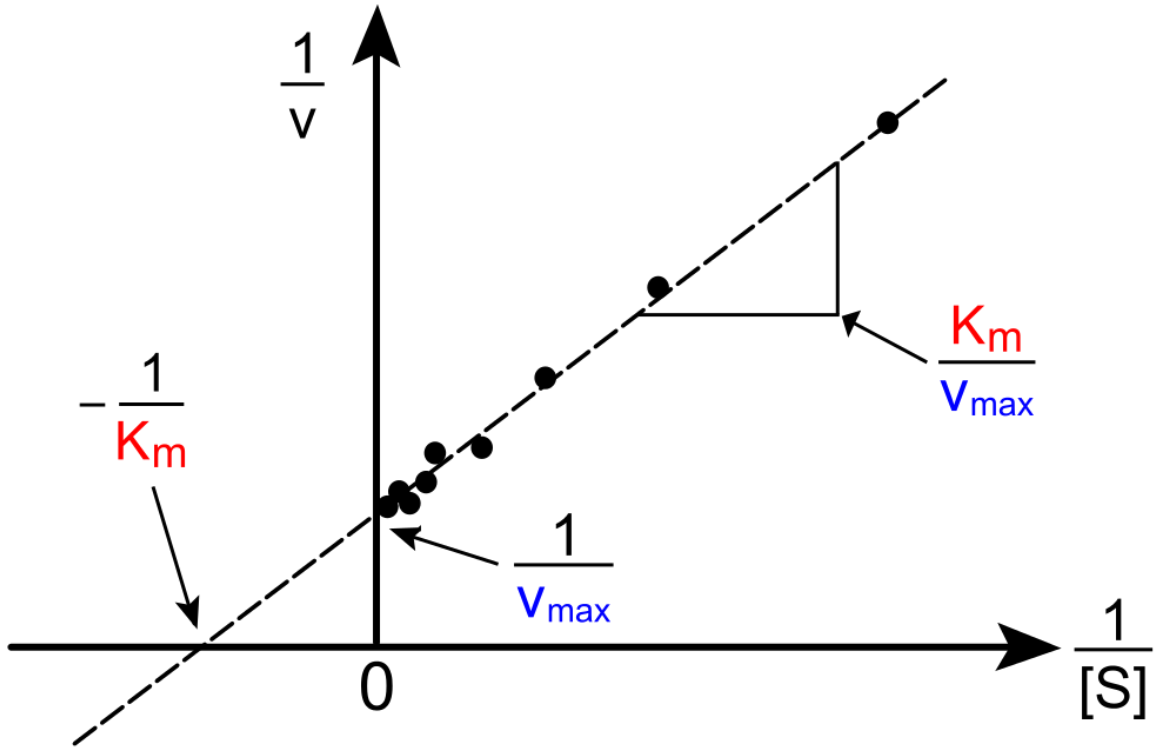
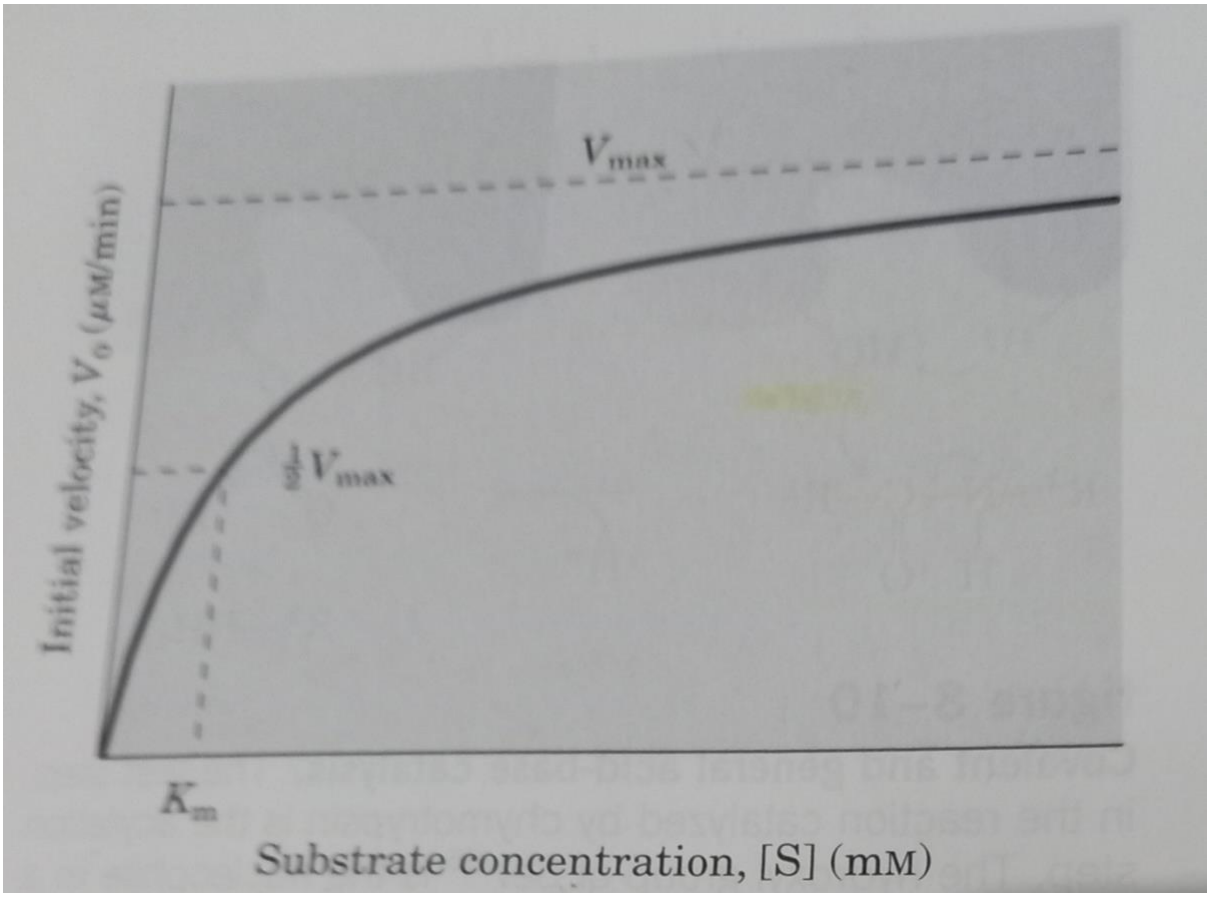


Significance of Km and Vmax value

- Km value is used as a **measure of an enzyme's affinity** for its substrate. The lower the Km value the higher the enzyme's affinity for the substrate and vice versa
- Km value also provides **an idea of the strength of binding of the substrate** to the enzyme molecule. The lower the Km value the more tightly bound the substrate is to the enzyme for the reaction to be catalyzed and vice versa.
- Km value indicates the **lowest concentration of the substrate [S] the enzyme can recognize** before reaction catalysis can occur.



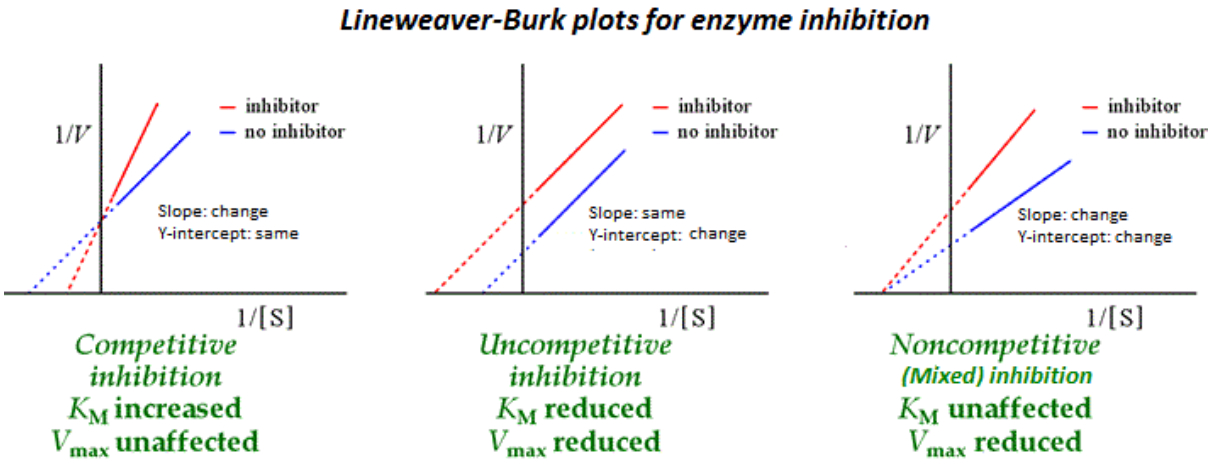
Unit Of Km = M (molar) / mM (milli molar) / µM (micromolar)

Unit of Vmax = Amount of product formed per unit time : micromole / minute

Biochemical reactions involving a single substrate are assumed to follow Michaelis-menten equation.

Kcat / turn over number: number of substrate molecules converted to product in a given unit time on a single enzyme molecule when the enzyme is fully saturated with substrate.

Kcat = Vmax/[ET]., unit = Second^{-1}



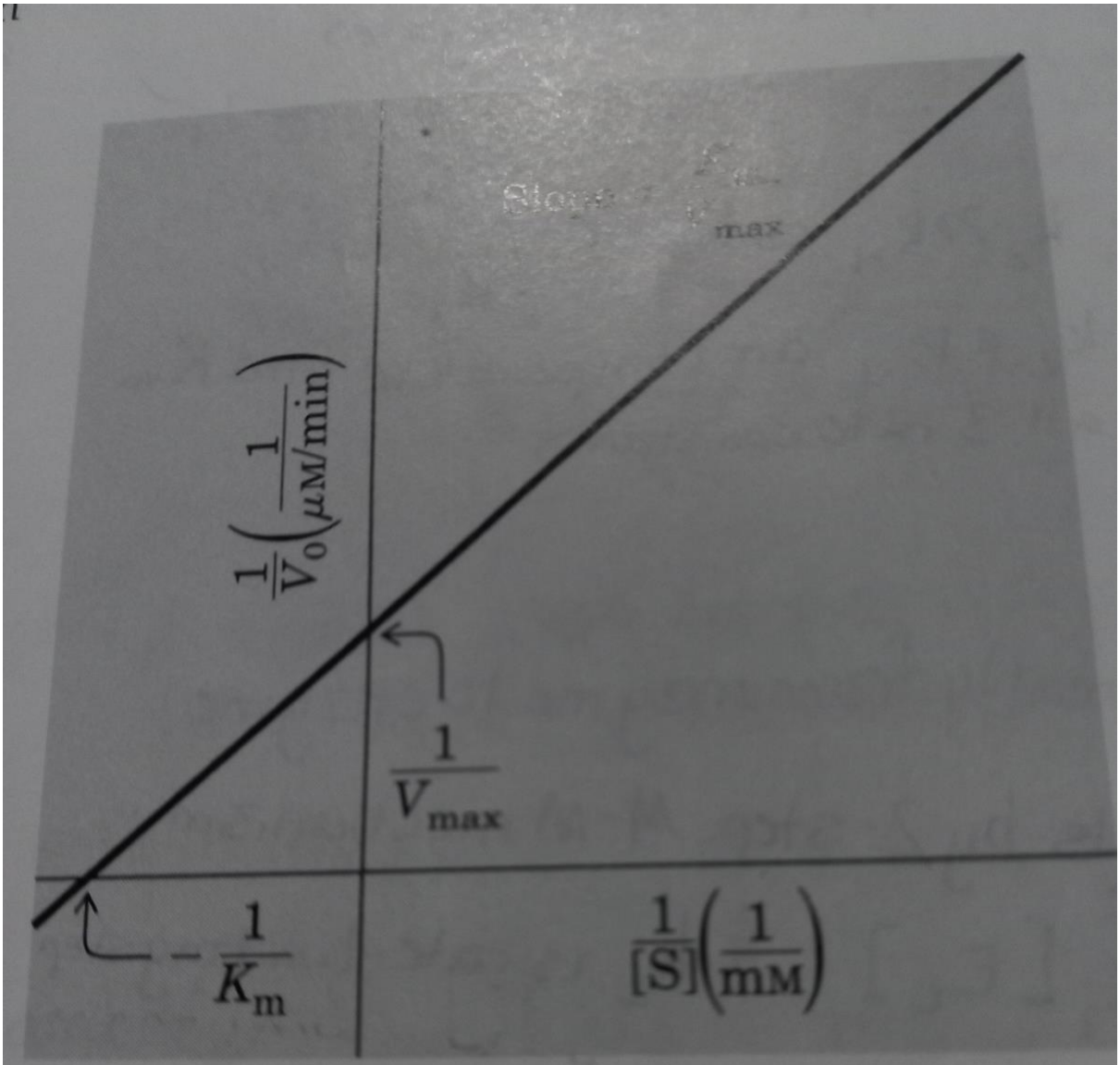
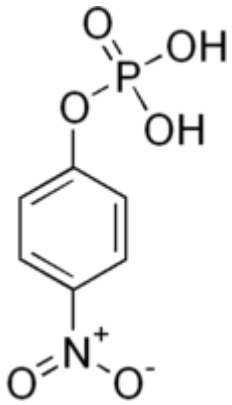


figure 1

A double-reciprocal or Lineweaver-Burk plot.



Substrate concentration = 1mM

371.15 g / 1000 substrate is present in 1000 ml solvent for preparation of 1Mm solution

1000ml 1mM substrate solution contains 371.15/1000 g

1ml 1mM substrate solution contains 0.37115/1000 g

50 ml 1mM substrate solution contains 0.37115/1000g x 50

0.1M Glycine-NaOH buffer, pH 9.8

Para nitro phenyl P + Buffer = Paranitro phenol + inorganic P (Pi)

Substrate concentration : 1 mM (milli molar) , Total volume = 50ml, how much substrate will be required to prepare 50 ml substrate solution?

Substrate concentration in tube no 1 =

V1 xS1 = V2 XS2

V1= 0.1ml

V2 = 1ml

S1 = 1mM

S2 = 0.1 x 1 ml x mM/ 1ml

1---S2 = 0.1mM 1/S2 = 1/0.1mM = $10mM^{-1}$

2---- S2 = 0.2 mM

3---- S2 = 0.4 mM

4----S2 = 0.6 mM

5----S2 =0 .8mM

Velocity = Product formed per minute = product formed / 30min

Micro mole

Tube no	Buffer (ml)	Enzyme (ml)	Substrate (ml)
Blank	0.8	---	0.2
1	0.7	0.2	0.1
2	0.6	0.2	0.2
3	0.4	0.2	0.4
4	0.2	0.2	0.6
5	0.1	0.2	0.7

Temp= 37degree C, Time of incubation = 30min

After 30 min of incubation 2ml 1M NaOH solution will be added to each tube

At 440nm optical density is observed.

Preparation of standard curve of para nitro phenol

Stock concentration of para nitro phenol = 0.1mM (milli molar)

Mol wt of para nitro phenol = 138.1 gm

Calculate the amount of para nitro phenol to prepare 0.1mM 25 ml solution.

0.1milli mole of paranitro phenol is present in 1000ml solution

138.1g/10000 paranitro phenol is present in 1000ml solution

1ml solution requires 138.1g/ 10000000 paranitro phenol

25 ml solution requires 138.1 x 25 g/ 10^7 =3452.5 g/ 10^7

Test tube no	Volume of paranitro phenol (ml)	Volume of NaOH (1M) (ml)
Blank	----	3
1	0.1	2.9
2	0.2	2.8
3	0.4	2.6
4	0.6	2.4
5	0.8	2.2

Tube No	[S] micro molar	1/ [S] micro molar-1	V (micromole/min)	1/V (micromole/min)-1
1	100			
2	200			
3	400			
4	600			
5	800			

Para nitro phenol standard curve

TubeNo	Amount of Para nitro phenol (µmole/ml) micromole per millilitre	Optical density at 440 nm
Blank	0.0	0.0
1		0.102
2		0.202
3		0.350
4		0.452
5		0.660

TubeNo	Concentration of paranitro phenol (µg/ml)	
Blank	0.0	
1	50	
2	100	
3	200	
4	400	
5	800	
unknown	---	

$$\frac{1}{V_0} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

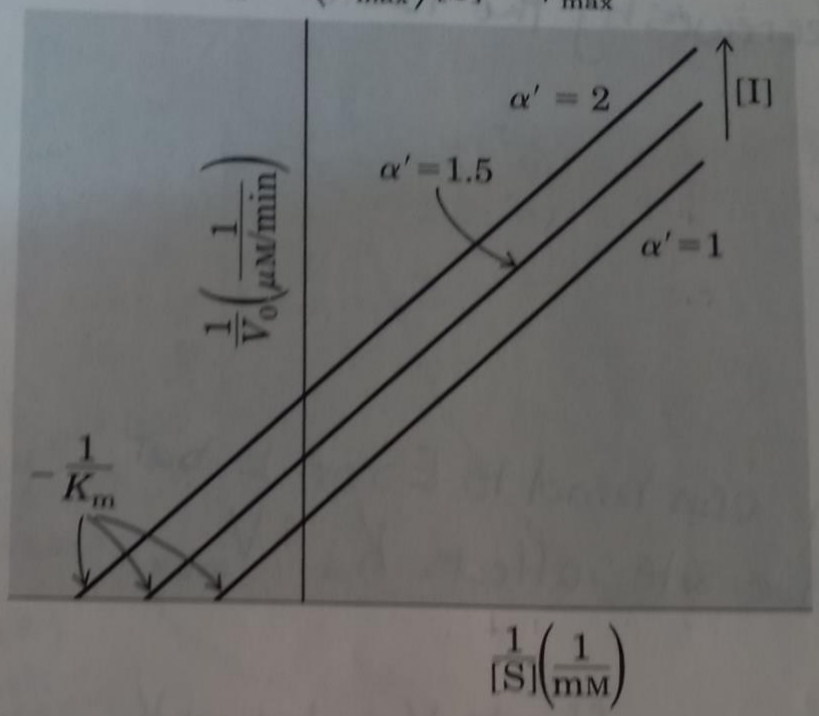


figure 2
Uncompetitive inhibition.

$$\frac{1}{V_0} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

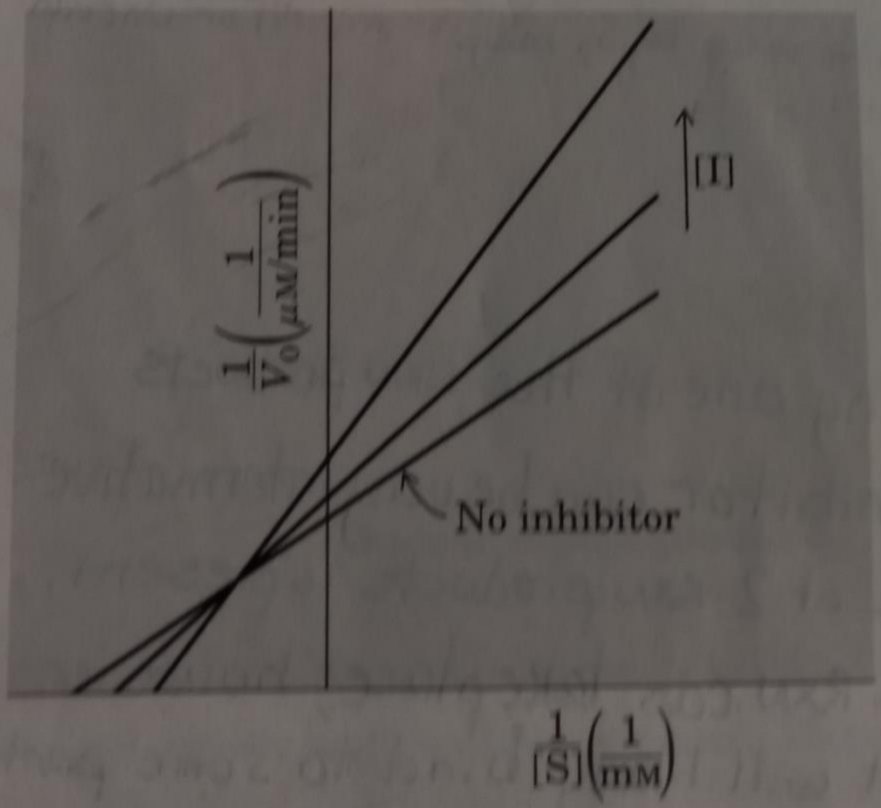


figure 3

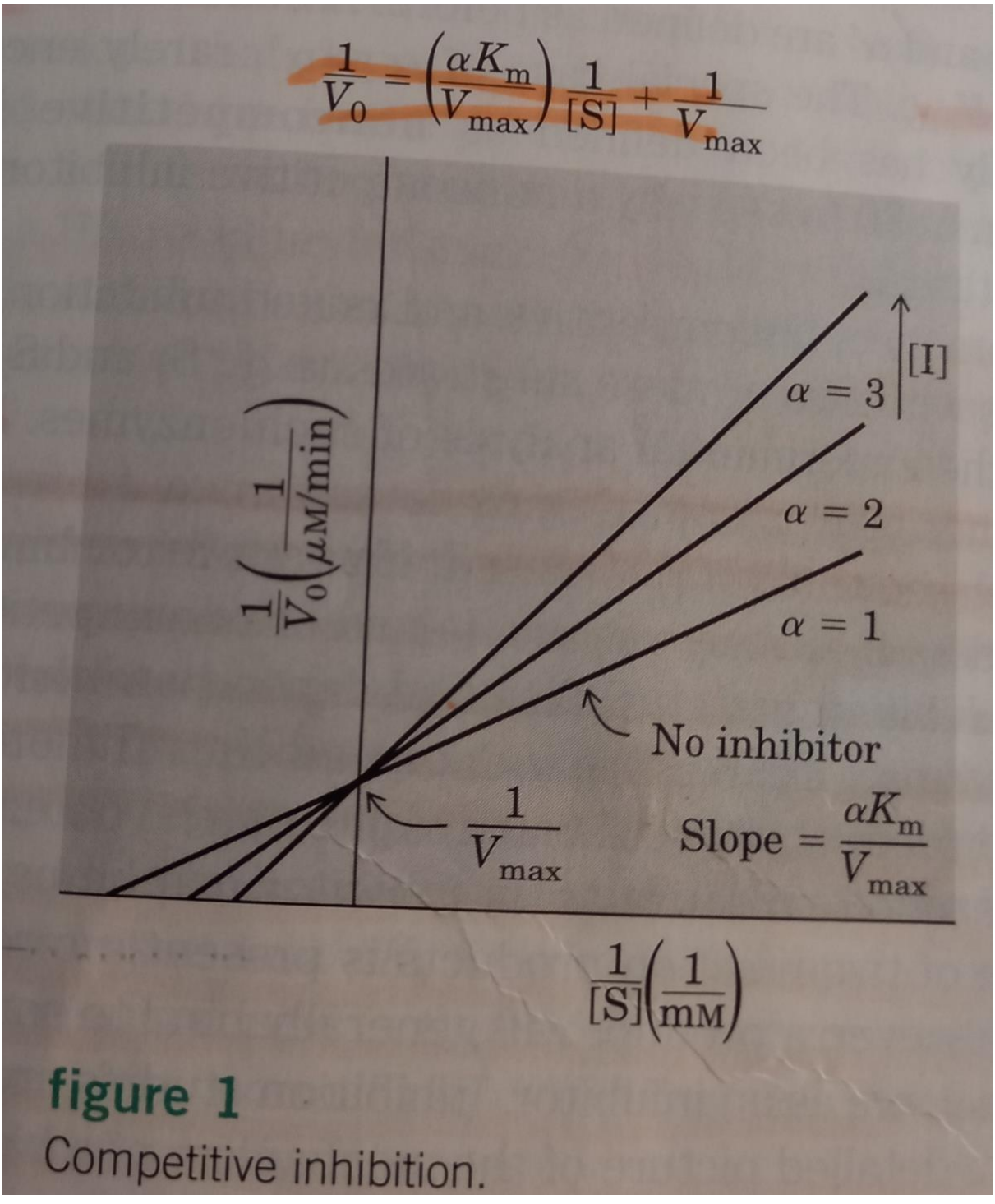
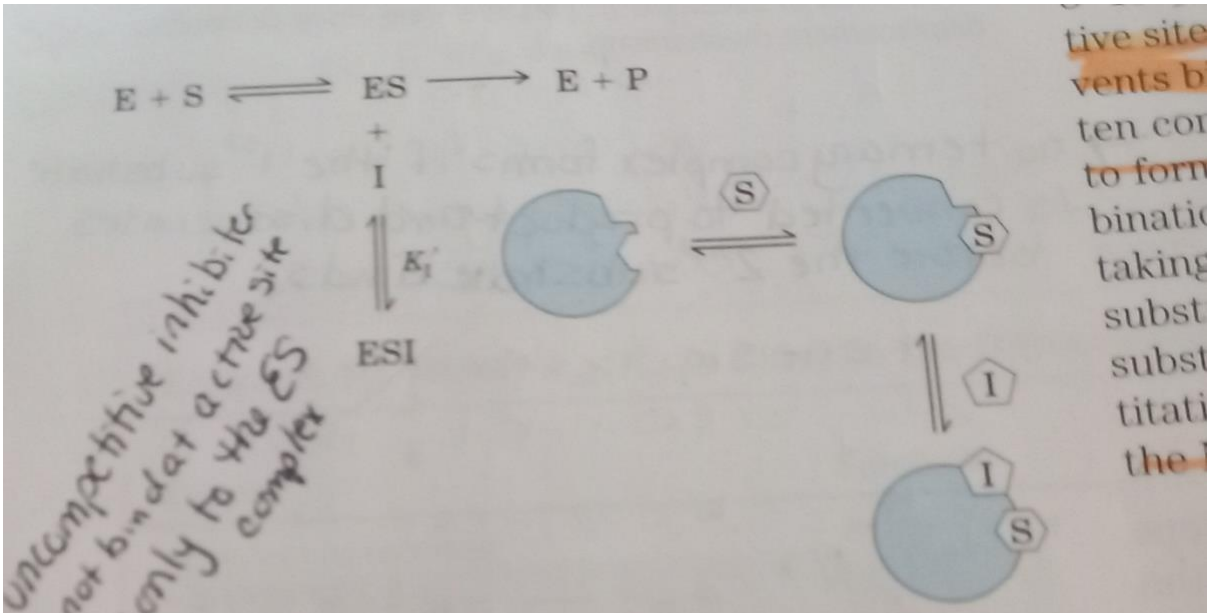
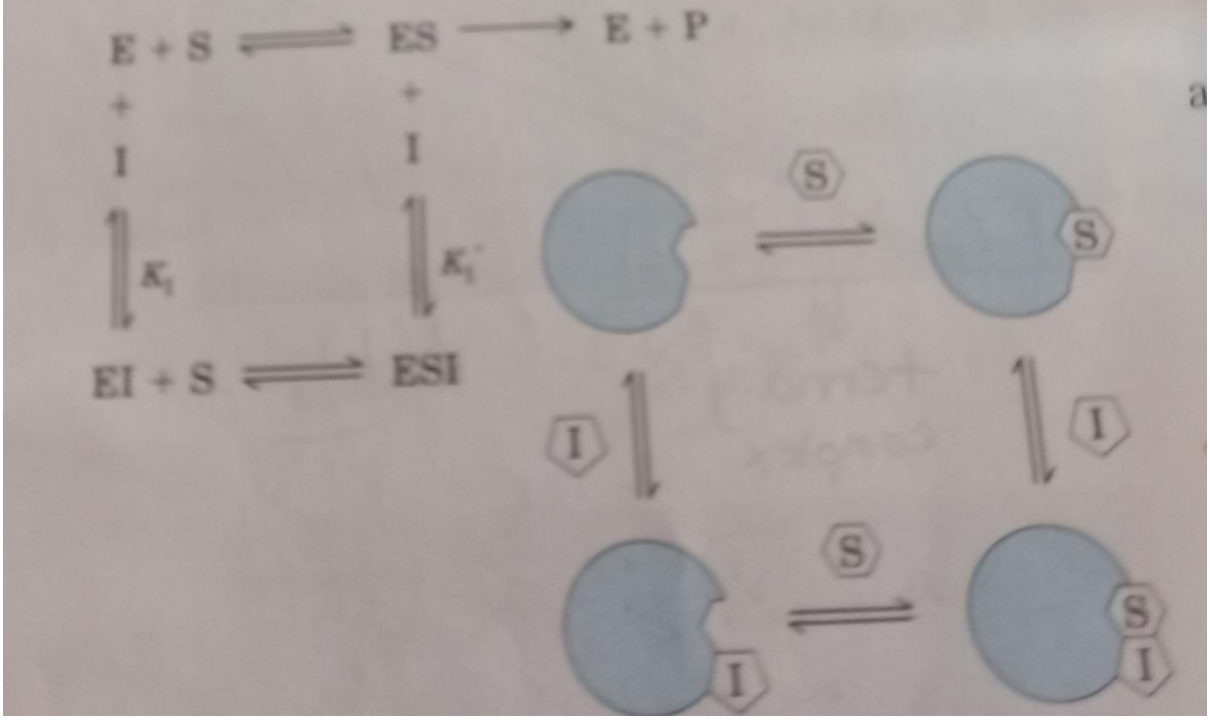


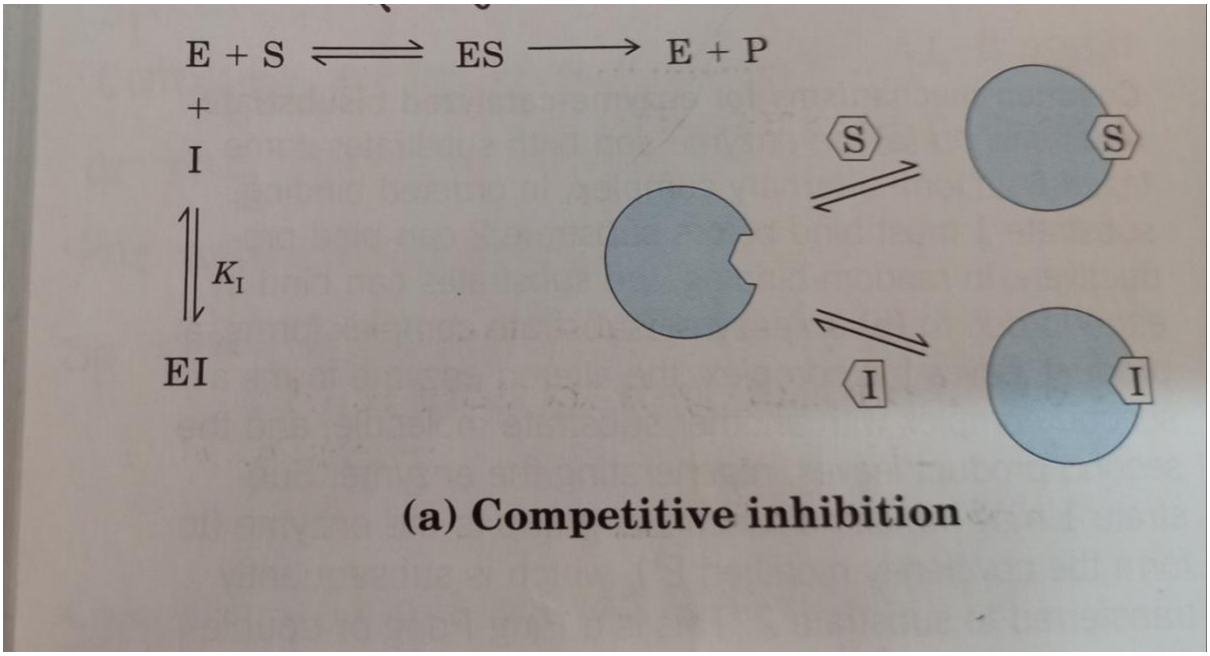
figure 1
Competitive inhibition.



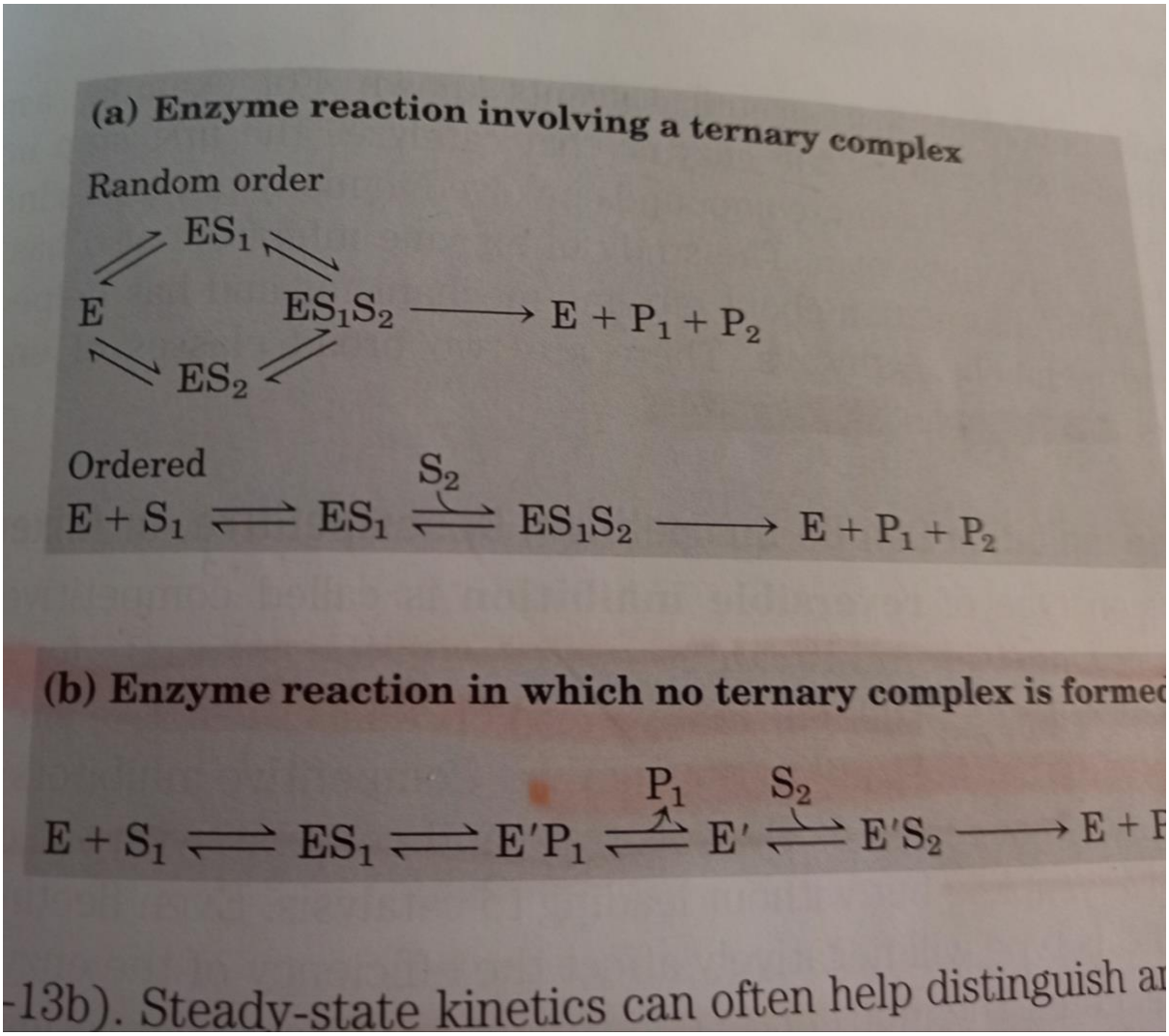
uncompetitive inhibitor does not bind at active site and binds only to the ES complex

(b) Uncompetitive inhibition





(a) Competitive inhibition



	Competitive	unncompetitive	Noncompetitive	
Inhibitor binding	Occupies the active site	Binds at a site distinct from the substrate active site, binds only ES complex	Binds at a site distinct from the substrate active site, it will bind either E or ES	
Michaelis Menten Equation	$V0 = \frac{Vmax[S]}{\alpha Km + S}$ $\alpha = 1 + \frac{[I]}{K_I}$ $K_I = \frac{[E][I]}{[EI]}$			
Apparent Km (αKm)	Increses by the factor α	Deceases Km value	When binds E, effect is like	

			competitive inhibition and binds ES , the effect is like uncompetitive inhibition	
Apparent Vmax	Vmax remains same	Vmax value decreses		
Excess substrate concentration				

Irreversible inhibitors

An irreversible inhibitor will bind to an enzyme so that no other enzyme-substrate complexes can form. It will bind to the enzyme using a covalent bond at the active site which therefore makes the enzyme denatured. An example of an irreversible inhibitor is *diisopropyl fluorophosphate* which is present in nerve gas. It binds to the enzyme and stops nerve impulses being transmitted. An example of where we use irreversible inhibitors in medicine is penicillin. Penicillin works by inhibiting the activity of the enzyme responsible for the creation of the bacterial cell wall. This means that water can enter the bacterial cell, causing it to swell, burst and die termed lysis

Irreversible Inhibitor:

Group-specific reagents: Reacts with specific R group of amino acid.

Diisopropyl phosphofluoridate (DIPF)

Affinity labels: They are the molecules which are structurally similar to the substrate for the enzyme that covalently modify active site residue.

3-bromoacetol is an affinity label molecule for the enzyme Triose phosphate isomerise (TIM). It mimics the normal substrate, dihyroxy acetone phosphate., by binding at the active site, then it covalently modifies.

Suicide inhibitor: Generates a chemically reactive intermediate that inactivates the enzyme through covalent modification. N, N-dimethylproperagylamine is inhibitor of Mono amine Oxidase (MAO), the flavin prosthetic grp is being alkylated by this inhibitor at N5 position. MAO deaminates dopamine .

Penicillin irreversibly inactivates a key enzyme for bacterial cell wall synthesis.

Name of the inhibitors	Name of substrate	Enzyme	Type of inhibition
Methotrexate	Tetrahydrofolate	Dihydrofolate reductase	Competitive
Diisopropylphosphofluoridate	Inhibitors of enzymatic hydrolysis	Acetylcholinesterase	Irreversible
Bromoacetol phosphate	Conversion of Dihydroxyacetone phosphate to glyceraldehydes 3 phosphate	Triosephosphate isomerase	Irreversible
N,N-dimethylpropargyl amine	Deamination of Dopamine or serotonine	Monoamineoxidase	Irreversible
Penicillin	Cross-linking during bacterial cell wall synthesis	Transpeptidase	Irreversible
Malonate	Inhibits the conversion of succinate to fumarate in TCA cycle	Succinate dehydrogenase (Complex II)	Competitive inhibitor
Arsenate	Function of alkaline phosphatase	Alkaline phoshatase	Competitive inhibitor

Isozyme or Isoenzymes are enzymes that differ amino acid sequence yet catalyze the same reaction.Usually , these enzymes display different kinetic parameters, such as KM, or different regulatory properties. They are encoded by different genetic loci, which usually arise through gene duplication and divergence.

- LDH-1: Present primarily in cardiac myocytes and erythrocytes.
- LDH-2: Present mostly in white blood cells.
- LDH-3: Present in highest quantity in lung tissue.
- LDH-4: Highest amounts found in pancreas, kidney, and placenta.
- LDH-5: Highest amounts found in liver and skeletal muscle.

Effect of pH

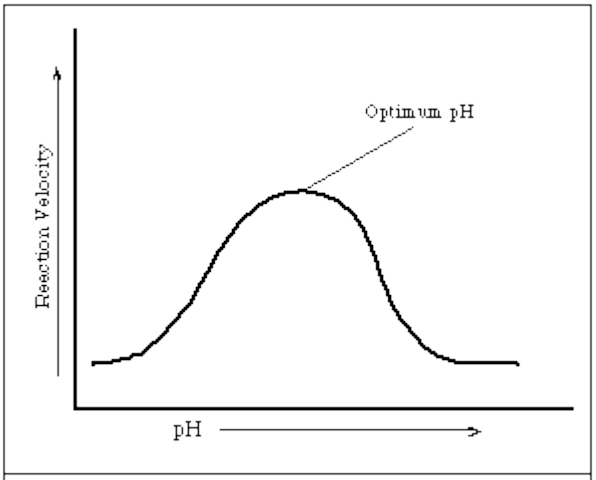
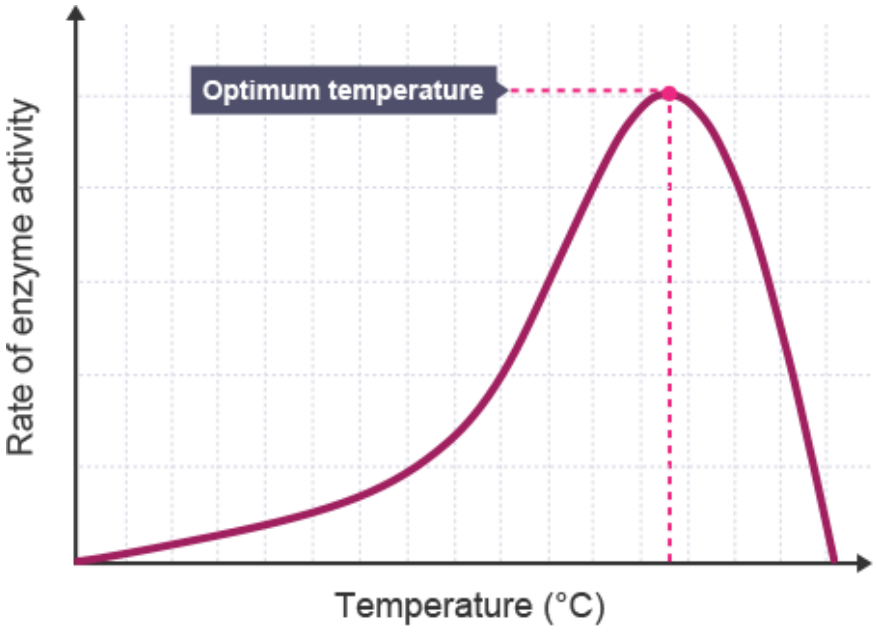
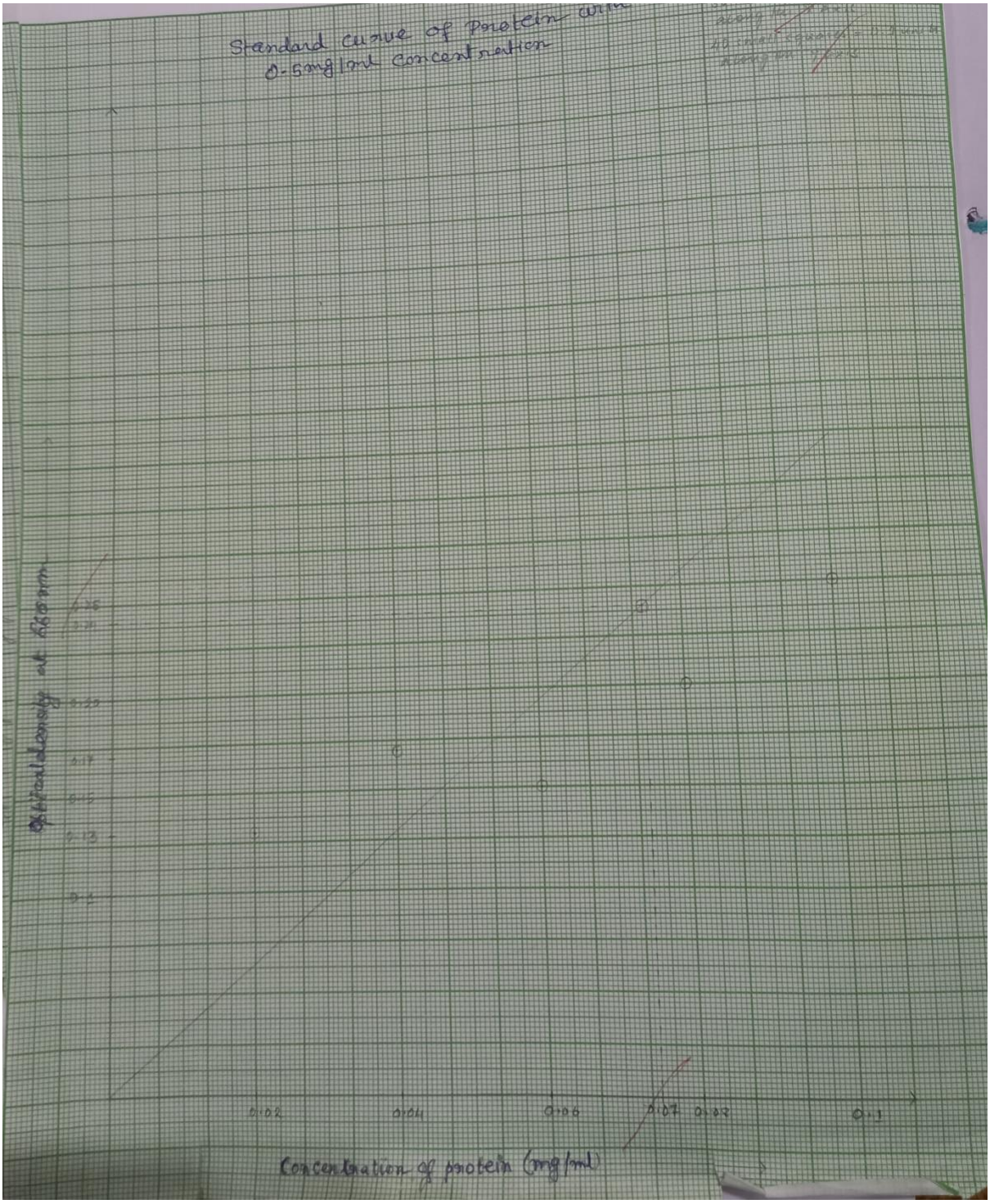


FIGURE 14
Effect of pH on reaction rate

Effect of temperature




Standard curve of Protein with
0-5mg/ml Concentration
Absorbance at 660nm
0.02
0.04
0.06
0.07
0.08
0.1
Concentration of protein (mg/ml)

TubeNo	Concentration of protein (µg/ml)	Optical density at 660 nm
Blank	0.0	0.0
1	50	0.040
2	100	0.070
3	200	0.125
4	400	0.220
5	800	0.390
unknown	---	0.270