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SCHOOL OF BIO & CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – Enzyme Technology – SBTA1401

Enzymes

Enzymes are protein catalysts that increase the velocity of a chemical reaction and are not consumed during the reaction they catalyze.

Differences between enzymes and chemical catalysts

a.Enzymes are proteins.
b.Enzymes are highly specific and produce only the expected products from the given reactants, or substrates (i.e., there are no side reactions).
c.Enzymes may show a high specificity toward one substrate or exhibit a broad specificity, using more than one substrate.
d.Enzymes usually function within a moderate pH and temperature range.

Active sites

Enzyme molecules contain a special pocket or cleft called the active site. The active site contains amino acid side chains that create a three-dimensional surface complementary to the substrate.

The active site binds the substrate, forming an enzyme-substrate (ES) complex. ES is converted to enzyme-product (EP), which subsequently dissociates to enzyme and product

Cofactors

Some enzymes associate with a nonprotein cofactor that is needed for enzymic activity. Commonly encountered cofactors include metal ions (for example, Zn^{2+}, Fe^{2+}) and organic molecules, known as coenzymes, that are often derivatives of vitamins (for example, NAD, FAD, coenzyme A.

Holoenzyme

Refers to the enzyme with its cofactor.

Apoenzyme

Refers to the protein portion of the holoenzyme. In the absence of the appropriate cofactor, the apoenzyme typically does not show biologic activity.

prosthetic group

A prosthetic group is a tightly bound coenzyme that does not dissociate from the enzyme. Most of enzymes can contain additional active site for interaction with regulated molecule. It is called allosteric site.

Allosteric binding sites

Allosteric enzymes are regulated by molecules called effectors (also modifiers or modulators) that bind noncovalently at a site other than the active site. The presence of an allosteric effector can alter the affinity of the enzyme for its substrate or modify the maximal catalytic activity of the enzyme or both. Effectors that inhibit enzyme activity are termed negative effectors, whereas those that increase enzyme activity are called positive effector.

Specificity

The specificity of an enzyme is determined by the functional groups of the substrate, the functional groups of the enzyme, and the physical proximity of these functional groups. Two theories have been proposed to explain the specificity of enzyme action.

•**Lock and key theory**.

The enzyme active site is complementary in conformation to the substrate, so that enzyme and substrate recognize one another.

- active site has a rigid shape
- enzyme only binds substrates that exactly fit the active site
- enzyme is analogous to a lock

- substrate is the key that fits that lock

•**Induced-fit theory**.

The enzyme changes shape on binding substrate, so that the conformation of substrate and enzyme is only complementary after binding.

- enzyme structure is flexible, not rigid
- enzyme and substrate adjust the shape of the active site to bind substrate
- the range of substrate specificity increases
- shape changes improve catalysis during reaction

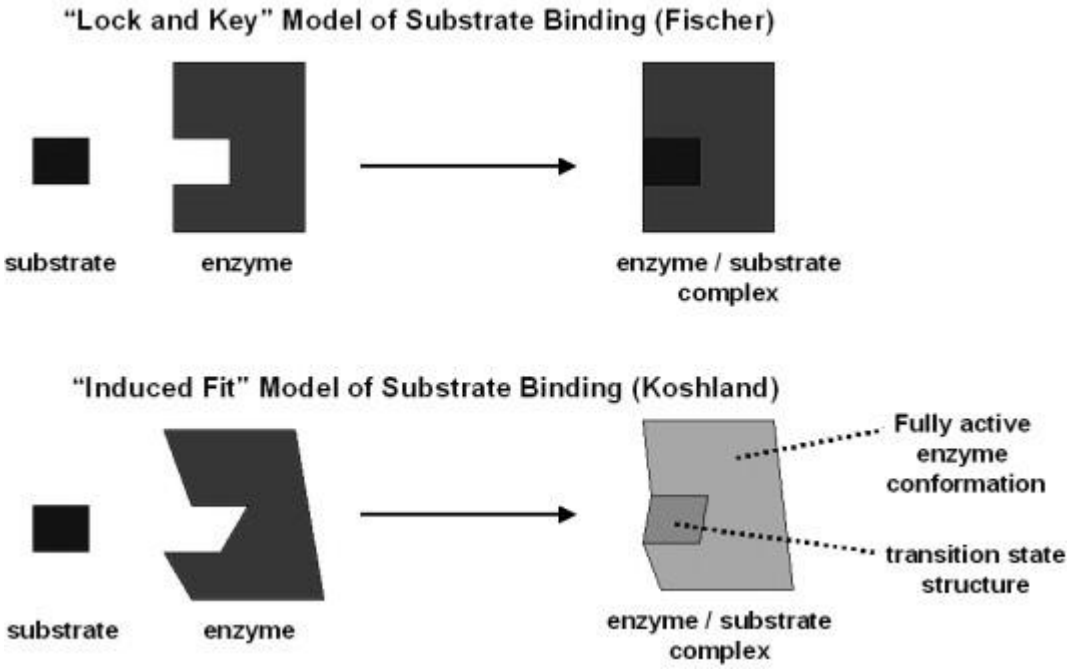


Fig.1. Model of substrate binding to enzyme

Four Steps of Enzyme Action

1. The enzyme and the substrate are in the same area. Some situations have more than one substrate molecule that the enzyme will change.

2. The enzyme grabs on to the substrate at a special area called the active site. The combination is called the **enzyme/substrate complex**. Enzymes are very, very specific and don't just grab on to any molecule. The active site is a specially shaped area of the enzyme that fits around the substrate. The active site is like the grasping claw of the robot on the assembly line. It can only pick up one or two parts.

3. A process called catalysis happens. Catalysis is when the substrate is changed. It could be broken down or combined with another molecule to make something new. It will break or build chemical bonds. When done, you will have the **enzyme/products complex**.

4. The enzyme releases the product. When the enzyme lets go, it returns to its original shape. It is then ready to work on another molecule of substrate.

Enzyme Nomencluature

Early enzymes were assigned arbitrary names (typically before the specific reaction being catalyzed was known) when discovered

eg. Catalase- dismutation of H_2O_2 to H_2O and O_2

Pepsin - Protease (Asp), Endopeptidase

Trypsin - Protease, Endopeptidase

Lysozyme- lyses bacterial cell walls

To standardize enzyme nomenclature, the Enzyme Commission (EC) of the International Union for Biochemistry (IUB) adopted a classification system in 1964; the standards were revised in 1972, 1978 ans 1984.

The IUB system assigns a systematic name to each enzyme, defining the substrate acted on, the reaction catalyzed, and, possibly,the name of any coenzyme involved in

the reaction. Because many systematic names are lengthy, a more usable, trivial, recommended name is also assigned by the IUB system.

In addition to naming enzymes, the IUB system identifies each enzyme by an EC numerical code containing four digits, separated by decimal points. The first digit places the enzyme in one of the following six classes:

1. Oxidoreductases

Catalyze an oxidation-reduction reaction between two substrates.

2. Transferases

Catalyze the transfer of a group other than hydrogen from one substrate to another.

3.Hydrolases

Catalyze hydrolysis of various bonds.

4.Lyases

Catalyze removal of groups from substrates without hydrolysis. The product contains double bonds

5. Isornerases

Catalyze the interconversion of geometric, optical, or positional isomers.

6. Ligases

Catalyze the joining of two substrate molecules, coupled with breaking of the pyrophosphate bond in adenosine triphosphate (ATP) or a similar compound.

The second and third digits of the EC code number represent the subclass and sub-subclass of the enzyme, respectively, divisions that are made according to criteria specific to the enzymes in the class. The final number is the serial number specific to each enzyme in a sub subclass.

Enzyme classification

The 6 major classes of enzymes with some important examples from some subclasses are described below

1. Oxidoreductases.

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.

Second digit	*Hydrogen or electron donor*
1	alcohol (>CHOH)
2	aldehyde or ketone (>C=O)
3	–CH.CH–
4	primary amine (–$CHNH_2$ or –$CH\overset{+}{N}H_3$)
5	secondary amine (>CHNH–)
6	NADH or NADPH (only where some other redox catalyst is the acceptor)

The third-digit refers to the hydrogen or electron acceptor, as follows:

Third digit	*Hydrogen or electron acceptor*
1	NAD^+ or $NADP^+$
2	Fe^{3+} (e.g. cytochromes)
3	O_2
99	An otherwise unclassified acceptor

Trivial names of oxidsoreductases include oxidases (transfer of H to O_2) and dehydrogenases (transfer of H) to an acceptor other than O_2). These often indicate the identity of the donor and/or acceptor.

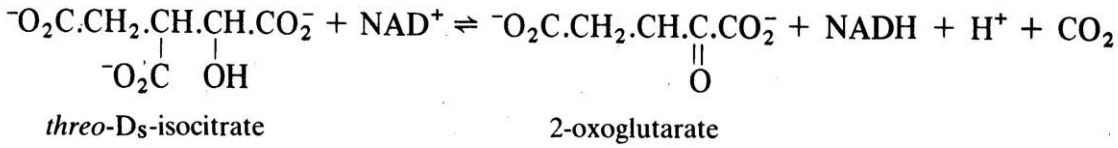
Here are some examples:

L-lactate: NAD^+ oxidoreductose (E.C. 1.1.1.27) (trivial name lactate dehydrogenase) catalyses:

$$CH_3.\underset{\underset{OH}{|}}{CH}.CO_2^- + NAD^+ \rightleftharpoons CH_3.\underset{\underset{O}{||}}{C}.CO_2^- + NADH + H^+$$

L-lactate pyruvate

threo-D_s isocitrate: NAD^+ oxidoreductase (decarboxylating) (E.C. 1.1.1.41) (trivial name isocitrate dehydrogenase) catalyses:



threo-D_S-isocitrate — 2-oxoglutarate

Transferases

These catalyse reactions of the type:

$$AX + B \rightleftharpoons BX + A,$$

but specifically exclude oxidoreductase and hydrolase reactions. In general, the Enzyme Commission recommends that the names of transferases should end 'X-transferase', where X is the group transferred, although a name ending 'trans-X-ase' is an acceptable alternative. The second digit in the classification describes the type of group transferred. For example:

Second digit	*Group transferred*
1	1-carbon group
2	aldehyde or ketone group (>C=O)
3	acyl group (–C(=O)–R)
4	glycosyl (carbohydrate) group
7	phosphate group

In general, the third digit further describes the group transferred. Thus,

E.C. 2.1.1 enzymes are methyltransferases (transfer $-CH_3$) whereas
E.C. 2.1.2 enzymes are hydroxymethyltransferases (transfer $-CH_2OH$) and
E.C. 2.1.3 enzymes are carboxyl- or carbamoyl-transferases (transfer $-\overset{\overset{O}{\|}}{C}-OH$ or $-\overset{\overset{O}{\|}}{C}-NH_2$).

Similarly, E.C. 2.4.1 enzymes are hexosyltransferases (transfer hexose units) and E.C. 2.4.2 enzymes are pentosyltransferases (transfer pentose units).

The exception to this general rule for transferases is where there is transfer of phosphate groups: these cannot be described further, so there is opportunity to indicate the acceptor.

E.C. 2.7.1 enzymes are phosphotransferases with an alcohol group as acceptor,
E.C. 2.7.2 enzymes are phosphotransferases with a carboxyl group as acceptor,
E.C. 2.7.3 enzymes are phosphotransferases with a nitrogenous group as acceptor.

Phosphotransferases usually have a trivial name ending in '-kinase'.

Some examples of transferases are:

Methylmalonyl–CoA: pyruvate carboxyltransferase (E.C. 2.1.3.1) (trivial name: methylmalonyl–CoA carboxyltransferase, formerly transcarboxylase) which catalyses the transfer of a carboxyl group from methylmalonyl–CoA to pyruvate:

$$\underset{\text{methylmalonyl-CoA}}{CH_3.\underset{\displaystyle CO_2^-}{\underset{|}{CH}}.COSCoA} + \underset{\text{pyruvate}}{CH_3.CO.CO_2^-} \rightleftharpoons \underset{\text{propionyl-CoA}}{CH_3.CH_2.COSCoA} + \underset{\text{oxaloacetate}}{\underset{\displaystyle CO_2^-}{\underset{|}{CH_2}}.CO.CO_2^-}$$

ATP: D-hexose-6-phosphotransferase (E.C. 2.7.1.1) (trivial name: hexokinase) which catalyses:

$$\underset{\text{D-hexose}}{C_5H_9O_5.CH_2OH} + ATP \rightleftharpoons \underset{\text{D-hexose-6-phosphate}}{C_5H_9O_5.CH_2OPO_3^{2-}} + ADP$$

Hydrolases

These enzymes catalyse hydrolytic reactions of the form:

$$A{-}X + H_2O \rightleftharpoons X{-}OH + HA.$$

They are classified according to the type of bond hydrolysed. For example:

Second digit	*Bond hydrolysed*
1	ester
2	glycosidic (linking carbohydrate units)
4	peptide $\left(-\overset{\displaystyle O}{\overset{\|\|}{C}}-\overset{\displaystyle H}{\overset{\|}{N}}-\right)$
5	C–N bonds other than peptides

The third digit further describes the type of bond hydrolysed. Thus,

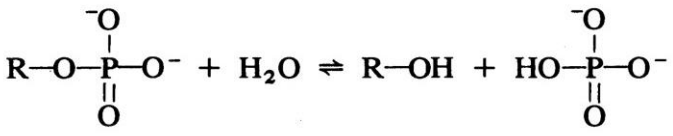
E.C.3.1.1. enzymes are carboxylic ester $\left(-\overset{\displaystyle O}{\overset{\|\|}{C}}-O-\right)$ hydrolases,

E.C. 3.1.2 enzymes are thiol ester $\left(-\overset{\displaystyle O}{\overset{\|\|}{C}}-S-\right)$ hydrolases,

E.C. 3.1.3 enzymes are phosphoric monoester $(-O-PO_3^{2-})$ hydrolases,

E.C. 3.1.4 enzymes are phosphoric diester $\left(-O-\overset{\displaystyle O}{\overset{\|\|}{\overset{\displaystyle -O}{\overset{|}{P}}}}-O-\right)$ hydrolases

For example, orthophosphoric monoester phosphohydrolase (E.C. 3.1.3.1) (alkaline phosphatase) catalyses:



organic phosphate inorganic phosphate

Alkaline phosphatases are relatively non-specific, and act on a variety of substrates at alkaline pH.

The trivial names of hydrolases are recommended to be the only ones to consist simply of the name of the substrate plus '-ase'.

Lyases

These enzymes catalyse the non-hydrolytic removal of groups from substrates, often leaving double bonds.

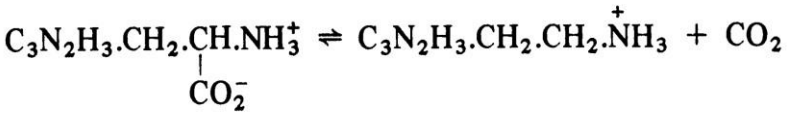
The second digit in the classification indicates the bond broken, for example,

Second digit	*Bond broken*
1	C–C
2	C–O
3	C–N
4	C–S

The third digit refers to the type of group removed. Thus, for the C–C lyases:

Third digit	*Group removed*
1	carboxyl group (i.e. CO_2)
2	aldehyde group (–CH=O)
3	ketoacid group (–C.CO_2^-) ‖ O

For example, L-histidine carboxy-lyase (E.C. 4.1.1.22) (trivial name: histidine decarboxylase, catalyses:



histidine histamine

Isomerases

Enzymes catalysing isomerization reactions are classified according to the type of reaction involved. For example:

Second digit	*Type of reaction*
1	Racemization or epimerization (inversion at an asymmetric carbon atom)
2	cis–trans isomerization
3	intramolecular oxidoreductases
4	intramolecular transfer reaction

The third digit describes the type of molecule undergoing isomerization. Thus, for racemases and epimerases:

Third digit	*Substrate*
1	amino acids
2	hydroxy acids
3	carbohydrates

An example is alanine racemase (E.C. 5.1.1.1) which catalyses:

L-alanine $\rightleftharpoons$ D-alanine

Ligases

These enzymes catalyse the synthesis of new bonds, coupled to the breakdown of ATP or other nucleoside triphosphates. The reactions are of the form:

$$X + Y + ATP \rightleftharpoons X - Y + ADP + P_i$$

or $$X + Y + ATP \rightleftharpoons X - Y + AMP + (PP)_i$$

The second digit in the code indicates the type of bond synthesized. For example:

Second digit	*Bond synthesized*
1	C–O
2	C–S
3	C–N
4	C–C

The third digit further describes the bond being formed. Thus,

E.C. 6.3.1 enzymes are acid–ammonia ligases (amide, $-\overset{\overset{O}{\|}}{C}-NH_2$, synthetases) and

E.C. 6.3.2 enzymes are acid–amino acid ligases (peptide, $-\overset{\overset{O}{\|}}{C}-\underset{\underset{H}{|}}{N}-$, synthetases).

An example is L-glutamate: ammonia ligase (E.C. 6.3.1.2) (trivial name: glutamine synthetase) which catalyses:

$$O{=}\underset{\underset{^-O}{|}}{C}.CH_2.CH_2.\underset{\underset{^+NH_3}{|}}{CH}.CO_2^- + ATP + NH_3 \rightleftharpoons O{=}\underset{\underset{NH_2}{|}}{C}.CH_2.CH_2.\underset{\underset{^+NH_3}{|}}{CH}.CO_2^- + ADP + P_i$$

L-glutamate L-glutamine

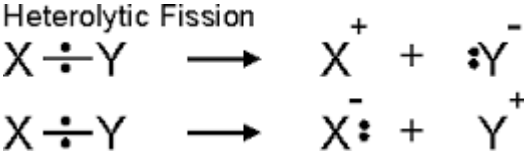
Chemical catalysis

Homolytic fission

Homolytic fission is chemical bond dissociation of a molecule by a process where each of the fragments retains one of the originally bonded electrons.

Heterolytic fission involves cleavage of a chemical bond in a process where both of the electrons involved in the original bond remain with only one of the fragment species.

Heterolytic Fission



In heterolytic fission if both electrons are retained by a carbon atom, a carbonion is produced

$$R_3C{:}X \rightleftharpoons R_3C{:}^{-} + X^{+}$$

carbanion

If neither electron is retained by the carbon atom, a **carbonium ion** is produced

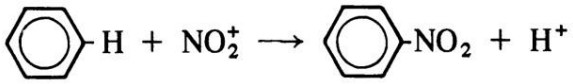
$$R_3C{:}X \rightleftharpoons R_3C^{+} + {:}X^{-}$$

carbonium ion

Organic compound can participate in four different types of reactions

1. Displacement or Substitution reaction
2. Addition reaction
3. Elimination reaction
4. Rearrangements

Substitution reactions may be **nucleophilic**, when the group attacking the carbon atom is an electron donor called a nucleophile since it is attracted to nuclei; alternatively they may be **electrophilic**, when the attacking group is an electron acceptor, or electrophile. Electrophilic substitution reactions often involve the displacement of hydrogen. For example:



benzene nitrobenzene

In **nucleophilic substitution**, an atom other than hydrogen is usually displaced. Such reactions may have a unimolecular or a bimolecular mechanism.

In **unimolecular nucleophilic substitution (S_N1)** reactions, the rate-limiting step is the ionization of a single molecule to form a carbonium ion which then reacts with a nucleophile, as in the following example:

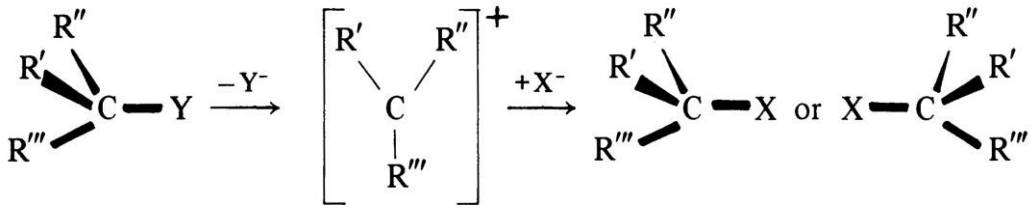


Fig 2 Mechanism of unimolecular nucleophilic substitution

In **bimolecular nucleophilic substitution (S_N2)** reactions, the attacking nucleophile adds to the carbon atom at a point diametrically opposite the leaving group, which it displaces in one rapid step:

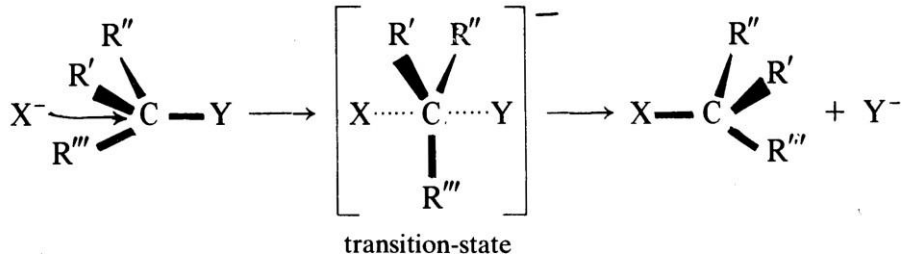


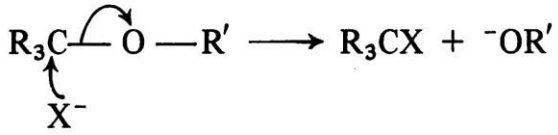
Fig 3 Mechanism of biimolecular nucleophilic substitution

It is unnecessary here to go into details about the other types of reaction. **Addition reactions** involve the addition of an electrophile or a nucleophile to form a relatively stable compound without any group being displaced; **elimination reactions** involve the removal of a group, usually in a strongly basic solution, without it being replaced by another group; **rearrangements** are reactions where bonds are formed and broken within a single molecule.

Acid–base catalysis

Acid–base catalysis, acceleration of a chemical reaction by the addition of an acid or a base, the acid or base itself not being consumed in the reaction. The catalytic reaction may be acid-specific (acid catalysis), as in the case of decomposition of the sugar sucrose into glucose and fructose in sulfuric acid; or base-specific (base catalysis), as in

the addition of hydrogen cyanide to aldehydes and ketones in the presence of sodium hydroxide.



In the presence of an acid, however, conditions are much more favourable:

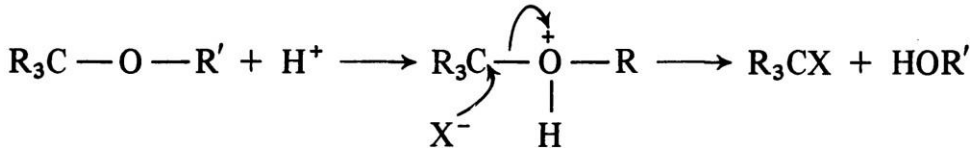
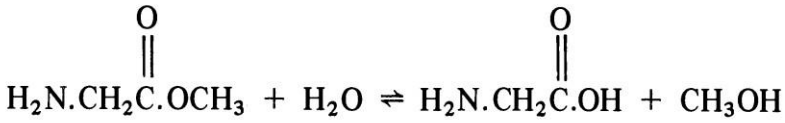


Fig 4 Mechanism of acid catalysis

Electrostaticcatalysis

A transition-state may be stabilized by electrostatic interaction between its charged groups and charged groups on a catalyst. Thus, the positive charge on a carbonium ion can be stabilized by interaction with a negatively charged carboxylate ion; similarly, the negative charge on an oxyanion can be stabilized by a positively charged metal ion. For example, the hydrolysis of glycine esters:



may be catalysed by cupric ions, the mechanism probably involving the following steps:

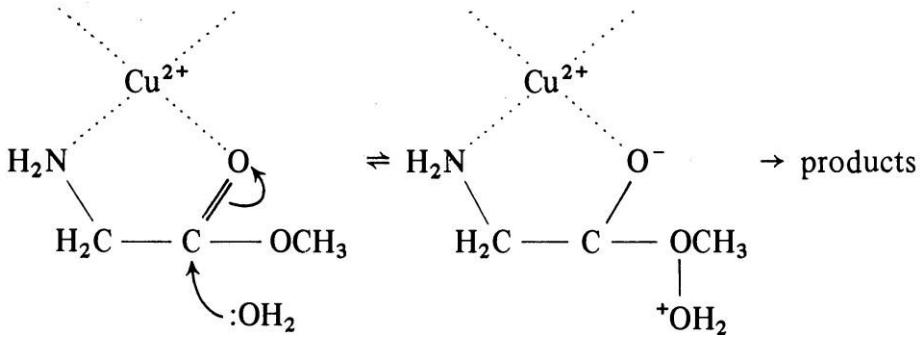


Fig 5 Mechanism of electroststic catalysis

Covalent catalysis

In contrast to acid–base and electrostatic catalysis, where the transition-state is merely modified, covalent catalysis introduces a different reaction mechanism and is sometimes termed **alternative pathway catalysis**. In the case of nucleophilic catalysis, the catalyst is more nucleophilic than the normal attacking groups and so rapidly forms an intermediate which itself rapidly breaks down to give the products. For example, Bender and co-workers (1957) have shown that a variety of tertiary amines catalyse the hydrolysis of esters, as follows:

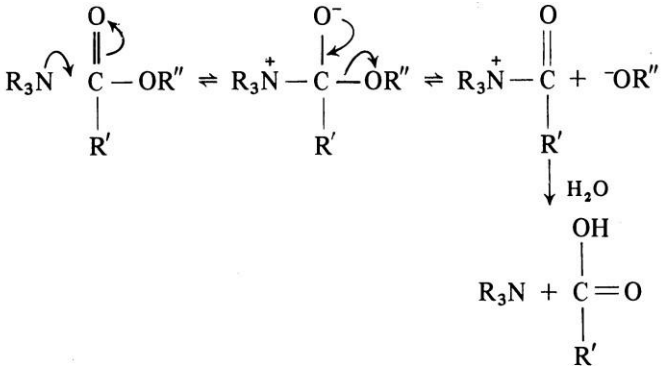


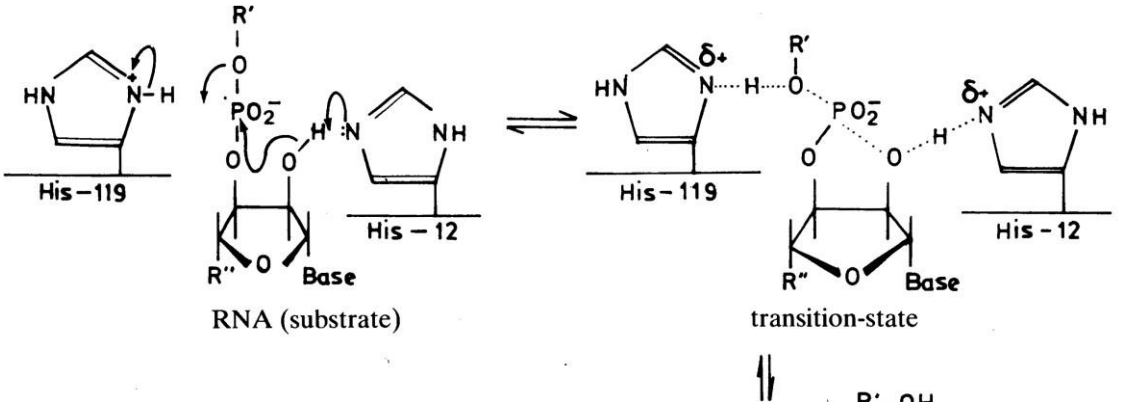
Fig 6 Mechanism of covalent catalysis

Enzyme catalysis

All of the above mechanisms of catalysis are seen in enzyme-catalysed reactions. However, enzymes, because of their great size and range of properties are able to impose their presence on a reaction to a far greater extent than most catalysts.

Ribonuclease A

An example of concerted acid-base catalysis - reaction subject to both general acid and general base catalysis



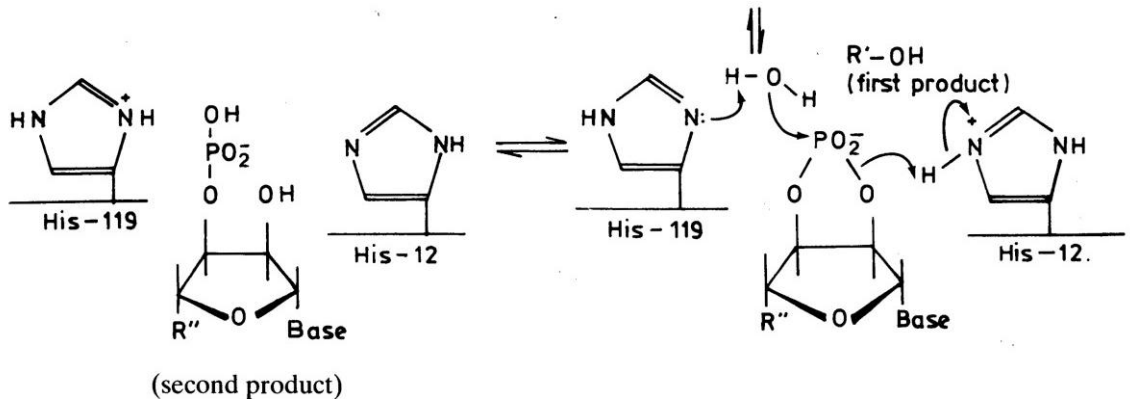
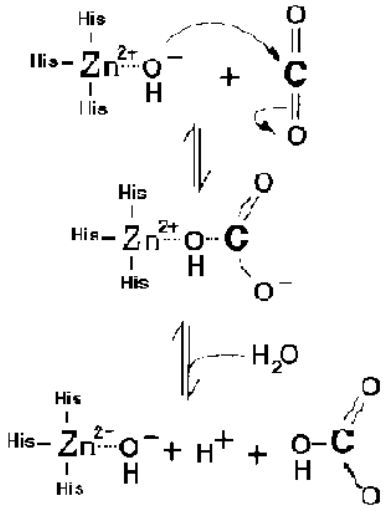


Fig 7 Mechanism of acid base catalysis

Example for electrostatic catalysis

Carbonic anhydrase

- $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$
- balance the pH in the kidney during filtration of urine from the serum
- Zn^{2+} → help activate the bound water molecule to generate a hydroxide ion which can attack carbon dioxide



Example for covalent catalysis is chymotripsin

Chymotrypsin is a digestive enzyme that breaks down proteins (i.e., it is a proteolytic enzyme; it can also be referred to as a protease). It is naturally produced by the pancreas in the human body. Chymotrypsin cleaves the peptide bond at carboxyl side of aromatic amino acids residue.

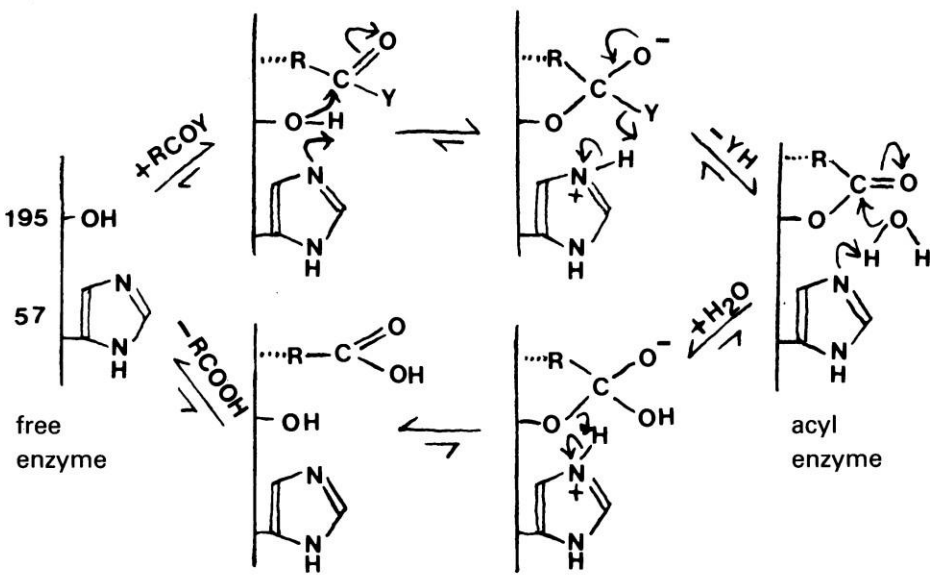
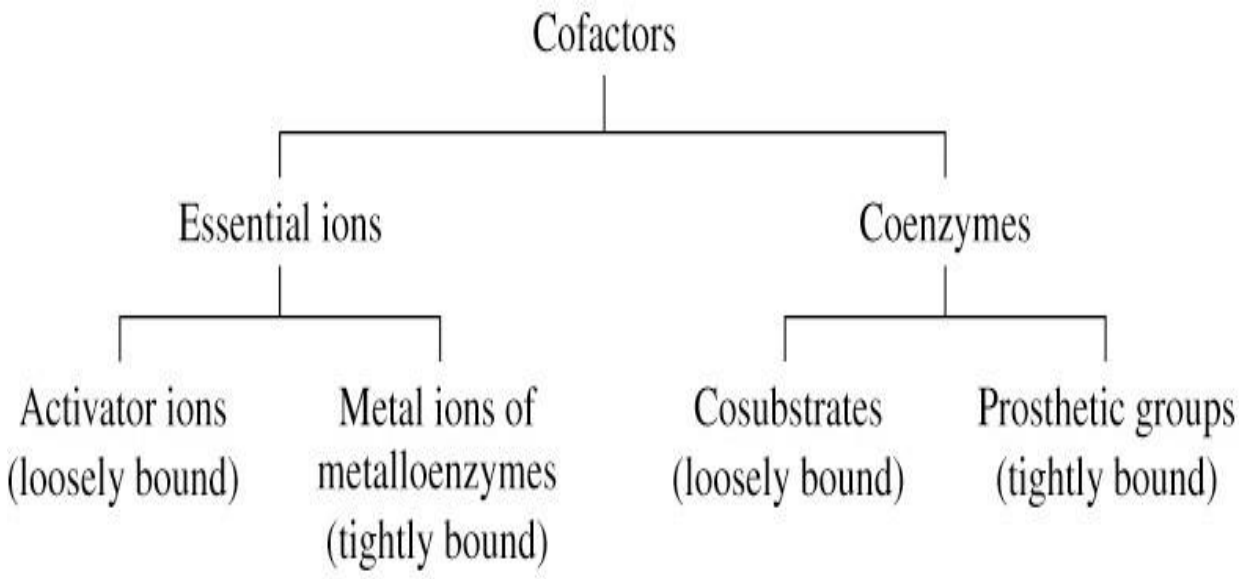


Fig 8 Mechanism of covalent catalysis

CO-ENZYMES AND COFACTORS

- A cofactor is a small non-protein molecules that is bound (either tightly or loosely) to an enzyme and is required for catalysis.
- Catalytic activity of many enzymes depends on the presence of cofactors.

Types of cofactors



Essential Ion Cofactors

- **Activator ions** – bind reversibly to enzyme and often participate in substrate binding.
- **Metal ions of metalloenzymes** – cations that are tightly bound to enzyme and participate directly in catalysis (Fe, Zn, Cu, Co).
- **Metal activated enzymes** – require or are stimulated by addition of metal ions (i.e. Mg^{2+}, is required by many ATP requiring enzymes)

Inorganic Cofactors

S.No.	**Ion**	**Examples of enzymes containing this ion**
1.	Cupric	Cytochrome oxidase
2.	Ferrous or Ferric	Cytochrome (via Heme) Hydrogenase
3.	Magnesium	Glucose 6-phosphatase

		Hexokinase
4.	Manganese	Arginase
5.	Molybdenum	Nitrate reductase Nitrogenase
6.	Nickel	Urease
7.	Zinc	Alcohol dehydrogenase Carbonic anhydrase

Coenzyme

- Coenzymes are derivatives of vitamins without which the enzyme cannot exhibit any reaction. One molecule of coenzyme is able to convert a large number of substrate molecules with the help of enzyme.
- Coenzyme accepts a particular group removed from the substrate or donates a particular group to the substrate (The function of coenzymes is to transport groups between enzymes)
- Chemical groups include hydride ions which are carried by coenzymes such as NAD, phosphate groups which are carried by coenzymes such as ATP and acetyl groups which are carried by coenzymes such as coenzyme A.
- Coenzymes which lose or gain these chemical groups in the course of the reaction are often reformed in the same metabolic pathway. For example NAD+ used in glycolysis and the citric acid cycle is replaced in the electron transport chain of respiration.
- Coenzymes are called co substrate because the changes that take place in substrates are complimentary to the changes in coenzymes.

- Loosely attached to apoenzymes, seperated easily by dialysis they are often called **cosubstrate or secondary substrate.**
- A coenzyme is a necessary helper for enzymes that assist in biochemical transformations.
- A coenzyme Transport a variety of chemical groups (Such as Hydride, Acetyl, Formyl, Methenyl or methyl). By using following reaction
    - Oxidoreduction,
    - Group transfer,
    - Isomerization and
    - Covalent bond formation reaction.

Vitamins of B complex group acting as co-enzymes

S.no.	Vitamins	Co-enzyme
1.	Thiamine Vitamin B 1	TPP (thiamine pyrophosphate)
2.	Riboflavin Vitamin B 2	FMN, FAD
3.	Niacin Vitamin B 3	NAD,NADH
4.	Pantothenic acid Vitamin B 5	component of coenzyme A
5.	Pyridoxine Vitamin B 6	PLP (pyridoxal phosphate)
6.	Biotin	Biotin
7.	Folic acid	THF (Tetrahydrofolate)
8.	Cobalamine Vitamin B 12	cobamide

1. Adenosine triphosphate (ATP)

- The function of ATP is to transport chemical energy within cells for metabolism, and as such ATP is often referred to as the energy currency of cells.
- Adenosine triphosphate is composed of an adenine nucleotide base, a ribose sugar and three phosphate groups.
- Energy can be released from ATP when the terminal phosphate group is released in a hydrolysis reaction.
- This is because the energy of ATP is held in the bonds between the phosphate groups and when the bonds are broken it is accompanied by a release of energy.

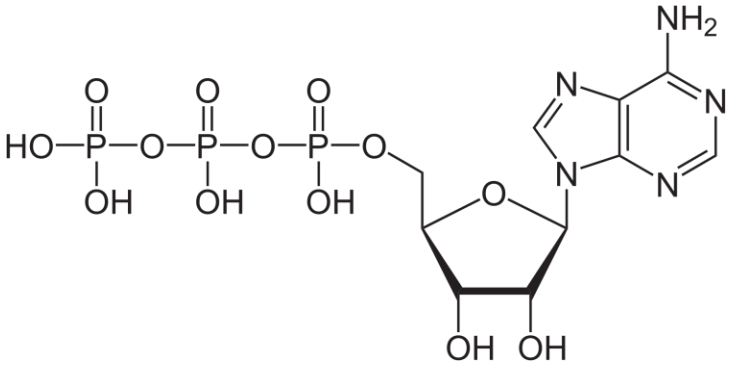


Fig 9 Structure of ATP

2. THIAMIN PYROPHOSPHATE (TPP)

- Thiamin is rapidly converted to thiamin pyrophosphate (TPP) in small intestine, brain and liver.
- TPP is formed from thiamin by the action of thiamine diphosphotransferase.
- TPP coenzyme is required by enzymes in the decarboxylation of α-keto acids.

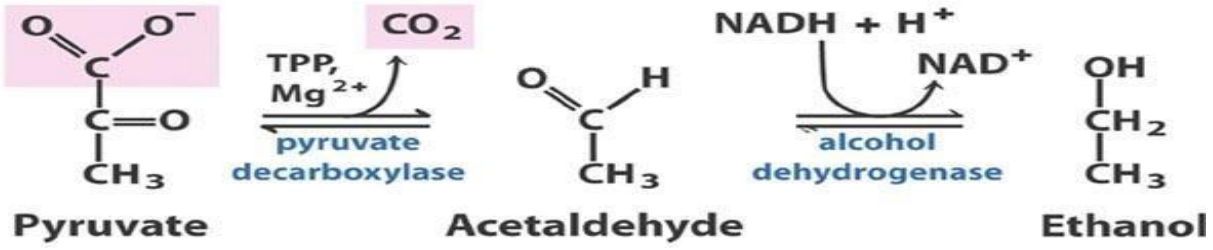


Fig 10 Mechanism of action of TPP

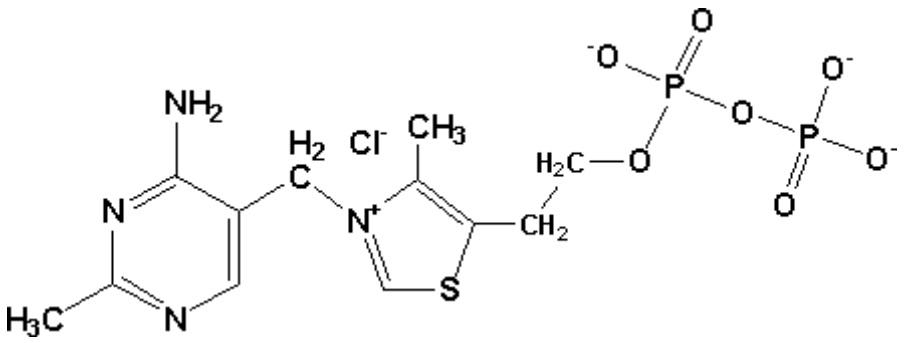


Fig 11 Structure of TPP

3. FLAVIN ADENINE DINUCLEOTIDE (FAD) AND FLAVIN MONONUCLEOTIDE (FMN)

- FAD is composed of an adenine nucleotide, a ribose sugar and two phosphate groups.
- FAD can also exist as a monophosphate and is called flavin adenine monophosphate (FMN).
- The primary role of FAD is in oxidative phosphorylation.
- FAD is involved with redox reactions and like NAD, FAD can exist in two redox states; FAD and FADH. The two states are interconvertable as a result of the addition or removal of electrons.
- This is possible because FAD is able to accept hydride ions with their electron pairs.

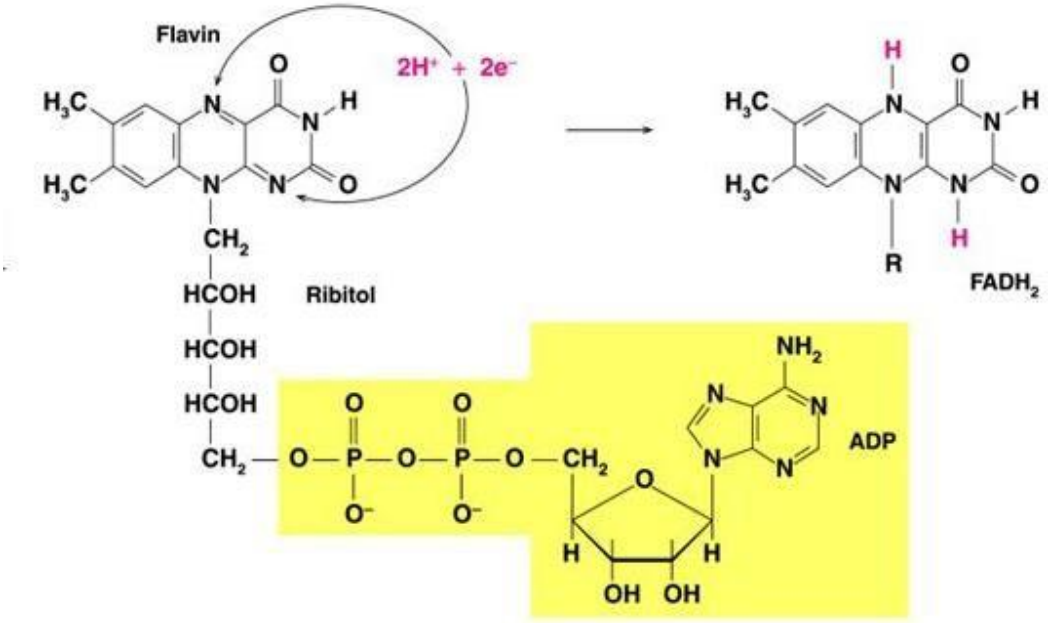
- 

Fig 12 Structure of FAD & reduced state

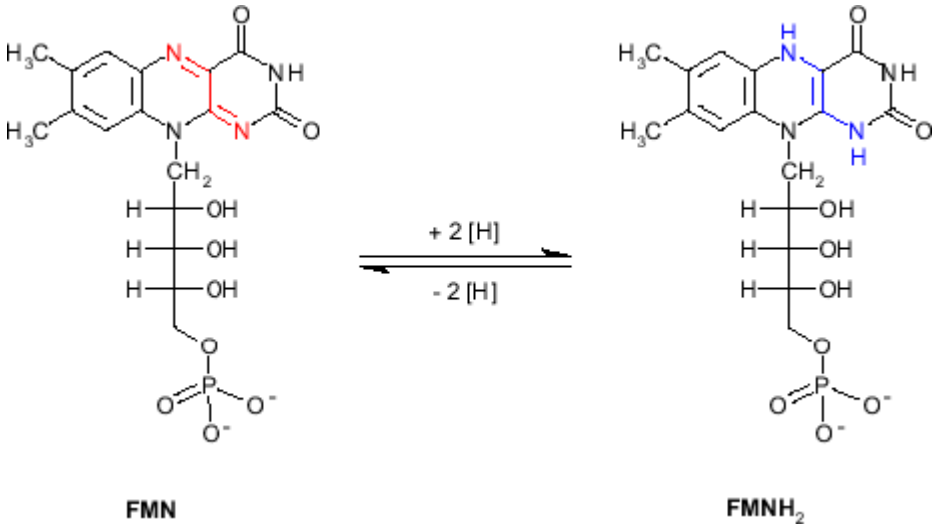


Fig 13 Structure of FMN & reduced state

- For example, FAD is a coenzyme used by the enzyme succinate dehydrogenase to help catalyse a reaction. The role of FAD in this reaction is to accept two electrons from succinate which results in the production of fumarate. FAD is

reduced to FADH2 but remains tightly bound to succinate dehydrogenase. No further reactions can occur until FAD is regenerated.

- They play key roles in hydrogen transfer reactions associated with
  - Glycolysis
  - TCA cycle
  - Oxidative phosphorylation.

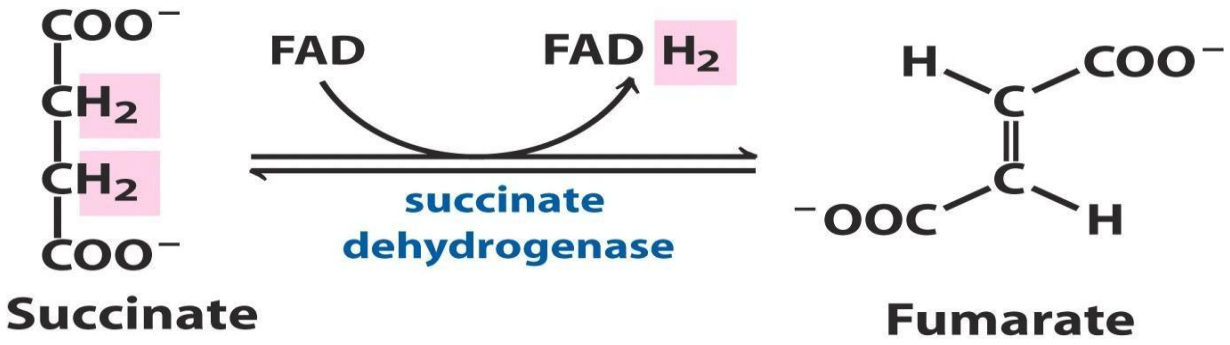


Fig 14 Mechanism of action of FAD

4. Nicotine Adenine Dinucleotide (NAD) & Nicotine Adenine Dinucleotide Phosphate (NADP)

- NAD is composed of two nucleotides, adenine and nicotinamide. The nucleotides are held together by a pair of phosphate groups which act as a bridge and are also bonded to a ribose sugar each.
- The function of NAD is to carry electrons from one enzyme controlled reaction to another.
- As such NAD is involved with redox reactions because substrates are either oxidised, in which they lose electrons or are reduced in which they gain electrons.

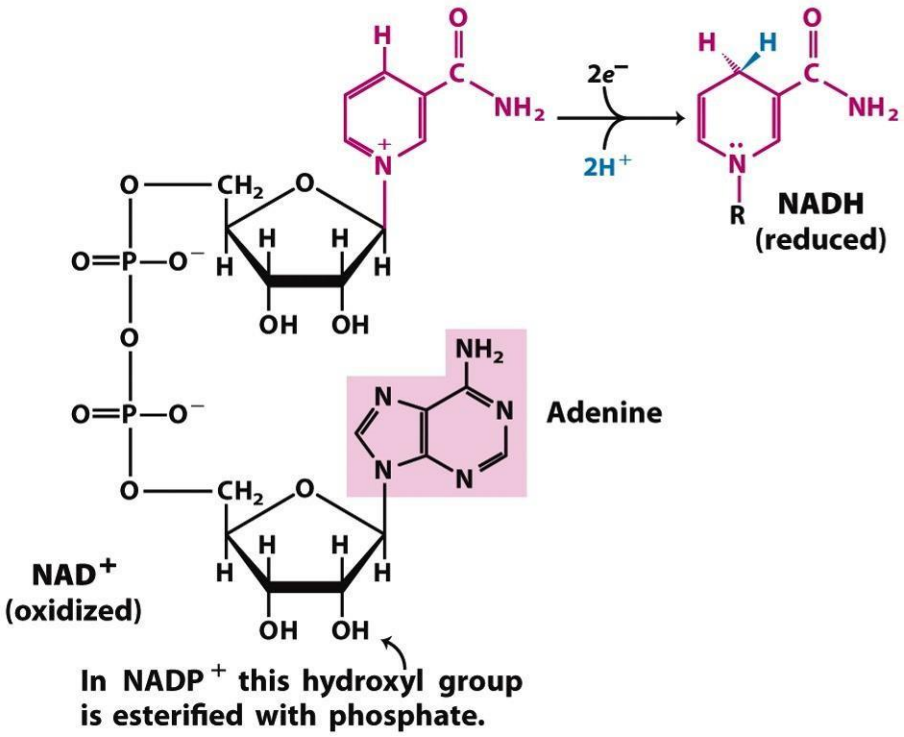


Fig 15 Structure of NAD

- NAD is either found as NAD+, which is an oxidising agent and is involved with accepting electrons from other molecules, or NADH which is used as a reducing agent to donate electrons to other molecules.

5. Coenzyme A

- Coenzyme A is not tightly bound to the enzymes to which it is associated and is able to freely be released.
- It plays an important role in the metabolism of protiens, carbohydrates and fats which are important reactions that allow the energy from food to be released.
- For example coenzyme A is required for the oxidation of pyruvate in the citric acid cycle.

- In addition coenzyme A is involved with acetylation reactions. These reactions are important in proper protein function and as a result many of the proteins in the body have undergone such modification reactions in which an acetate group is added to the protein.
- The acetate group is donated from coenzyme A. The addition of an acetate group alters the 3D structure of the protiens to which it is added and as a result thier function is also altered.
- In some cases a long chain fatty acid is also donated to the protien. This addition is needed for the cell signalling properties of various membrane protiens.
- Acetate groups from coenzyme A are also added to various substrates in reactions involved with gene expression and cell division.
- Coenzyme A is also important in the synthesis of cholesterol and steroid hormones, and is required for the detoxification of a range of harmful drugs that can accumulate in the liver.

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SCHOOL OF BIO & CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – II – Enzyme Technology – SBTA1401

Michaelis Menten Kinetics

The Michaelis-Menten model is one of the simplest and best-known approaches to enzyme kinetics. It takes the form of an equation relating reaction velocity to substrate concentration for a system where a substrate *S* binds reversibly to an enzyme *E* to form an enzyme-substrate complex *ES*, which then reacts irreversibly to generate a product *P* and to regenerate the free enzyme *E*. This system can be represented schematically as follows:

$$E + S \rightleftharpoons ES \rightarrow E + P$$

The Michaelis-Menten equation arises from the general equation for an enzymatic reaction:

$$E + S \leftrightarrow ES \leftrightarrow E + P$$

where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex, and P is the product. Thus, the enzyme combines with the substrate in order to form the ES complex, which in turn converts to product while preserving the enzyme.

The rate of the forward reaction from E + S to ES may be termed k_1, and the reverse reaction as k_{-1}. Likewise, for the reaction from the ES complex to E and P, the forward reaction rate is k_2, and the reverse is k_{-2}. Therefore, the ES complex may dissolve back into the enzyme and substrate, or move forward to form product.

In this model, the substrate S reversibly associates with the enzyme E in a first step, and some of the resulting complex ES is allowed to break down and yield the product P and the free enzyme back. We would like to know how to recognize an enzyme that behaves according to this model. One way is to look at the enzyme's kinetic behavior -- at how substrate concentration affects its rate. So we want to know what rate law such an enzyme would obey. If a newly discovered enzyme obeys that rate law, then we can assume that it acts according to this model. Let's derive a rate law from this model.

For this model, let v_0 be the initial velocity of the reaction. The latter stands for the appearance of the product P in solution (+ d[P]/dt) whose**phenomenological rate equation** (first-order) is given by

$v_0 = k_{cat}[ES]$ (2),

containing an experimentally measurable (dependent) variable - v_0, a kinetic parameter - k_{cat}, and another variable unknown to us - [ES].

Before proceeding, one should state (and remember) some implicite assumptions:

- As long as **initial velocity** is considered, the concentration of product can be neglected (compared to that of the substrate, thus [P] << [S]), and
- The concentration of substrate is in large excess over that of the enzyme ([E] << [S]).

These assumptions, which hold in most kinetic experiments performed in test tubes at low enzyme concentration, are convenient when considering the mass conservation equations for the reactants $[S]_0 = [S]_{free} + [ES] + [P]$ which now approximates to **$[S]_0 = [S]$**, while that for the enzyme is $[E]_{total} = [E]_{free} + [ES]$ (the possible formation of a complex EP is not considered here).

We want to express v_0 in terms of measurable (experimentally defined, independent) variables, like [S] and $[E]_{total}$, so we can see how to test the mechanism by experiments in kinetics. So we must replace the unknown [ES] in (2) with measurables.

During the initial phase of the reaction, *as long as the reaction velocity remains constant*, the reaction is in a **steady state**, with ES being formed and consumed at the same rate. During this phase, the rate of formation of [ES] (one second order kinetic step) equals its rate of consumption (two first orderkinetic steps). According to model (1),

Rate of formation of [ES] = $k_1[E][S]$.

Rate of consumption of [ES] = k_{-1}[ES] + k_{cat} [ES].

So in the **steady state**,

$k_{-1}[ES] + k_{cat}[ES] = k_1[E][S]$ (3)

Remember that we are trying to solve for [ES] in terms of measurables, so that we can replace it in (2). First, collect the kinetic constants, and the concentrations (variables) in (3):

$(k_{-1} + k_{cat})\,[ES] = k_1\,[E][S]$,

and (4)

$(k_{-1} + k_{cat})/k_1 = [E][S]/[ES]$

To simplify (4), first group the kinetic constants by defining them as K_m:

$K_m = (k_{-1} + k_{cat})/k_1$ (5)

and then express [E] in terms of [ES] and $[E]_{total}$, to limit the number of unknowns:

$[E] = [E]_{total} - [ES]$ (6)

Substitute (5) and (6) into (4)

$K_m = ([E]_{total} - [ES])\,[S]/[ES]$ (7)

Solve (7) for [ES]:

First multiply both sides by [ES] (no Black Magic involvement here...):

$[ES]\,K_m = [E]_{total}[S] - [ES][S]$

Then collect terms containing [ES] on the left:

$[ES]\,K_m + [ES][S] = [E]_{total}[S]$

Factor [ES] from the left-hand terms:

$[ES](K_m + [S]) = [E]_{total}[S]$

and finally, divide both sides by (K_m + [S]):

$[ES] = [E]_{total}\,[S]/(K_m + [S])$ (8)

Substitute (8) into (2):

$v_0 = k_{cat}[E]_{total}\,[S]/(K_m + [S])$ (9)

The maximum velocity V_{max} occurs when the enzyme is saturated -- that is, when all enzyme molecules are tied up with S, or $[ES] = [E]_{total}$. Thus,

$V_{max} = k_{cat}\,[E]_{total}$ (10)

Substitute V_{max} into (9) for $k_{cat}\,[E]_{total}$:

$v_0 = V_{max}\,[S]/(K_m + [S])$ (11)

This equation expresses the initial rate of reaction in terms of a measurable quantity, the **initial** substrate concentration. The two kinetic parameters, $V_{max}$and K_m , will be different for every enzyme-substrate pair.

Reaction Order Note

When [S]<<Km[S]<<Km,

v=Vmax[S]Kmv=Vmax[S]Km

This means that the rate and the substrate concentration are directly proportional to each other. The reaction is first-order kinetics.

When [S]>>Km[S]>>Km,

v=Vmaxv=Vmax

This means that the rate is equal to the maximum velocity and is independent of the substrate concentration. The reaction is zero-order kinetics.

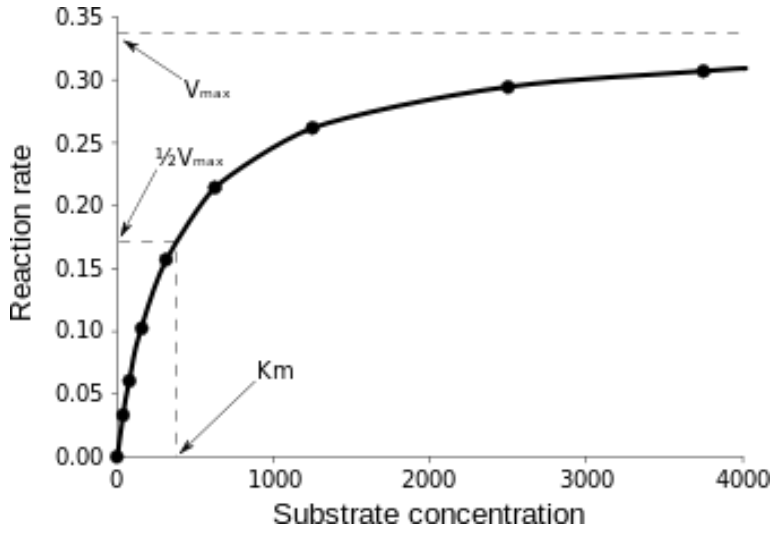


Fig 1 Reaction of zero order kinetics

Then, at

v=Vmax2v=Vmax2, Km=[S]Km=[S]

v=Vmax2=Vmax[S]Km+[S]v=Vmax2=Vmax[S]Km+[S]

Therefore, Km is equal to the concentration of the substrate when the rate is half of the maximum velocity. From the Michaelis Menten Kinetic equation, we have many different ways to find Km and Vmax such as the Lineweaver-Burk plot, Hanes- Woolf plot, and Eadie-Hofstee plot, etc.

Determination of V_{max} and K_m

It is important to have as thorough knowledge as is possible of the performance characteristics of enzymes, if they are to be used most efficiently. The kinetic parameters V_{max}, K_m and k_{cat}/K_m should, therefore, be determined. There are twoapproaches to this problem using either the reaction progress curve (integral method) or the initial rates of reaction (differential method). Use of either method depends on prior knowledge of the mechanism for the reaction and, at least approximately, the optimum conditions for the reaction. If the mechanism is known and complex then the data must be reconciled to the appropriate model (hypothesis),

usually by use of a computer-aidedanalysis involving a weighted least-squares fit. Many such computer programs are currently available and, if not, the programming skill involved is usually fairly low. If the mechanism is not known, initial attempts are usually made to fit the data to the Michaelis-Menten kinetic model. Combining equations (1.1) and (1.8),

$$\frac{d[P]}{dt} = V = \frac{V_{max}[S]}{K_m + [S]} \qquad (1.99)$$

which, on integration, using the boundary condition that the product is absent at time zero and by substituting [S] by ($[S]_0$ - [P]), becomes

$$t = \frac{[P]}{V_{max}} - \frac{K_m}{V_{max}} Ln\left(\frac{[S]_0 - [P]}{[S]_0}\right) \qquad (1.100)$$

If the fractional conversion (***X***) is introduced, where

$$\boldsymbol{X} = \frac{[S]_0 - [P]}{[S]_0} \qquad (1.101)$$

then equation (1.100) may be simplified to give:

$$t = \frac{\boldsymbol{X}[S]_0}{V_{max}} - \frac{K_m Ln(1-\boldsymbol{X})}{V_{max}} \qquad (1.102)$$

Use of equation (1.99) involves the determination of the initial rate of reaction over a wide range of substrate concentrations. The initial rates are used, so that [S] = $[S]_0$, the predetermined and accurately known substrate concentration at the start of the reaction. Its use also ensures that there is no effect of reaction reversibility or product inhibition which may affect the integral method based on equation (1.102). Equation (1.99) can be utilised directly using a computer program, involving a weighted least-squares fit, where the parameters for determining the hyperbolic relationship between the initial rate of reaction and initial substrate concentration (i.e. K_m and V_{max}) are chosen in order to

minimise the errors between the data and the model, and the assumption is made that the errors inherent in the practically determined data are normally distributed about their mean (error-free) value.

Alternatively the direct linear plot may be used. This is a powerful non-parametric statistical method which depends upon the assumption that any errors in the experimentally derived data are as likely to be positive (i.e. too high) as negative (i.e. too low). It is common practice to show the data obtained by the above statistical methods on one of three linearised plots, derived from equation (1.99). Of these, the double reciprocal plot is preferred to test for the qualitative correctness of a proposed mechanism, and the Eadie-Hofstee plot is preferred for discovering deviations from linearity.

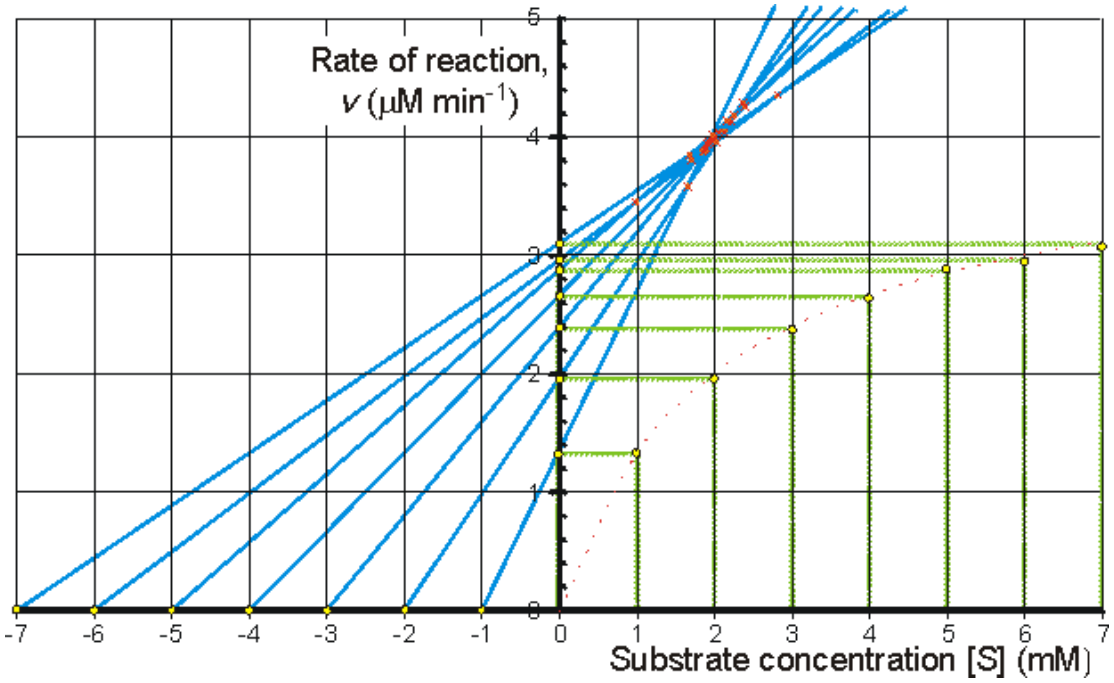


Fig 2 Rate of enzymatic reaction

The direct linear plot. A plot of the initial rate of reaction against the initial substrate concentration also showing the way estimates can be directly made of the K_m and V_{max}. Every pair of data points may be utilised to give a separate estimate of these parameters (i.e. n(n-1)/2 estimates from n data points with differing $[S]_0$). These estimates are determined from the intersections of lines passing through the (x,y) points $(-[S]_0,0)$ and

(0,v); each intersection forming a separate estimate of K_m and V_{max}. The intersections are separately ranked in order of increasing value of both K_m and V_{max} and the median values taken as the best estimates for these parameters. The error in these estimates can be simply determined from sub-ranges of these estimates, the width of the sub-range dependent on the accuracy required for the error and the number of data points in the analysis. In this example there are 7 data points and, therefore, 21 estimates for both K_m and V_{max}. The ranked list of the estimates for K_m (mM) is 0.98,1.65, 1.68, 1.70, 1.85, 1.87, 1.89, 1.91, 1.94, 1.96, **1.98**, 1.99, 2.03, 2.06, 1.12, 2.16, 2.21, 2.25, 2.38, 2.40, 2.81, with a median value of 1.98 mM. The K_m must lie between the 4th (1.70 mM) and 18th (2.25 mM) estimate at a confidence level of 97% (Cornish-Bowden *et al.*, 1978). The list of the estimates for V_{max}($M.min^{-1}$) is ranked separately as 3.45, 3.59, 3.80, 3.85, 3.87, 3.89, 3.91, 3.94, 3.96, 3.96, **3.98**, 4.01, 4.03, 4.05, 4.13, 4.14, 4.18, 4.26, 4.29, 4.35, with a median value of 3.98 $M.min^{-1}$. The V_{max} must lie between the 4th (3.85 $M.min^{-1}$) and 18th (4.18 $M.min^{-1}$) estimate at a confidence level of 97%. It can be seen that outlying estimates have little or no influence on the results. This is a major advantage over the least-squared statistical procedures where rogue data points cause heavily biased effects.

Three ways in which the hyperbolic relationship between the initial rate of reaction and the initial substrate concentration

$$V = \frac{V_{max}[S]_0}{K_m + [S]_0}$$

can be rearranged to give linear plots. The examples are drawn using K_m = 2 mM and V_{max} = 4 M min^{-1}.

(a) Lineweaver-Burk (double-reciprocal) plot of 1/v against $1/[S]_0$ giving intercepts at $1/V_{max}$ and $-1/K_m$

$$\frac{1}{v} = \frac{K_m}{V_{max}} x \frac{1}{[S]_0} + \frac{1}{V_{max}} \qquad (1.103)$$

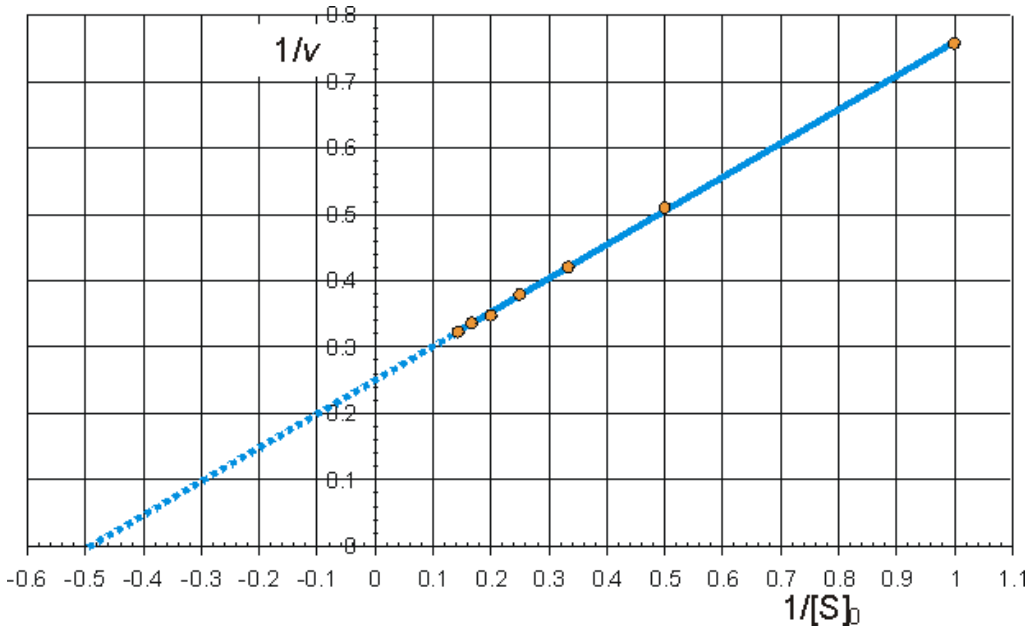


Fig 3 LB plot

(b) Eadie-Hofstee plot of v against v/$[S]_0$ giving intercepts at V_{max} and V_{max}/K_m

$$v = -K_m x \frac{v}{[S]_0} + V_{max} \qquad (1.104)$$

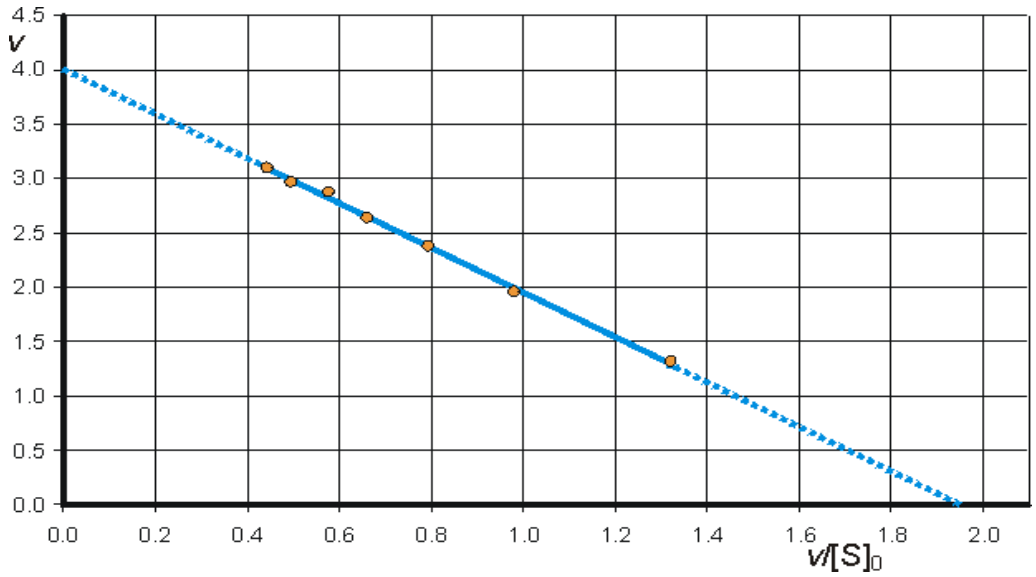


Fig 4 Eadie-Hofstee plot

c) Hanes-Woolf (half-reciprocal) plot of $[S]_0/v$ against $[S]_0$ giving intercepts at

K_m/V_{max} and K_m.

$$\frac{[S]_0}{v} = \frac{1}{V_{max}} x[S]_0 + \frac{K_m}{V_{max}} \qquad (1.105)$$

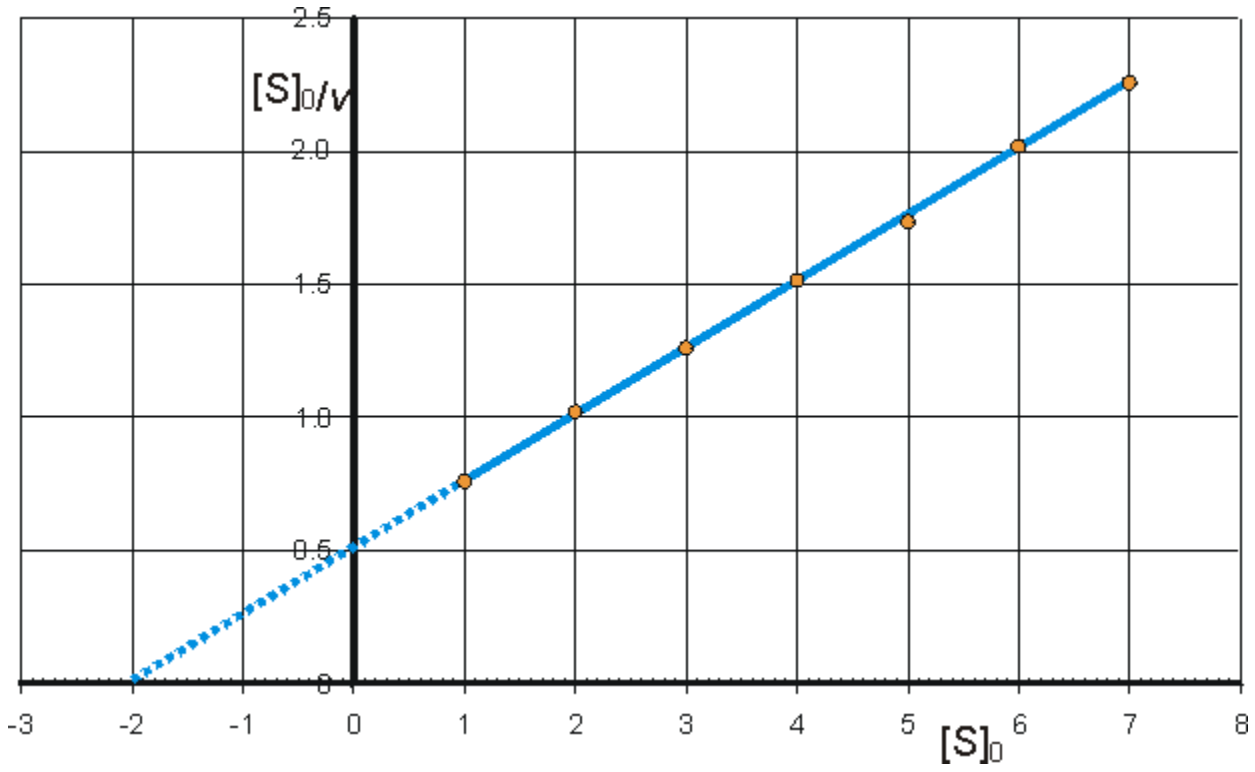


Fig 5 Hanes-Woolf plot

The progress curve of the reaction can be used to determine the specificity constant (k_{cat}/K_m) by making use of the relationship between time of reaction and fractional conversion (see equation (1.102). This has the advantage over the use of the initial rates (above) in that fewer determinations need to be made, possibly only one progress curve is necessary, and sometimes the initial rate of reaction is rather difficult to determine due to its rapid decline. If only the early part of the progress curve, or its derivative, is utilised in the analysis, this procedure may even be used in cases where there is competitive inhibition by the product, or where the reaction is reversible.

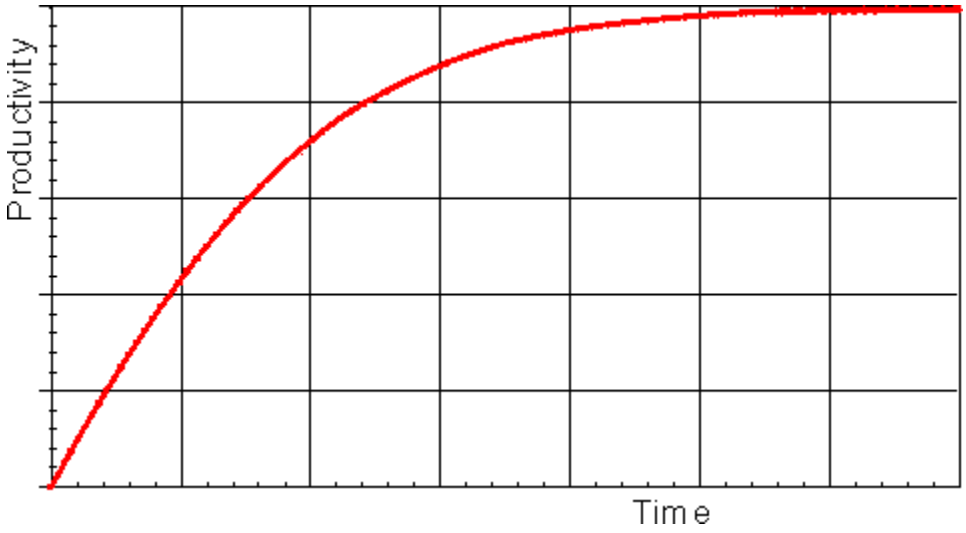


Fig 6 Hyperbolic curve

A schematic plot showing the amount of product formed (productivity) against the time of reaction, in a closed system. The specificity constant may be determined by a weighted least-squared fit of the data to the relationship given by equation (1.102).

The type of inhibition and the inhibition constants may be determined from the effect of differing concentrations of inhibitor on the apparent K_m, V_{max} and k_{cat}/K_m, although some more specialised plots do exist (e.g. Cornish-Bowden, 1974).

Lineweaver-Burk Plot

For example, by taking the reciprocal of the Michaelis Menten Kinetics Equation, we can obtain the Lineweaver-Burk double reciprocal plot:

vo=(Vmax[S])(KM+[S])vo=(Vmax[S])(KM+[S])

1v=(kM+[S])vmax[S]1v=(kM+[S])vmax[S]

1v=(KmVmax)(1[S])+1Vmax1v=(KmVmax)(1[S])+1Vmax

Apply this to equation for a straight line y=mx+by=mx+b and we have:

y=1vy=1v

x=1[S]x=1[S]

m=slope=KmVmaxm=slope=KmVmax

b=y−intercept=1Vmaxb=y−intercept=1Vmax

When we plot y=1vy=1v versus x=1[S]x=1[S], we obtain a straight line.

x−intercept=−1Kmx−intercept=−1Km

y−intercept=1Vmaxy−intercept=1Vmax

slope=KmVmaxslope=KmVmax

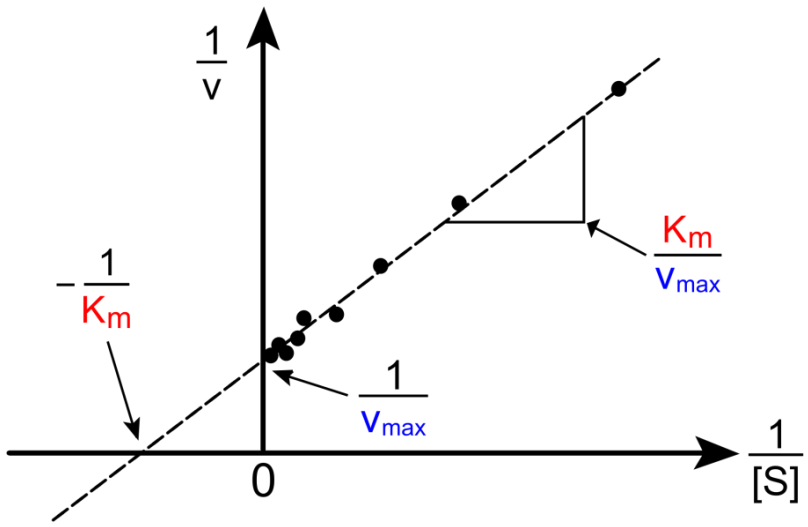


Fig 7 An example of a Lineweaver-Burke plot.

Eadie-Hofstee

In biochemistry, an **Eadie–Hofstee diagram** is a graphical representation of enzyme kinetics in which reaction rate is plotted as a function of the ratio between rate andsubstrate concentration:

$$v = -K_m \frac{v}{[S]} + V_{\max}$$

where *v* represents reaction rate, K_m is the Michaelis–Menten constant, [*S*] is the substrate concentration, and V_{max} is the maximum reaction rate.

It can be derived from the Michaelis–Menten equation as follows:

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

invert and multiply with $V_{\max}$:

$$\frac{V_{\max}}{v} = \frac{V_{\max}(K_m + [S])}{V_{\max}[S]} = \frac{K_m + [S]}{[S]}$$

Rearrange:

$$V_{\max} = \frac{vK_m}{[S]} + \frac{v[S]}{[S]} = \frac{vK_m}{[S]} + v$$

Isolate v:

$$v = -K_m \frac{v}{[S]} + V_{\max}$$

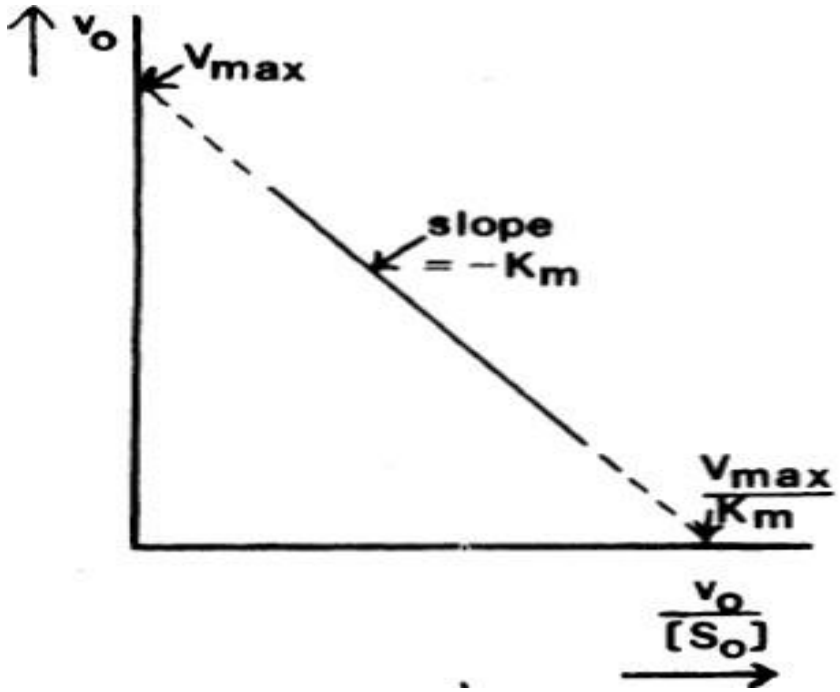


Fig 8 EH plot

A plot of v against *v*/[S] will hence yield V_{max} as the y-intercept, V_{max}/K_m as the x-intercept, and K_m as the negative slope. Like other techniques that linearize the Michaelis–Menten equation, the Eadie-Hofstee plot was used historically for rapid

identification of important kinetic terms like K_m and V_{max}, but has been superseded by nonlinear regression methods that are significantly more accurate and no longer computationally inaccessible. It is also more robust against error-prone data than the Lineweaver–Burk plot, particularly because it gives equal weight to data points in any range of substrate concentration or reaction rate. (The Lineweaver–Burk plot unevenly weights such points.) Both plots remain useful as a means to present data graphically.

One drawback from the Eadie–Hofstee approach is that neither ordinate nor abscissa represent independent variables: both are dependent on reaction rate. Thus any experimental error will be present in both axes. Also, experimental error or uncertainty will

propagate un evenly and become larger over the abscissa thereby giving more weight to smaller values of$v$/[S]. Therefore, the typical measure of goodness of fit for linear regression, the correlation coefficient R, is not applicable.

Hanes–Woolf plot

In biochemistry, a **Hanes–Woolf plot** is a graphical representation of enzyme kinetics in which the ratio of the initial substrate concentration [S] to the reaction velocity v is plotted against [S]. It is based on the rearrangement of the Michaelis–Menten equationshown below:

$$\frac{[S]}{v} = \frac{[S]}{V_{\max}} + \frac{K_m}{V_{\max}}$$

where K_m is the Michaelis–Menten constant and V_{max} is the maximum reaction velocity.

J B S Haldane stated that this method was due to Barnet Woolf.[1] It was also used by Charles Samuel Hanes, even though he neither mentions nor cites Woolf.[2] Hanes pointed out that the use of linear regression to determine kinetic parameters from this type of linear transformation is flawed, because it generates the best fit between observed and calculated values of 1/v, rather than v.

The equation can be derived from the Michaelis–Menten equation as follows:

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

invert and multiply by [S]:

$$\frac{[S]}{v} = \frac{[S](K_m + [S])}{V_{\max}[S]} = \frac{K_m + [S]}{V_{\max}}$$

Rearrange:

$$\frac{[S]}{v} = \frac{1}{V_{\max}}[S] + \frac{K_m}{V_{\max}}$$

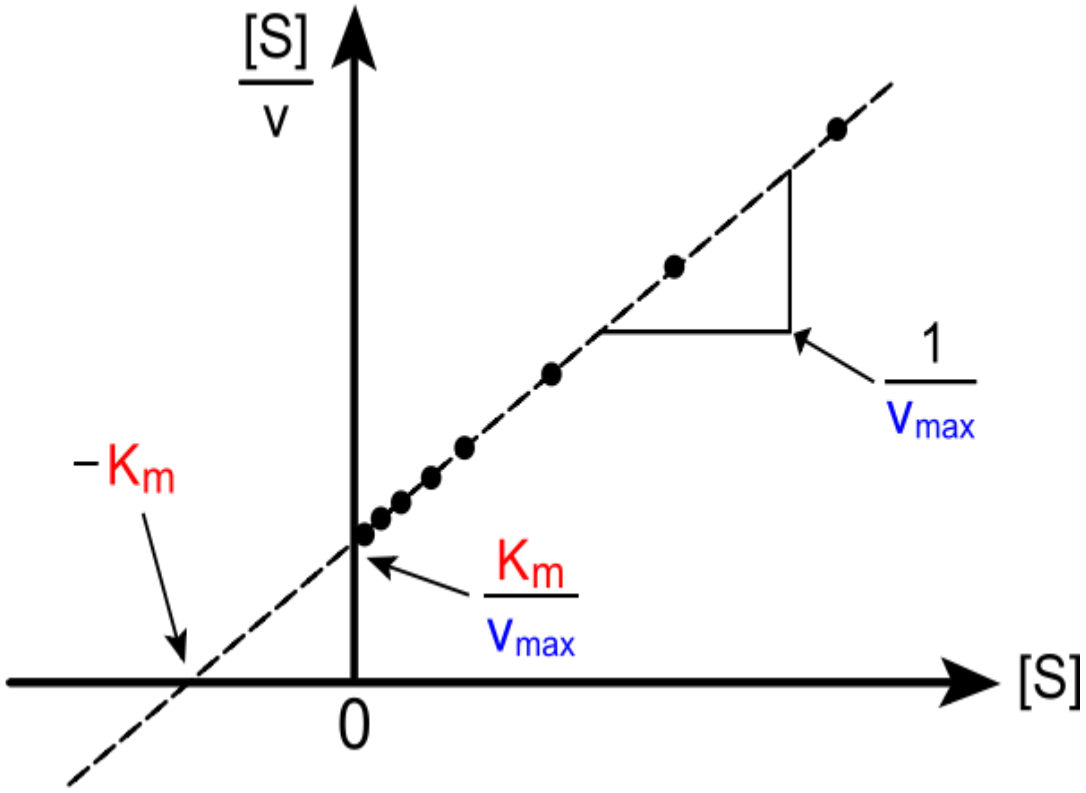


Fig 9 Hanes plot

As is clear from the equation, perfect data will yield a straight line of slope $1/V_{max}$, a *y*-intercept of K_m/V_{max} and an *x*-intercept of $-K_m$.

Like other techniques that linearize the Michaelis–Menten equation, the Hanes–Woolf plot was used historically for rapid determination of the important kinetic parameters K_m, V_{max} and V_{max}/K_m, but it has been superseded by nonlinear regression methods that are significantly more accurate and no longer computationally inaccessible. It remains useful, however, as a means to present data graphically.

One drawback of the Hanes–Woolf approach is thatneither ordinate nor

abscissa represent independent variables: both are dependent on substrate concentration. As a result, the typical measure of goodness of fit, the correlation coefficient *R*, is not applicable.

Enzyme inhibitors

Various compounds can reduce the activity of enzymes. They may act in a variety of different ways, and indeed may be reversible or irreversible inhibitors of the enzyme.

Irreversible inhibitor

An irreversible inhibitor causes covalent modification of the enzyme, so that its activity is permanently reduced. Compounds that act as irreversible inhibitors are often useful as drugs that need be taken only every few days, although adjusting the dose to suit the patient's response is a lengthy process with such compounds. By contrast, the effect of a reversible inhibitor can be reversed by removing the inhibitor, e.g. by dialysis or gel filtration.

The normal sequence of an enzyme reaction can be represented as:

$$E + S \rightleftharpoons E\text{-}S \rightleftharpoons E\text{-}P \rightleftharpoons E + P$$

where:
E=enzyme
S=substrate
E-S=enzyme-substrate complex
E-P=enzyme-product complex

P = product

Reversible inhibitors

There are three main types of reversible inhibitor:

- competitive inhibitor
- non-competitive inhibitor
- uncompetitive inhibitor

They interact with the enzyme or enzyme-substrate complex at different stages in the sequence

Competitive inhibition

A competitive inhibitor competes with the substrate for the active site of the enzyme:

$$E\text{-}I \rightleftharpoons E + S + I \rightleftharpoons E\text{-}S \rightleftharpoons E\text{-}P \rightleftharpoons E + P$$

This means that increasing the concentration of substrate will decrease the chance of inhibitor binding to the enzyme. Hence, if the substrate concentration is high enough the enzyme will reach the same Vmax as without the inhibitor. However, it will require a higher concentration of substrate to achieve this and so the Km of the enzyme will also be higher.

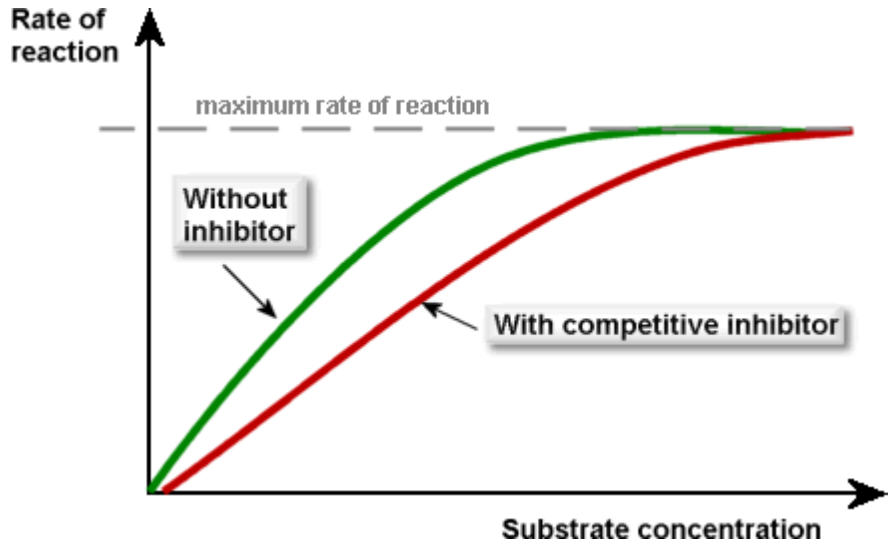


Fig 10 Inhibition reaction

Reacting the enzyme with a range of concentrations of substrate at different concentrations of a competitive inhibitor will give a family of curves as shown below:

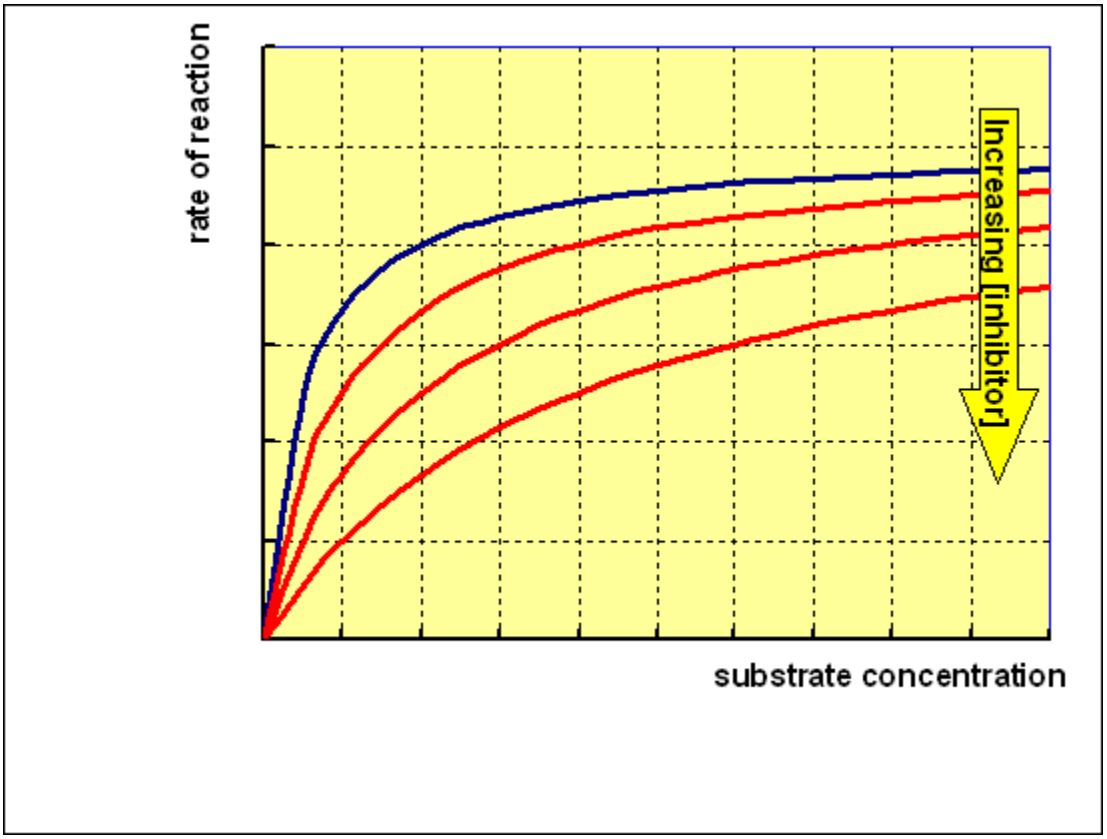


Fig 11 Increasing concentration of inhibitors

The Lineweaver-Burk double reciprocal plot for this set of data shows a series of lines crossing the y (1/v) axis at the same point - i.e. Vmax is unchanged, but with a decreasing value of 1/Km (and hence a higher Km) in the presence of the inhibitor:

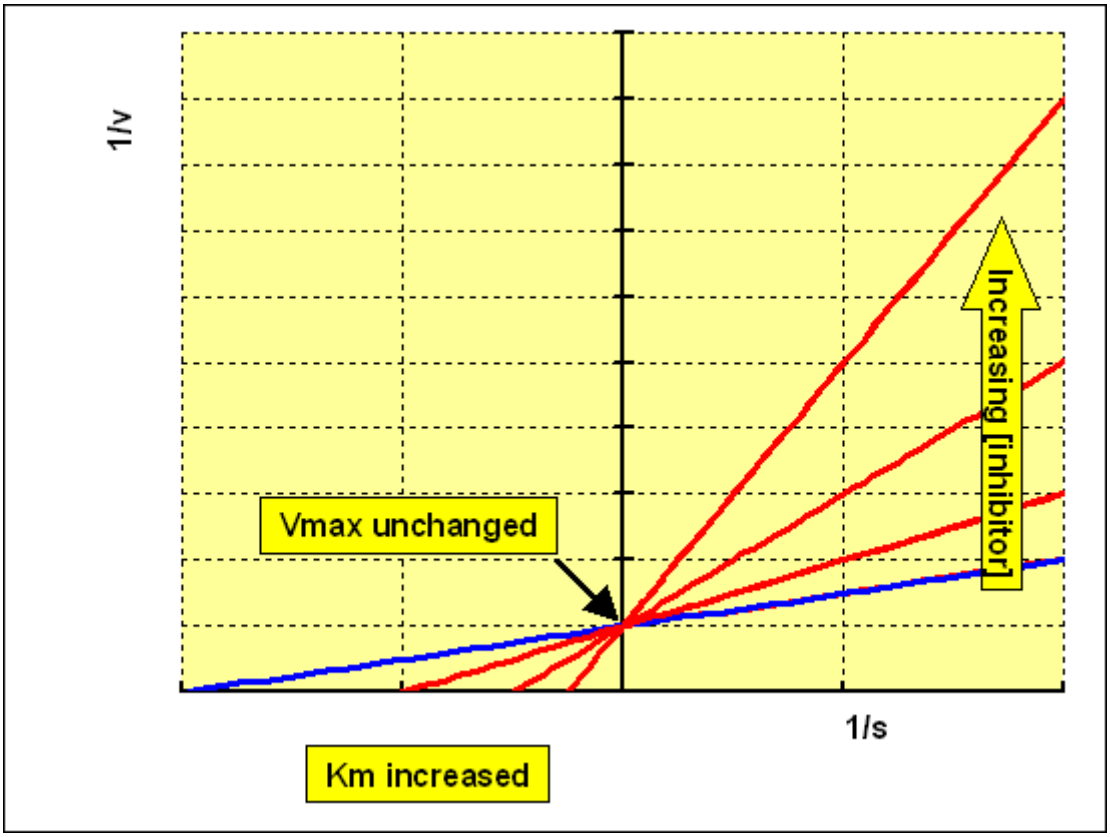


Fig 12 LB plot of inhibited enzyme reaction

Non-competitive inhibition

A non-competitive inhibitor reacts with the enzyme-substrate complex, and slows the rate of reaction to form the enzyme-product complex.

$$E + S + I \rightleftharpoons E\text{-}S + I \rightleftharpoons E\text{-}S\text{-}I \rightleftharpoons E\text{-}P\text{-}I \rightleftharpoons E + P + I$$

This means that increasing the concentration of substrate will not relieve the inhibition, since the inhibitor reacts with the enzyme-substrate complex.

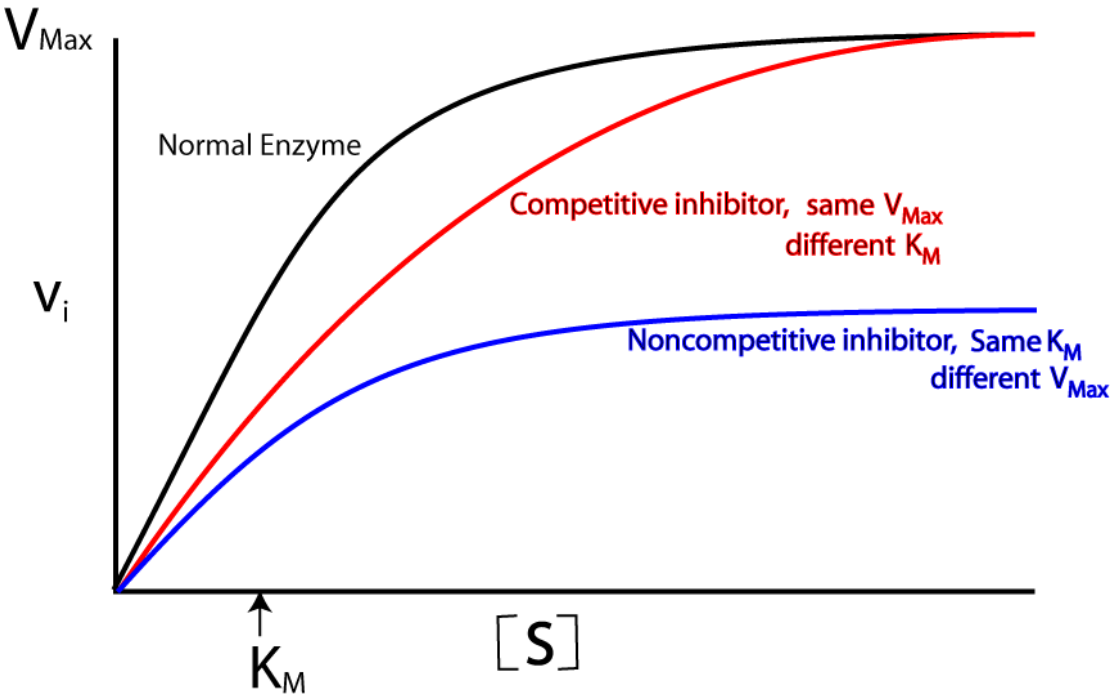
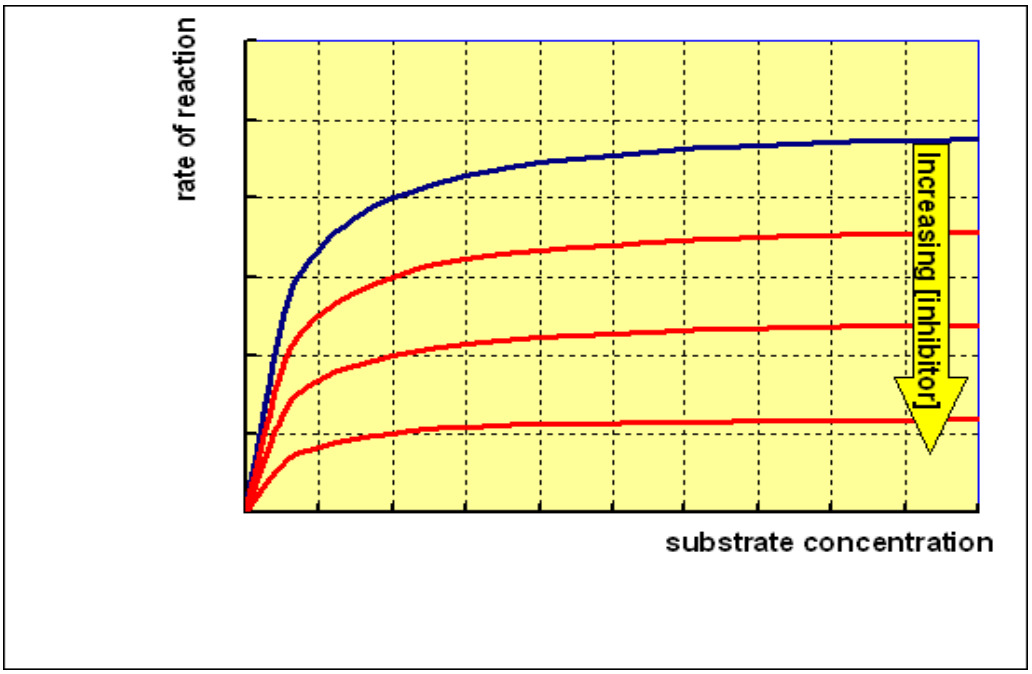


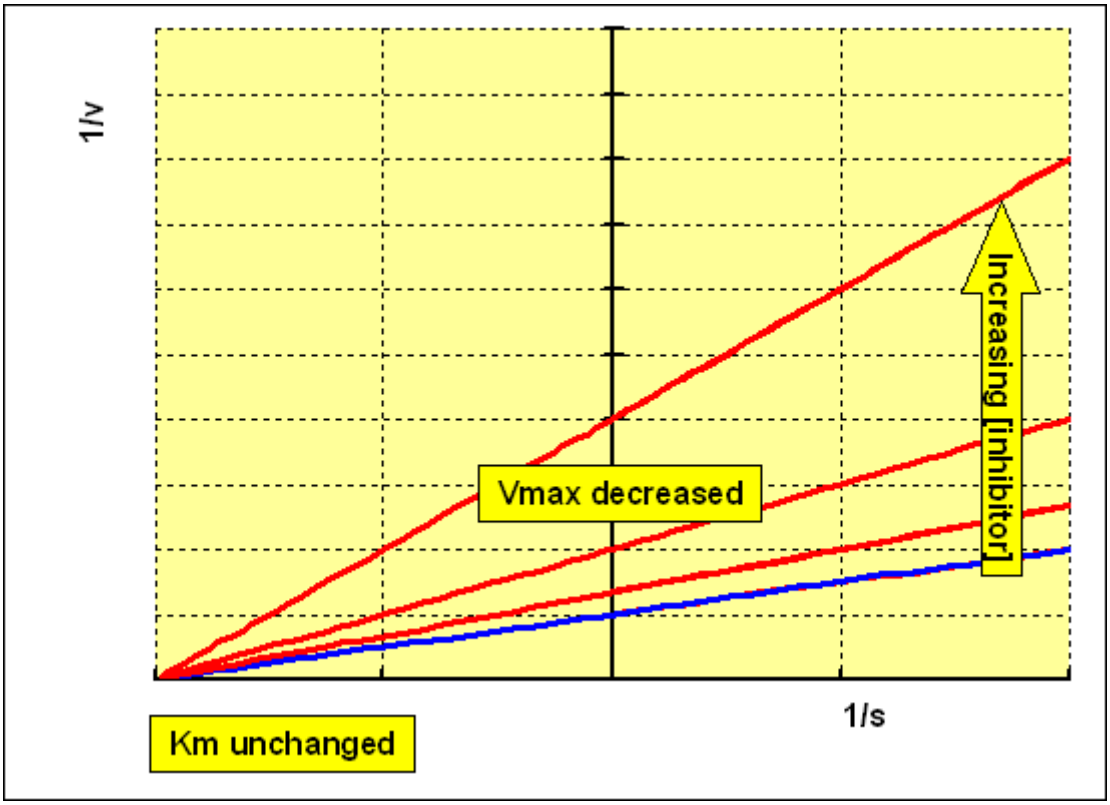
Fig 13 Non-competitive inhibition

Reacting the enzyme with a range of concentrations of substrate at different concentrations of a non-competitive inhibitor will give a family of curves as shown below:



The Lineweaver-Burk double reciprocal plot for this set of data shows a series of lines

converging on the same point on the X (1/S) axis - i,.e. Km is unchanged, but Vmax is reduced:

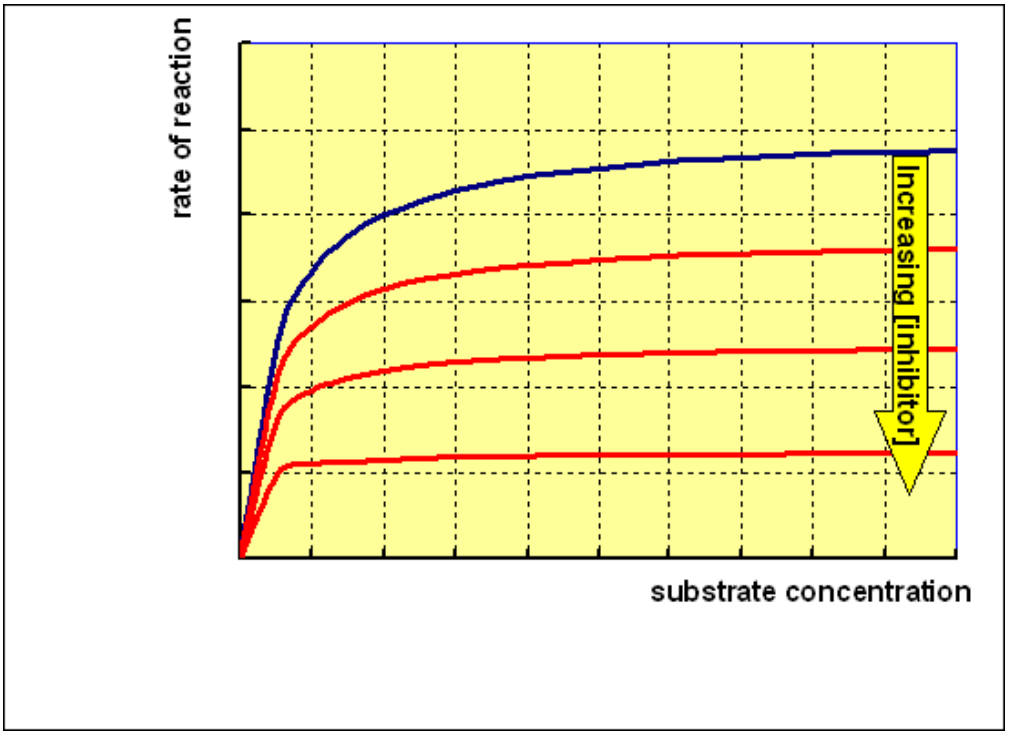


Uncompetitive inhibition

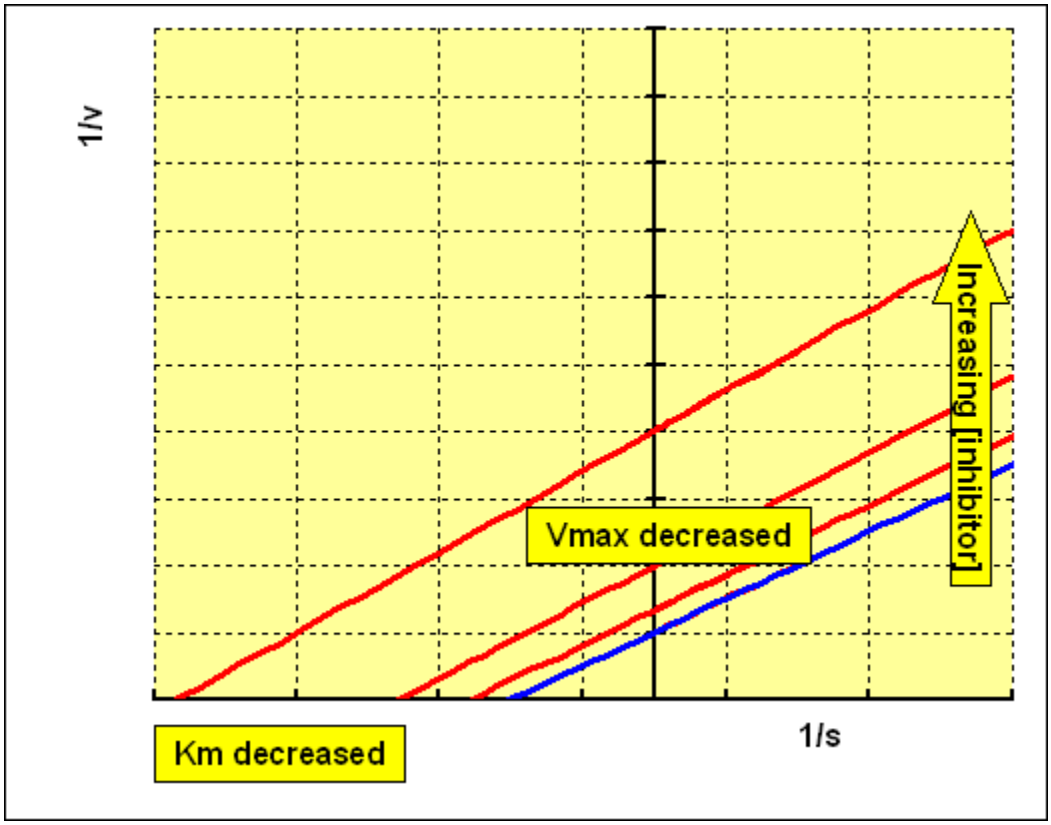
This is a very rare class of inhibition. An uncompetitive inhibitor binds to the enzyme and enhances the binding of substrate (so reducing Km), but the resultant enzyme-inhibitor-substrate complex only undergoes reaction to form the product slowly, so that Vmax is also reduced:

$$E + S + I \rightleftharpoons E\text{-}I + S \rightleftharpoons E\text{-}I\text{-}S \rightleftharpoons E\text{-}I\text{-}P \rightleftharpoons E + P + I$$

Reacting the enzyme with a range of concentrations of substrate at different concentrations of an uncompetitive inhibitor will give a family of curves as shown below:



The Lineweaver-Burk double reciprocal plot for this set of data shows a series of parallel lines - both Km and Vmax are reduced:

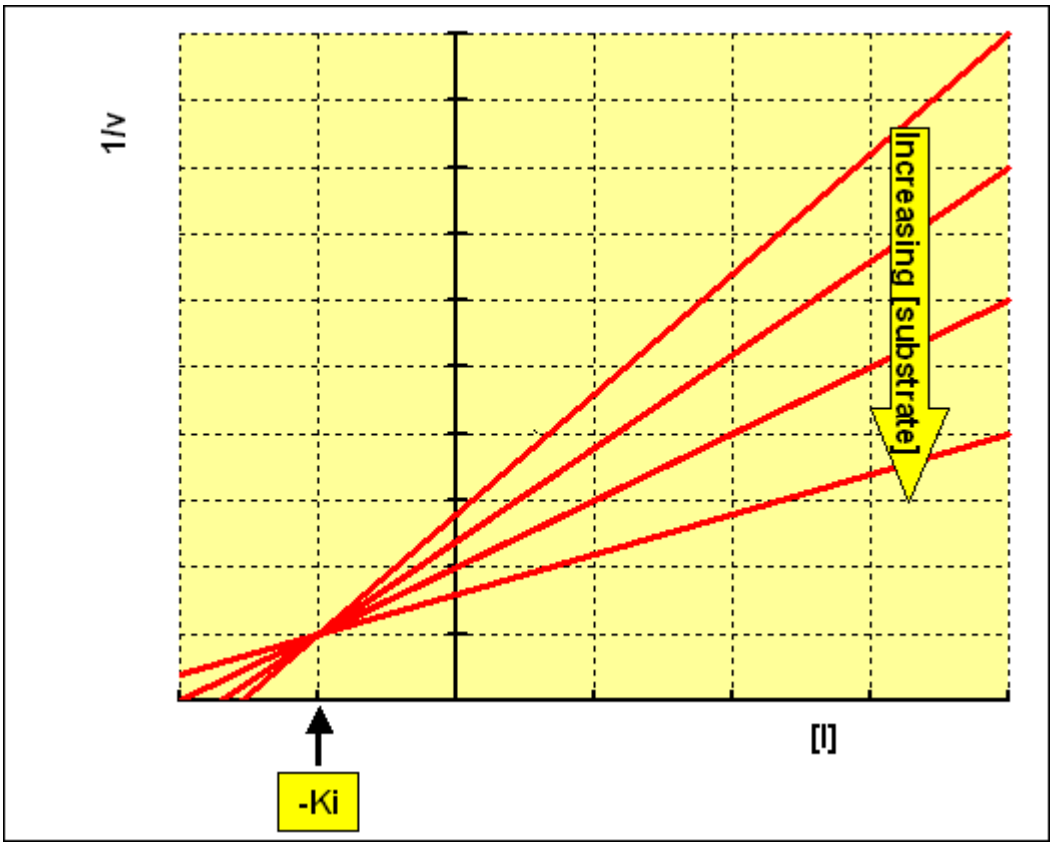


The choice of a competitive or non-competitive inhibitor as a drug

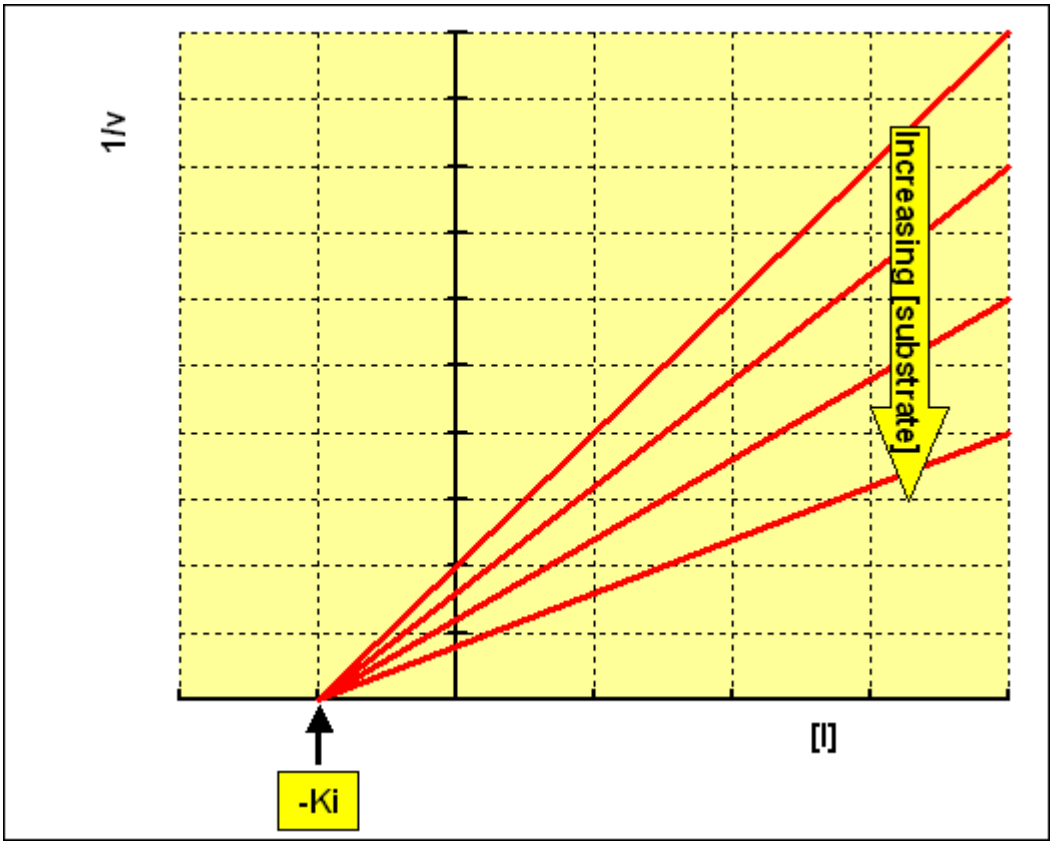
If the requirement is to increase the intracellular concentration of the substrate, then either a competitive or non-competitive inhibitor will serve, since both will inhibit the utilisation of substrate, so that it accumulates.

However, if the requirement is to decrease the intracellular concentration of the product, then the inhibitor must be non-competitive. As unused substrate accumulates, so it will compete with a competitive inhibitor, and the final result will be a more or less normal rate of formation of product, but with a larger pool of substrate. Increasing the concentration of substrate does not affect a non-competitive inhibitor. Ki, the inhibitor constant

The inhibitor constant, Ki, is an indication of how potent an inhibitor is; it is the concentration required to produce half maximum inhibition. Plotting 1/v against concentration of inhibitor at each concentration of substrate (the Dixon plot) gives a family of intersecting lines. For a competitive inhibitor, the lines converge above the x axis, and the value of [I]where they intersect is -Ki



For a non-competitive inhibitor, the lines converge on x axis, and the value of [I] where they intersect is -Ki



Mixed Type Inhibition

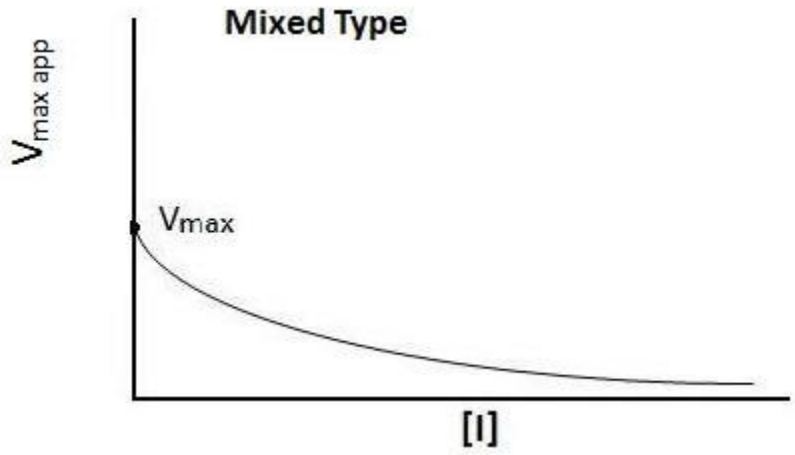
Mixed type inhibition is similar to noncompetitive inhibition except that binding of the substrate or the inhibitor affect the enzyme's binding affinity for the other. The change in binding affinity is included in the chemical equation by the term ki. For mixed type inhibition ki>1, which means that binding affinity for the substrate is decreased when the inhibitor is present.

$$\begin{array}{ccc} E + S & \overset{k_s}{\longleftrightarrow} & ES \overset{k_p}{\longrightarrow} E + P \\ \updownarrow ki & & \updownarrow \alpha ki \\ EI + S & \overset{\alpha k_s}{\longleftrightarrow} & EIS \end{array}$$

Note that it is possible to completely deactivate the enzye activity by saturating it with I

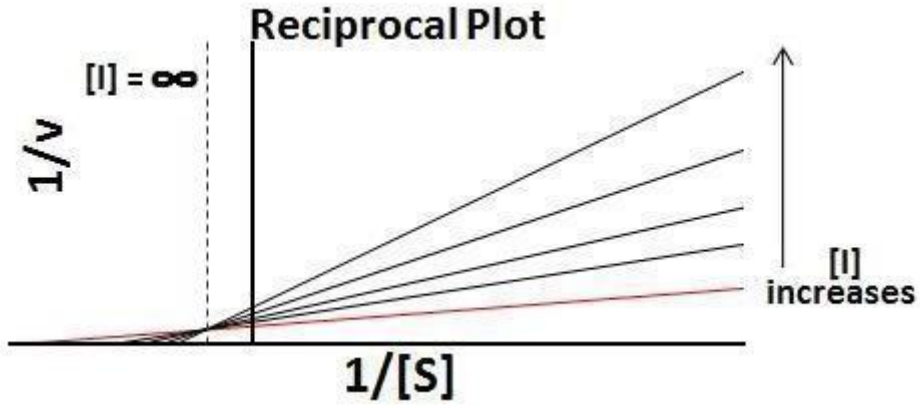
because the EIS complex does not form product.The velocity equation for mixed type is:

v=vmax[S]ks(1+[I]ki)+[S](1+[I]αki)v=vmax[S]ks(1+[I]ki)+[S](1+[I]αki)



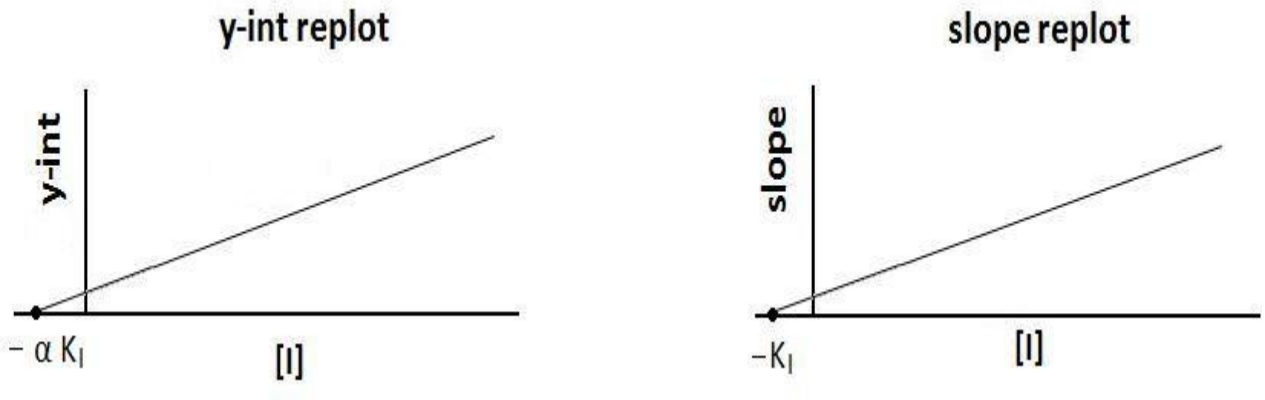
As usual, the basic velocity graph is not very useful, so we take the double reciprocal of the velocity equation. The Lineweaver-Burke equation for mixed type is:

$$\frac{1}{v} = \frac{ks}{v\max}\left(1+\frac{[I]}{ki}\right)\frac{1}{[S]} + \frac{1}{v\max}\left(1+\frac{[I]}{\alpha ki}\right)$$



Due to the ? factor, we cannot solve for all of the kinetic constants from the Lineweaver-Burke plot alone. To solve this problem, we make re-plots of the slope and the y-intercept at varying concentrations of I.

$$\text{slope} = \frac{ks}{v\max \bullet ki}\frac{1}{[I]} + \frac{ks}{v\max^{ix}}$$



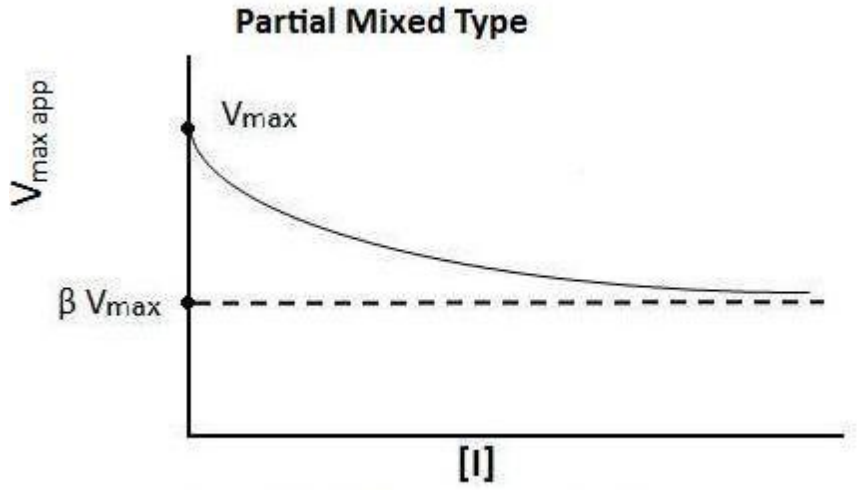
Partial Mixed Type Inhibition

Partial mixed type inhibition is similar to mixed type inhibition except that the inhibited, substrate-bound EIS complex maintains a reduced level of catalytic activity. This means that an inhibitor can never stop the reaction, even at saturating concentration. Partial mixed type still has the ? factor that raises ks, but it also has a ? factor that decreases v_{max}. At saturating [I], ks approaches ?ks and v_{max} approaches ?v_{max}.

The general and velocity equations are:

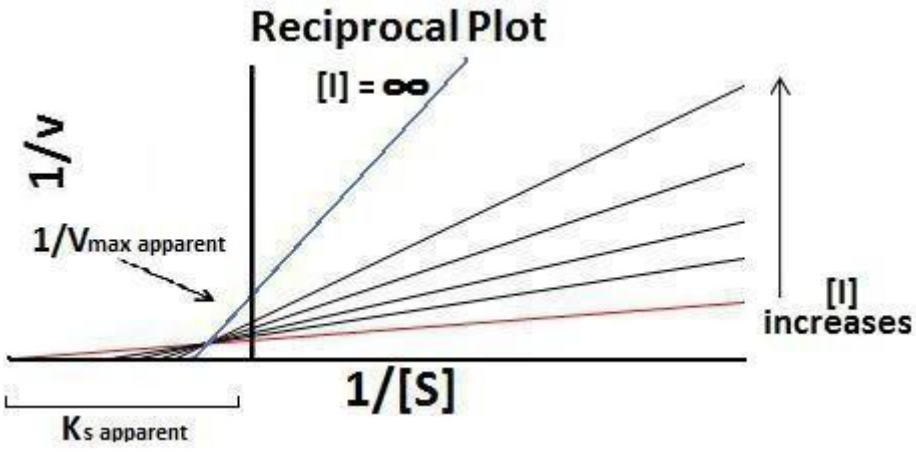
$$\begin{array}{ccccc} E+S & \xleftrightarrow{ks} & ES & \xrightarrow{kp} & E+P \\ \updownarrow ki & & \updownarrow \alpha ki & & \\ EI+S & \xleftrightarrow{\alpha ks} & EIS & \xleftrightarrow{\beta kp} & E+P+I \end{array}$$

$$v = \frac{v\max[S] + \beta v\max[S]}{ks(1+\frac{[I]}{ki}) + [S](1+\frac{[I]}{\alpha ki})}$$



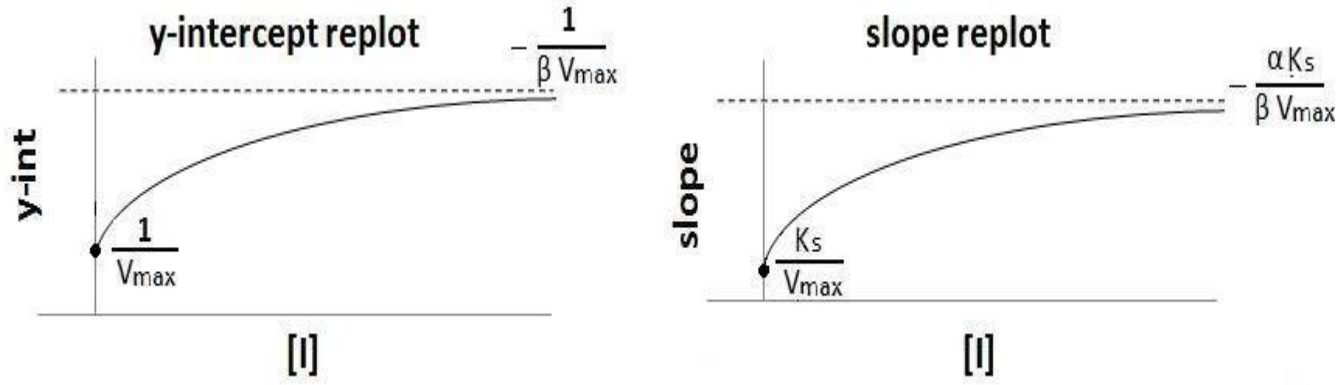
Again, we can take the double reciprocal and look at the line with no inhibitor to determine ks and vmax.

$$\frac{1}{v} = \frac{vmax[S]}{ks\left(\frac{1+\frac{[I]}{ki}}{1+\frac{\beta[I]}{\alpha ki}}\right)+[S]\left(\frac{1+\frac{[I]}{\alpha ki}}{1+\frac{\beta[I]}{\alpha ki}}\right)}$$



Similar to mixed type inhibition, re-plots of the slope and intercept are needed to

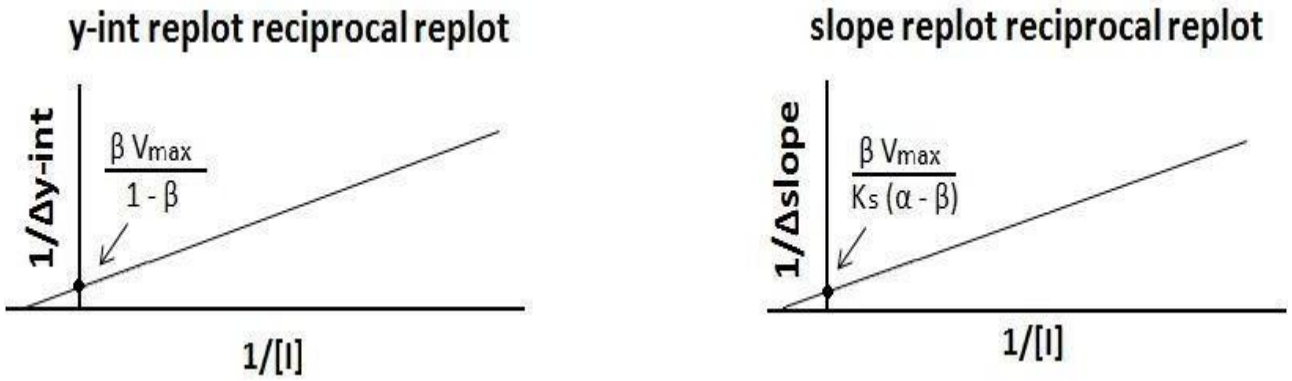
determine ?, ?, and Ki. However, in this case, the re-plots are curved, so a few extra manipulations are necessary to get information from these graphs.



First, the y-intercept is subtracted from the equation to force the curves to go through the origin. These equations are called ?slope and ?y-intercept, and the double reciprocals of these ?plots can be taken to solve for ?, ?, and Ki.

$$\frac{1}{\Delta y - \text{intercept}} = \frac{\alpha ki \bullet v\,\text{max}}{1-\beta}\frac{1}{[I]} + \frac{\beta v\,\text{max}}{1-\beta} \qquad \frac{1}{\Delta \text{slope}} = \frac{\alpha ki}{ks(\alpha-\beta)}\frac{1}{[I]} + \frac{\beta v\,\text{max}}{ks(\alpha-\beta)}$$

\

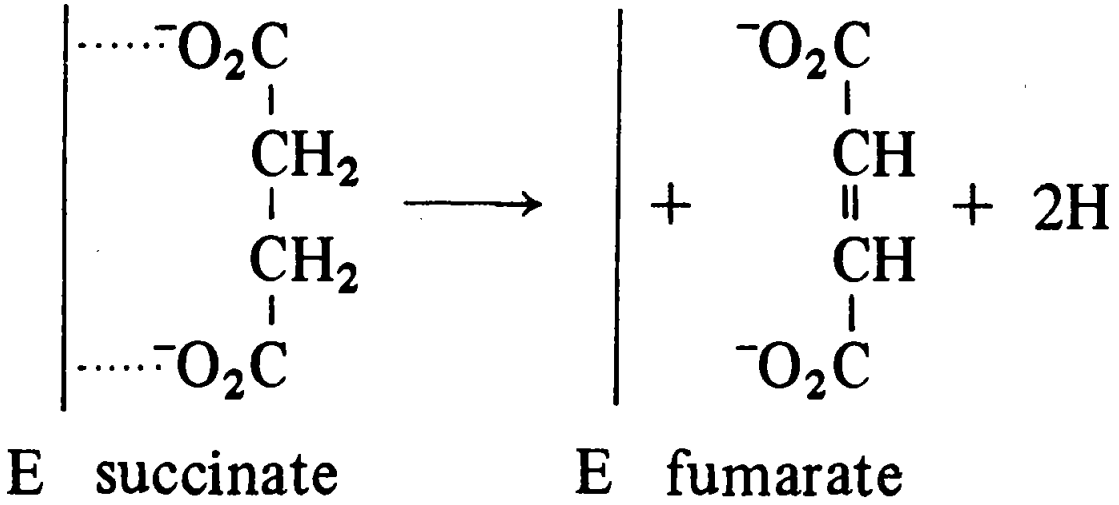


Substrate inhibition

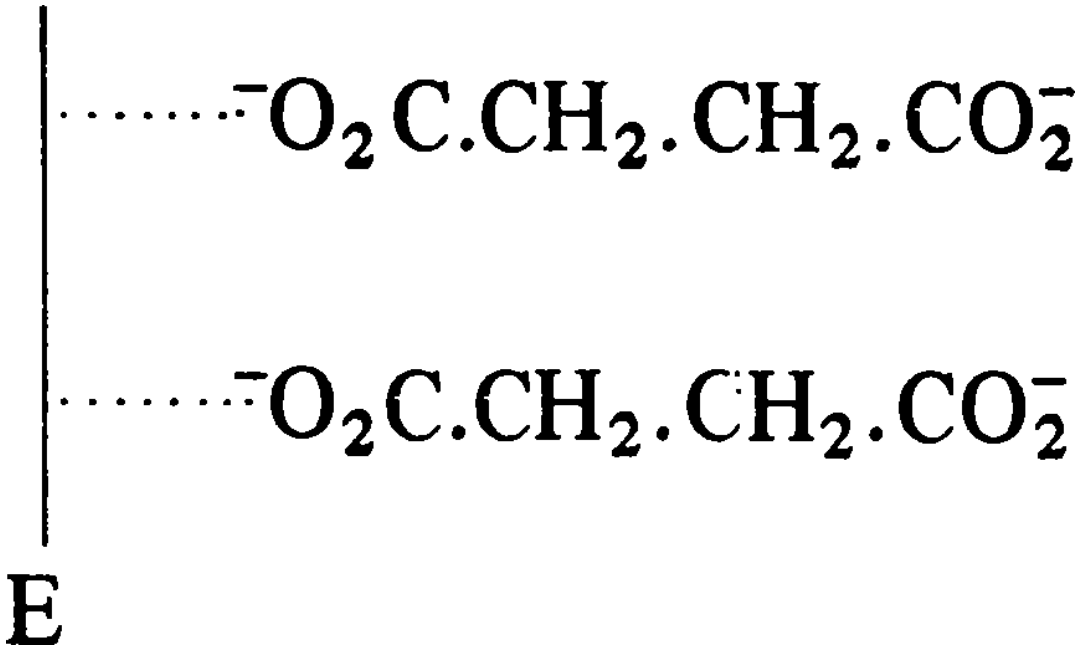
For a given enzyme concentration, the initial reaction velocity increases with increasing initial substrate concentration to a limiting value, Vmax. At still higher substrate concentrations, the initial velocity is sometimes found to be less than the maximum value. In some instances the observations may be explained away on the basis of interaction

between the detecting system and excess substrate, but in other cases it appears that the substrate, in very high concentrations, really can inhibit its own conversion to products.

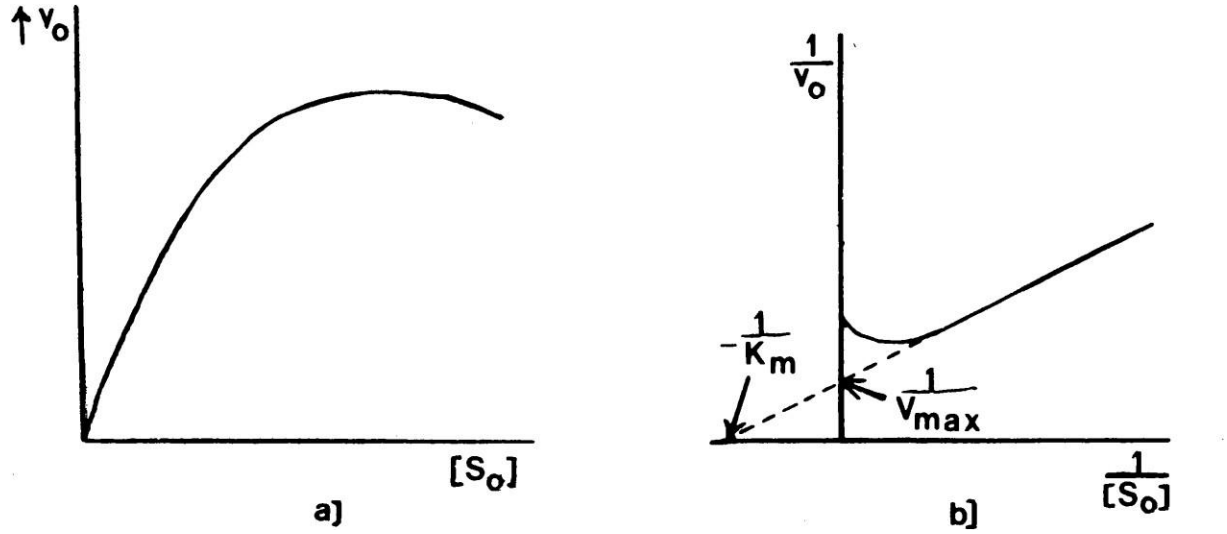
For example, in the reaction catalysed by succinate dehydrogenase, where succinate is converted to fumerate, both the carboxyl groups of the substrate have to bind to the enzyme.



At very high substrate concentrations there is an increased possibility of carboxyl groups from two separate substrate molecules binding to the same enzyme. If this happens, the reaction cannot take place until one of them has dissociated away.



The characteristic features of substrate inhibition are shown below;



In general, it can be seen that substrate inhibition occurs when a molecule of substrate binds to one site on the enzyme and then another molecule of substrate binds to a separate site on the enzyme to form a dead-end complex. This can be regarded as a form of uncompetitive inhibition, the extra substrate molecule being the inhibitor.

The initial velocity equation for uncompetitive inhibition is

$$v_0 = \frac{\dfrac{V_{max}}{\left(1 + \dfrac{[I_0]}{K_i}\right)}[S_0]}{[S_0] + \dfrac{K_m}{\left(1 + \dfrac{[I_0]}{K_i}\right)}}$$

If the inhibitor is identical to the substrate, this becomes;

$$v_0 = \frac{\dfrac{V_{max}}{\left(1 + \dfrac{[S_0]}{K_i}\right)}[S_0]}{[S_0] + \dfrac{K_m}{\left(1 + \dfrac{[S_0]}{K_i}\right)}} = \frac{V_{max}[S_0]}{[S_0]\left(1 + \dfrac{[S_0]}{K_i}\right) + K_m}$$

At low [So] the term [So]/Ki is negligible and the expression reduces to the normal Michaelis-Menten equation. When [So] is very high, then,

$$[S_0]\left(1 + \frac{[S_0]}{K_i}\right) + K_m \simeq [S_0]\left(1 + \frac{[S_0]}{K_i}\right)$$

and the equation simplifies to;

$$v_0 = \frac{V_{max}}{1 + \dfrac{[S_0]}{K_i}}$$

Under these circumstances, v_o decreases as [So] increases, as observed for substrate inhibition at high substrate concentrations.

Allosteric inhibition

Allosteric inhibition plays a vital role in metabolic regulation. Consider a biosynthetic pathway:

A → B → C → D → E → F

Unnecessary production of excess F may be prevented, and supplies of A conserved, by feedback inhibition, where the end product F acts as an allosteric inhibitor of an early enzyme in the pathway, e.g. that catalyzing the reaction A →B.

An allosteric inhibitor, by definition, binds to the enzyme at a site distinct from the substrate-binding site. The term allosteric inhibition is usually reserved for the situation where the inhibitor rather than forming a dead-end complex with the enzyme, influences conformational changes which may alter the binding characteristics of the enzyme for the substrate or the subsequent reaction characteristics.

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SCHOOL OF BIO & CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – III – Enzyme Technology – SBTA1401

Enzyme Immobilization

- Immobilization of enzymes can be defined as the confinement of an enzyme (bio-catalyst) in a distinct phase, separated from the bulk phase but allowing it to exchange with the latter.
- Bulk Phase consists of a substrate, an effecter or inhibitor.
- Immobilized enzyme is either physically entrapped or covalently bonded by chemical means to an inert insoluble matrix or carrier.
- In other words, it involves the restrictive localization of enzymes.
- Matrix is generally a high molecular weight polymer. Ex : cellulose, polyacrlamide, alginate, etc.

Advantages of immobilized enzymes

1. Reuse
2. Continuous use
3. Less labour input
4. Saving in capital cost/ investment
5. Minimum reaction time
6. Less chance of contamination in products
7. More stability
8. Improved process control
9. High enzyme substrate ratio

Methods of Immobilization

- Physical Methods
    1. Adsorption
    2. Entrapping
    3. Membrane confinement (Encapsulation)
- Chemical Methods
    1. Covalent Bonding
    2. Cross Linking

3. Complexation & Chelation

Fig 1 Methods of Immobilization

Carrier for Immobilized Enzymes

Ideal Characteristics of the Carrier

- Low Cost
- Physical Strength
- Inertness
- Enhancement of enzyme specificity
- Regenerability
- Stability
- Reduction of product inhibition

Supports/ Matrix used in immobilization technology

Wide ranges of matrix are used in immobilization of enzyme. The matrix/ supports are grouped into three major categories

1. Natural polymers
   - Alginate: derived from algal cell wall (calcium or magnesium alginate)
   - Chitosan and chitin: enzyme bins to the — OH groups

- Collagen: protenaceous support
- Carrageenan: a sulfated polysaccharide obtained from algae
- Gelatin: partially hydrolyzed collagen, good water holding capacity
- Cellulose: cheapest support available
- Starch: good water holding capacity
- Pectin: good water holding capacity

2. Synthetic polymers

- They are ion exchange resins / polymers
- They are insoluble supports with porous surface
- The porous surface trap and hold the enzymes
- Example: DEAE cellulose, Polyvinyl chloride (PVC) , UV activated Polyethylene glycol (PEG)

3. Inorganic materials

- Zeolites
- Ceramics
- Diatomaceous earth (Trade name celite]
- Silica
- Glass
- Activated carbon
- Charcoal

1. Adsorption

- Oldest method of enzyme immobilization
- Simplest method of enzyme immobilization
- Nelson & Griffin used charcoal to adsorb invertase
- Enzymes are adsorbed to external surface of support Support/ carrier may be

1. Mineral support (aluminum oxide, clay)

2. Organic support (starch)

3. Modified sepharose and ion exchange resins

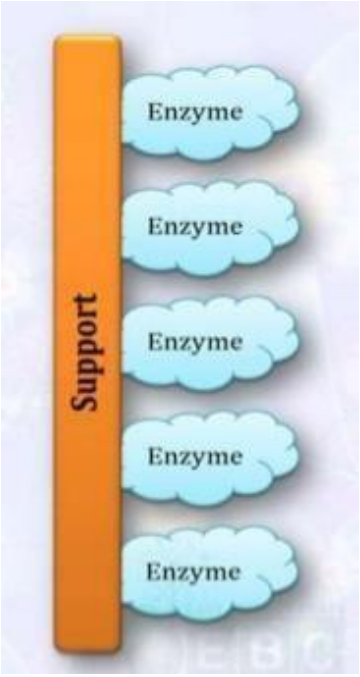


Fig -2 Adsorption

- Weak bonds stabilize enzymes to the support/ carrier
- Bonds involved are low energy bonds such as
    - Ionic interaction
    - Hydrogen bonds
    - Van der Waal forces
- Carrier particle size must be small (for appreciable surface bonding)
- Particle size used: 500 Å to 1 mm diameters
- No pore diffusion limitations (since enzyme are immobilized externally)

Methods of adsorption

1. Static process: Immobilization to carrier by allowing the solution containing enzyme to Contact the carrier (without stirring)

2. Dynamic batch process: Carrier is placed in the enzyme solution and mixed by stirring or agitation

3. Reactor loading process: Carrier is placed in the reactor, then enzyme solution is transferred to reactor

4. Electrode position process: Carrier is placed proximal to an electrode in an enzyme bath and the current is put on, the enzyme migrates to the carrier and deposited on the surface

Advantages

- Easy to carry out
- No reagents are required
- Minimum activation steps involved
- Comparatively cheap method

  Less disruptive to protein than chemical methods

Disadvantages

- Desorption of enzymes from the carrier
- Efficiency is less

2 Covalent bonding

- Involves the formation of covalent bonds between enzyme and support
- Widely used in method of enzyme immobilization
- Chemical groups in enzymes that forms covalent bonds with support are
  - Amino groups, Imino groups
  - Hydroxyl groups
  - Thiol groups and Methylthiol groups
  - Guanidyl groups and Imidazole groups
  - Phenol rings
- Important functional groups of enzyme that provide chemical groups to form covalent bonds with support/ carrier are
  - Alpha carboxyl group at ‘C’ terminal
  - Alpha amino group at ‘N’ terminal
  - Epsilon amino groups of Lysine and Arginine

- Beta and gamma carboxyl groups of Aspartate and Glutamate
- Phenol ring of Tyrosine
- Thiol group of Cysteine
- Hydroxyl groups of Serine and Threonine
- Imidazole group of Histidine
- Indole ring of Tryptophan

- Carriers / supports used for covalent bonding
  - Carbohydrates: Eg. Cellulose, DEAE cellulose, Agarose
  - Synthetic agents: Eg. Polyacrylamide
  - Protein carriers
  - Amino group bearing carriers: Eg. amino benzyl cellulose
  - Inorganic carriers: Porous glass, silica
  - Cyanogen bromide (CNBr)-agarose and CNBr Sepharose
- Hydroxyl and Amino groups form covalent bonds more easily

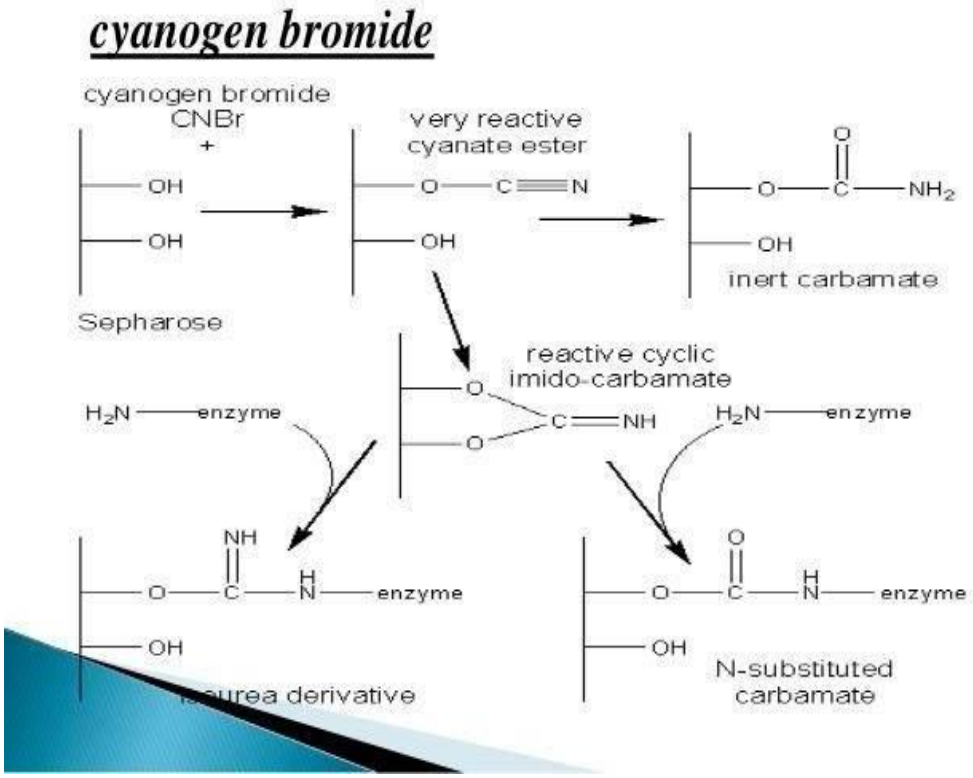


Fig 3 Covalent bonding of enzyme by cyanogens bromide

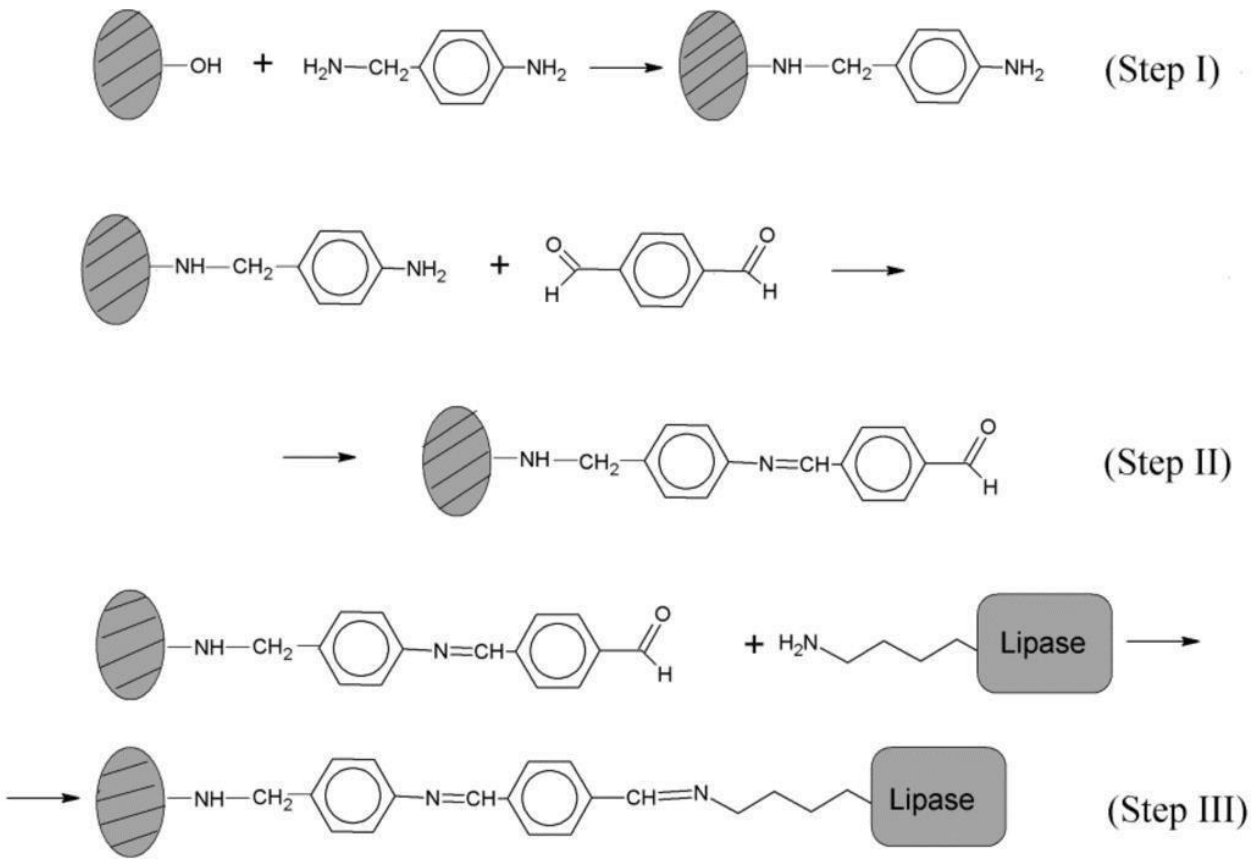


Fig 4 immobilization of *Rhizopus oryzae* lipase (ROL) through the ε-amino group of lysine residues to an organic linker bonded to the inorganic support.

Step 1 - surface OH groups of the supported $AlPO_4$/Sepiolite are activated by microwave heating with 4-aminobenzylamine

Step 2 - Terephtaldicarboxaldehyde is reacted through imines bonds also obtained by microwave heating

Step 3 – Covalent immobilization of the enzyme is obtained through the imines bonds produced with lysine residues.

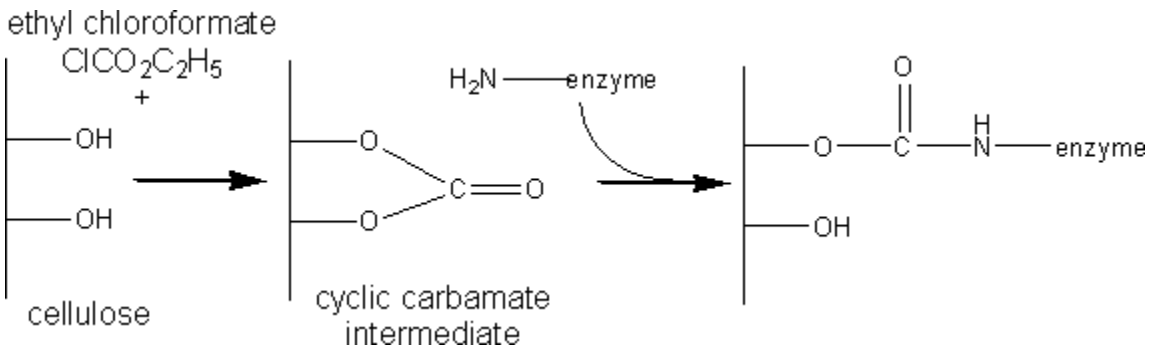


Fig 5 Covalent bonding of enzyme by Chloroformates

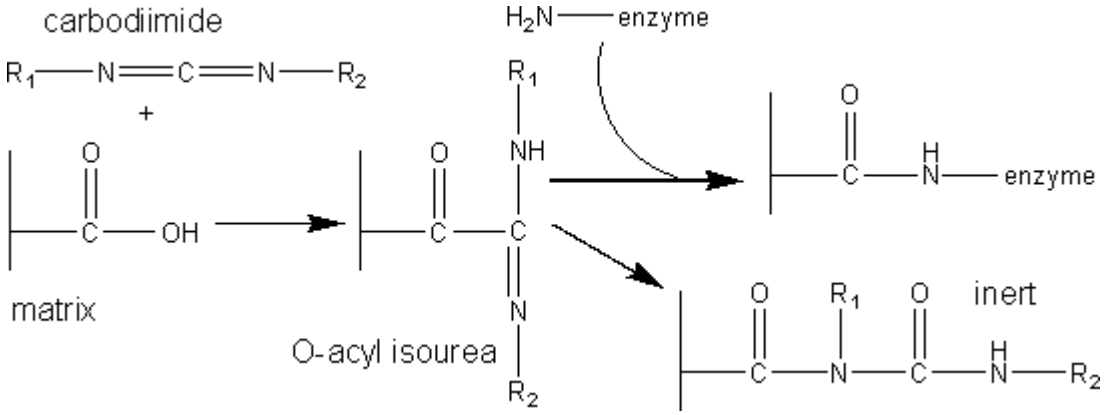


Fig 6 Covalent bonding of enzyme by carbodiimide

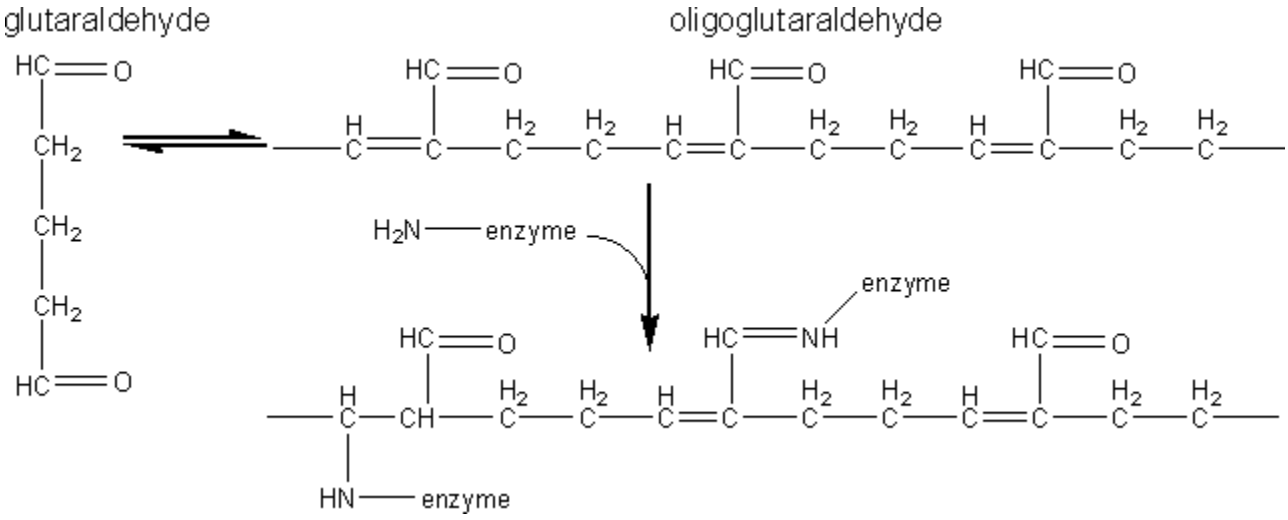


Fig 7 Covalent bonding of enzyme by glutaraldehyde

Methods of covalent bonding

1. Diazoation: Bonding between amino group of support and thyrosil or histidyl group of enzyme

2. Peptide bond: between amino / carboxyl groups of support and enzyme

3. Poly functional reagents: Use of a bi-functional or multifunctional reagent (glutaraldehyde) which forms bonding between the amino group of the support and amino group of the enzyme

Advantages

- Strong linkage of enzyme to the support
- No leakage or desorption problem
- Comparatively simple method
- A variety of support with different functional groups available
- Wide applicability

Disadvantages

- Chemical modification of enzyme leading to functional conformation loss
- Enzyme inactivation by changes in the conformation when undergoes reactions at active sites
- This can be overcome through immobilization in the presence of enzyme substrate or a competitive inhibitor

3. Entrapment

- Enzymes are physically entrapped inside a matrix
- Bonds involved may be covalent or non-covalent
- Matrix used will be water soluble polymer are
    - polyacrylamide gels
    - Cellulose triacetate
    - Agar
    - Gelatin

- Alginate

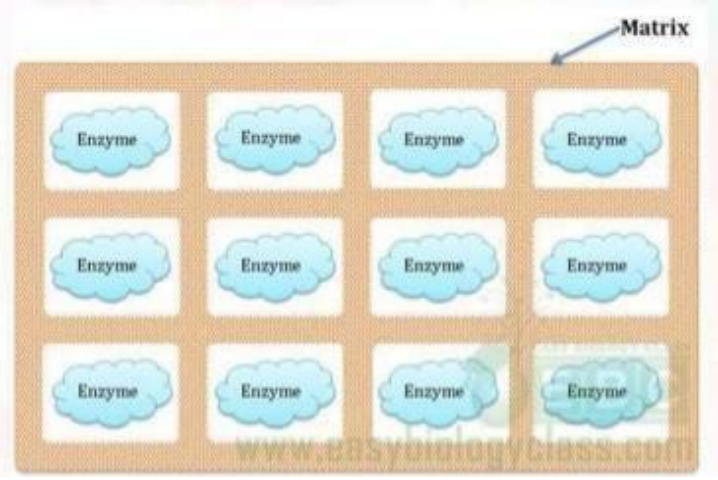


Fig 8 Entrapment of enzyme

- Form and nature of matrix varies
- Pore size of matrix is adjusted to prevent loss of enzyme
- Possibility of leakage of low molecular weight enzymes
- Agar and carrageenan have large pore sizes
- Pore size can be adjusted with the concentration of the polymer
- Entrapment of enzyme can be used for sensing application
- Not much success in industrial process
- Easy to practice at small scale

Methods of en trapment

1. Inclusion in the gels: enzymes trapped in gels

2. Inclusion in fibers: enzymes supported on fiber format

3. Inclusion in microcapsules: Enzymes entrapped in microcapsules formed by monomer mixtures such as polyamine, calcium alginate

Advantages

- Fast
- Cheap (low cost matrix available]

- Mild conditions are required
- Less chance of conformational changes in enzyme

Disadvantages

- Leakage of enzyme
- Pore diffusion limitation
- Chance of microbial contamination

4. Cross linking (Copolymerization)

- This method is also called as copolymerization.
- In this method of immobilization enzymes are directly linked by covalent bonds between various groups of enzymes via polyfunctional reagents.
- Unlike other methods, there is no matrix or support involved in this method.

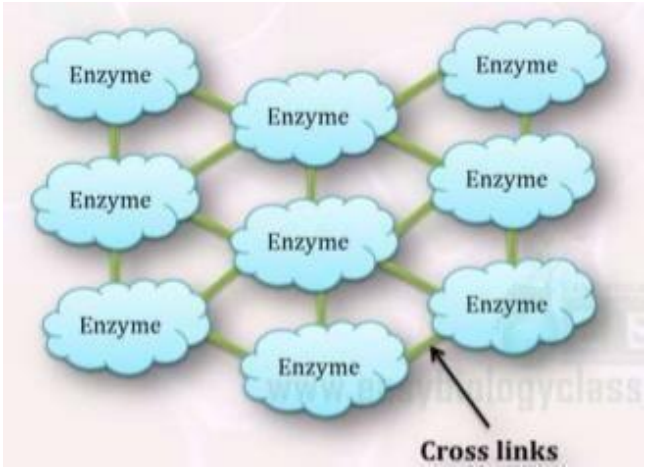


Fig 9 Crosslinking of enzyme

- Commonly used polyfunctional reagents are Glutaraldehyde and Diazonium salt
- Technique is cheap and simple but not often used with pure proteins
- It is widely used in commercial preparations

Demerit:

- Polyfunctional reagents can denature the enzyme

Fig 10 Chemicals used in crosslinking

5. Encapsulation

- Enclosing enzymes in a semi permeable membrane capsule
- Capsule is made up of nitro cellulose or nylon
- Effectiveness depends upon the stability of enzymes

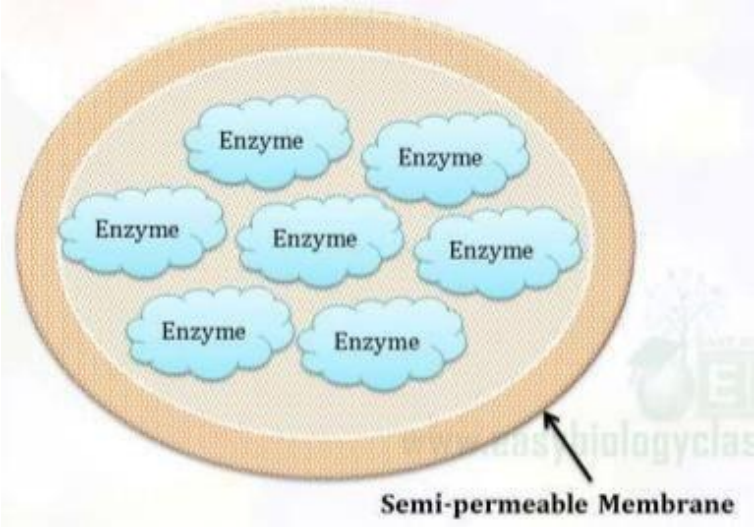


Fig 11 Encapsulation of enzyme

Advantages

- Cheap and simple method
- Large quantity of enzymes can be immobilized by encapsulation

Disadvantages

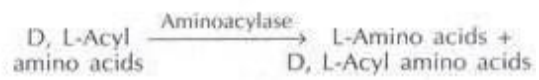
- Pore size limitation
- Only small substrate molecule is able to cross the membrane

Applications of enzyme immobilization:

1. **Industrial production:** Industrial production of antibiotics, beverages, amino acids etc. uses immobilized enzymes or whole cells.
2. **Biomedical applications:** Immobilized enzymes are widely used in the diagnosis and treatment of many diseases. Immobilized enzymes can be used to overcome inborn metabolic disorders by the supply of immobilized enzymes. Immobilization techniques are effectively used in drug delivery systems especially to oncogenic sites.
3. **Food industry:** Enzymes like pectinases and cellulases immobilized on suitable carriers are successfully used in the production of jams, jellies and syrups from fruits and vegetables.
4. **Research:** A Research activity extensively uses many enzymes. The use of immobilized enzyme allow researcher to increase the efficiency of different enzymes such as Horse Radish Peroxidase (HRP) in blotting experiments and different Proteases for cell or organelle lysis.
5. **Production of bio-diesel** from vegetable oils.
6. **Waste water management**: treatment of sewage and industrial effluents.
7. **Textile industry:** scouring, bio-polishing and desizing of fabrics.
8. **Detergent industry:** immobilization of lipase enzyme for effective dirt removal from cloths.

Production of L-Amino Acids:

mino acids (and not D-amino acids) are very important for use in food and feed supplements and medical purposes. The chemical methods employed for their production result in a racemic mixture of D- and L-amino acids. They can be acylated to form D, L-acyl amino acids. The immobilized enzyme aminoacylase (frequently immobilized on DEAE sephadex) can selectively hydrolyse D, L-acyl amino acids to produce L-amino acids.



The free L-amino acids can separated from the un-hydrolysed D-acyl amino acids. The latter can be recemized to D, L-acyl amino acids and recycled through the enzyme reactor containing immobilized aminoacylase. Huge quantities of L-methionine, L-phenylalanine L-tryptophan and L-valine are produced worldwide by this approach.

Production of High Fructose Syrup:

Fructose is the sweetest among the monosaccharide's, and has twice the sweetening strength of sucrose. Glucose is about 75% as sweet as sucrose. Therefore, glucose (the most abundant monosaccharide) cannot be a good substitute for sucrose for sweetening. Thus, there is a great demand for fructose which is very sweet, but has the same calorific value as that of glucose or sucrose.

High fructose syrup (HFS) contains approximately equivalent amounts of glucose and fructose. HFS is almost similar to sucrose from nutritional point of view. HFS is a good substitute for sugar in the preparation of soft drinks, processed foods and baking.

High fructose syrup can be produced from glucose by employing an immobilized enzyme glucose isomerase. The starch containing raw materials (wheat, potato, corn) are subjected to hydrolysis to produce glucose. Glucose isomerase then isomerizes glucose to fructose (Fig. 21.11). The product formed is HFS containing about 50% fructose. (Note: Some authors use the term high fructose corn syrup i.e. HFCS in place of HFS).

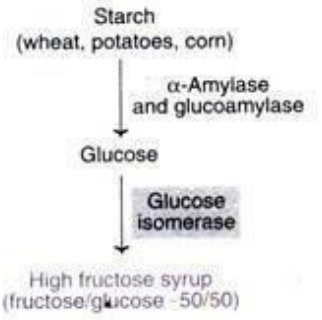


Fig. 21.11 : Production of high fructose syrup from starch (glucose isomerase is the immobilized enzyme).

Glucose isomerase:

This is an intracellular enzyme produced by a number of microorganisms. The species of *Arthrobacter, Bacillus* and *Streptomyces* are the preferred sources. Being an intracellular enzyme, the isolation of glucose isomerase without loss of biological activity requires special and costly techniques. Many a times, whole cells or partly broken cells are immobilized and used.

Mass transfer effect on immobilization

The kinetic behaviour of a bound enzyme can differ significantly from that of the same enzyme in free solution.

The properties of an enzyme can be modified by suitable choice of the immobilisation protocol, whereas the same method may have appreciably different effects on different enzymes.

These changes may be due to conformational alterations within the enzyme due to the immobilisation procedure, or the presence and nature of the immobilisation support.

If the immobilisation process introduces any strain into the enzyme, this is likely to encourage the inactivation of the enzymes under denaturing conditions (e.g. higher temperatures or extremes of pH).

The kinetic constants (e.g. K_m, V_{max}) of enzymes may be altered by the process of immobilisation due to internal structural changes and restricted access to the active site.

Thus, the intrinsic specificity ($k_.$/K_m) of such enzymes may well be changed relative to the soluble enzyme. An example of this involves trypsin where the freely soluble enzyme hydrolyses fifteen peptide bonds in the protein pepsinogen but the immobilised enzyme hydrolyses only ten.

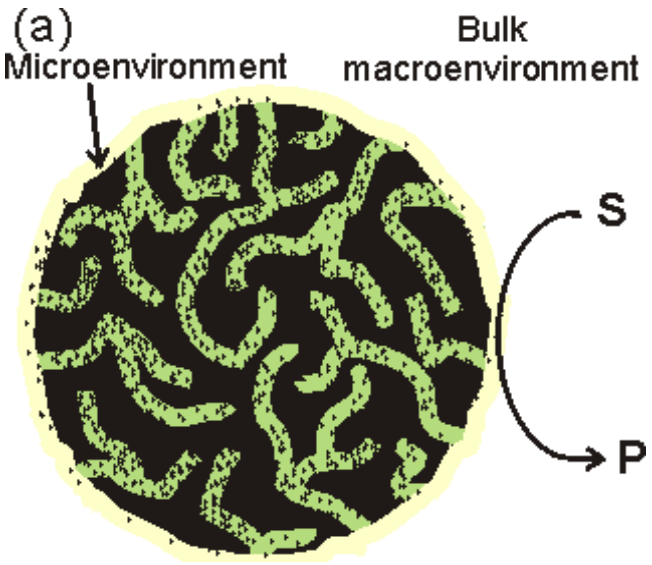


Fig 12 A schematic cross-section of an immobilised enzyme particle

- The above figure shows the macroenvironment and microenvironment.
- Triangular dots represent the enzyme molecules.
- The microenvironment consists of the internal solution plus that part of the surrounding solution which is influenced by the surface characteristics of the immobilised enzyme.
- Partitioning of substances will occur between these two environments.
- Substrate molecules (S) must diffuse through the surrounding layer (external transport) in order to reach the catalytic surface and be converted to product (P).
- In order for all the enzyme to be utilised, substrate must also diffuse within the pores in the surface of the immobilised enzyme particle (internal transport).
- The porosity (e) of the particle is the ratio of the volume of solution contained within the particle to the total volume of the particle.
- The tortuosity (t) is the average ratio of the path length, via the pores, between any points within the particle to their absolute distance apart.

Properties of immobilized enzymes

- As a consequence of enzyme immobilization, some properties of the enzyme molecule, such as its catalytic activity or thermal stability, become altered with respect to those of its soluble counterpart

- This modification of the properties may be caused either by changes in the intrinsic activity of the immobilized enzyme or by the fact that the interaction between the immobilized enzyme and the substrate takes place in a microenvironment that is different from the bulk solution.
- The observed changes in the catalytic properties upon immobilization may also result from changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix.
- Reduction in specific activity due to immobilization may cause denaturation.
- Carrier create a new environment this may alter the enzyme activity such as
    - Change in the nature of active site due to chemical and physical interaction between enzyme and carrier.
    - Carrier may show steric hindrance by inhibiting diffusion of substrate to enzyme.
    - Carrier may hold substrate or product by electrostatic interaction.
- Stability may be affected upon treating or storing.
- Alter in optimum pH may change upto 2 pH units.
- Km of immobilized enzyme is altered (decreased) when carrier is used in opposite charge to that of substrate (due to electrostatic interaction).

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SCHOOL OF BIO & CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – IV – Enzyme Technology – SBTA1401

Specificity of Enzymes

One of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

- Absolute specificity - the enzyme will catalyze only one reaction.
- Group specificity - the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
- Linkage specificity - the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
- Stereochemical specificity - the enzyme will act on a particular steric or optical isomer.

Though enzymes exhibit great degrees of specificity, cofactors may serve many apoenzymes. For example, nicotinamide adenine dinucleotide (NAD) is a coenzyme for a great number of dehydrogenase reactions in which it acts as a hydrogen acceptor. Among them are the alcohol dehydrogenase, malate dehydrogenase and lactate dehydrogenase reactions.

1. Relative, low or bond specificity

In this type the enzyme acts on substrates that are similar in structure and contain the same type of bonds e.g.

a. Amylase, which acts on α 1-4 glycosidic, bonds in starch, dextrin and glycogen.

b. Lipase that hydrolyzes ester bonds in different triglycerides

2. Moderate, structural or group specificity

In this type of specificity, the enzyme is specific not only to the type of bond but also to the structure surrounding it. For example:

a.Pepsin is an endopeptidase that hydrolyzes central peptide bonds in which the amino group belongs to aromatic amino acids e.g. phenyl alanine, tyrosine and tryptophan.

b.Trypsin is an endopeptidase that hydrolyzes central peptide bonds in which the amino group belongs to basic amino acids e.g. arginine, lysine and histidine.

c.Chymotrypsin is an endopeptidase that hydrolyzes central peptide bonds in which the carboxyl group belongs to aromatic amino acids.

d.Aminopeptidase is an exopeptidase that hydrolyzes peripheral peptide bond at the amino terminal (end) of polypeptide chain.

e.Carboxypeptidase is an exopeptidase that hydrolyzes peripheral peptide bond at the carboxyl terminal of polypeptide chain.

3. Absolute, high or substrate specificity

In this type of specificity, the enzyme acts only on one substrate e.g.

a)Uricase, which acts only on uric acid.

b) Arginase, which acts only on arginine.

c) Carbonic anhydrase, which acts only on carbonic acid.

d) Lactase, which acts on lactose.

e) Sucrase, which acts on sucrose.

f) Maltase, which acts on maltose.

4. Optical or stereo-specificity

In this type of specificity, the enzyme is specific not only to the substrate but also to its optical configuration e.g.

a. L amino acid oxidase acts only on L amino acids.

b. D amino acid oxidase acts only on D amino acids.

c . α- glycosidase acts only on α- glycosidic bonds, which are present in starch, dextrin and glycogen.

d. β- glycosidase acts only on β- glycosidic bonds that are present in cellulose. We can digest glycogen and starch due to presence of α-glycosidase, but we can not digest cellulose due to the absence of β-glycosidase

5. Dual specificity

There are two types of dual specificity:

A-The enzyme may act on two substrates by one reaction type.

e.g. xanthine oxidase enzyme acts on xanthine and hypoxanthine (two substrates) by oxidation (one reaction type).

B-The enzyme may act on one substrate by two different reaction types

e.g. isocitrate dehydrogenase enzyme acts on isocitrate (one substrate) by oxidation followed by decarboxylation (two different reaction types).

Active Site

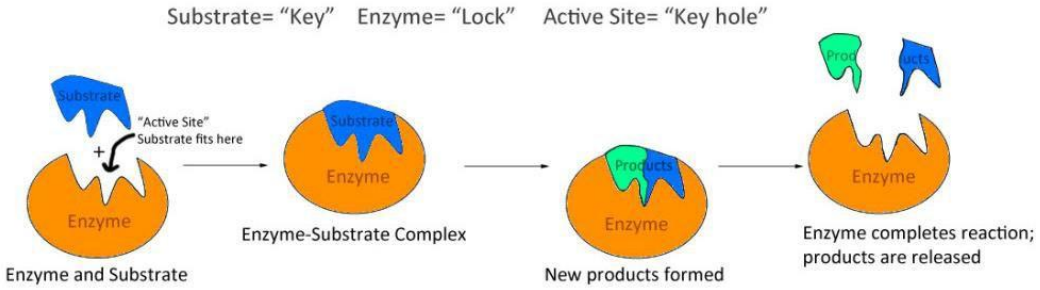
The basic mechanism by which enzymes catalyze chemical reactions begins with the binding of the substrate (or substrates) to the active site on the enzyme. The active site is the specific region of the enzyme which combines with the substrate. The binding of the substrate to the enzyme causes changes in the distribution of electrons in the chemical bonds of the substrate and ultimately causes the reactions that lead to the formation of products. The products are released from the enzyme surface to regenerate the enzyme for another reaction cycle.

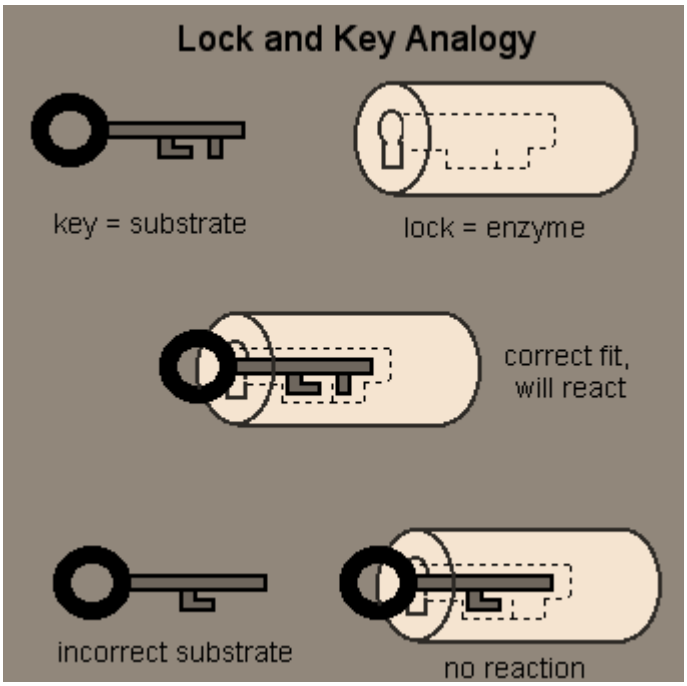
The active site has a unique geometric shape that is complementary to the geometric shape of a substrate molecule, similar to the fit of puzzle pieces. This means that enzymes specifically react with only one or a very few similar compounds.

Lock and Key Theory:

The specific action of an enzyme with a single substrate can be explained using a Lock and Key analogy first postulated in 1894 by Emil Fischer. In this analogy, the lock is the enzyme and the key is the substrate. Only the correctly sized key (substrate) fits into the key hole (active site) of the lock (enzyme).

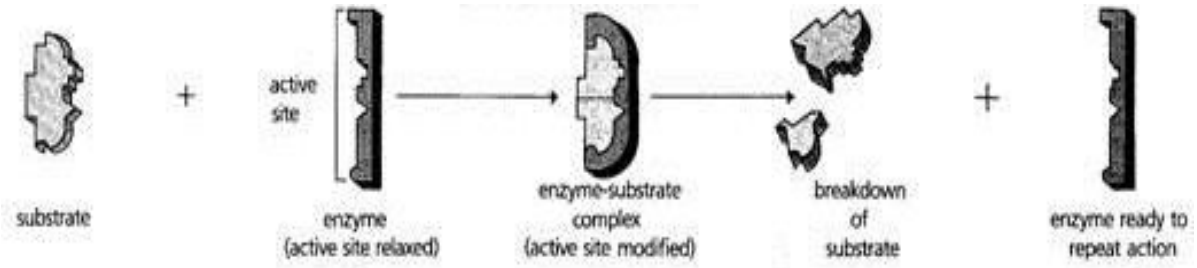
Smaller keys, larger keys, or incorrectly positioned teeth on keys (incorrectly shaped or sized substrate molecules) do not fit into the lock (enzyme). Only the correctly shaped key opens a particular lock. This is illustrated in graphic as follows.





Induced-Fit Theory

The induced-fit theory assumes that the substrate plays a role in determining the final shape of the enzyme and that the enzyme is partially flexible. This explains why certain compounds can bind to the enzyme but do not react because the enzyme has been distorted too much. Other molecules may be too small to induce the proper alignment and therefore cannot react. Only the proper substrate is capable of inducing the proper alignment of the active site.



Turnover number

Turnover number (also termed k_{cat}) is defined as the maximum number of chemical conversions of substrate molecules per second that a single catalytic site will execute for a given enzyme concentration .

Example

1. Carbonic anhydrase has a turnover number of 400,000 to 600,000 s^{-1}, which means that each carbonic anhydrase molecule can produce up to 600,000 molecules of product (bicarbonate ions) per second.
2. Acetylcholinesterase (AChE) may be one of the fastest enzymes. It hydrolyzes acetylcholine to choline and an acetate group. One of the earliest values of the turnover number was 3 x 10^7 (molecules of acetylcholine) per minute per molecule of enzyme. A more recent value at 25 °C, pH = 7.0, acetylcholine concentration of 2.5 x 10^{-3} M, was found to be 7.4 x 10^5 min^{-1}.

Enzyme	Turnover number (per second)
Carbonic anhydrase	600,000
3-Ketosteroid isomerase	280,000
Acetylcholinesterase	25,000
Penicillinase	2,000
Lactate dehydrogenase	1,000
Chymotrypsin	100
DNA polymerase I	15
Tryptophan synthetase	2
Lysozyme	0.5

Enzyme purification

Selection of organism

- It is always preferable to select a source enriched in that particular enzyme.
- To check from the literature whether the enzyme occurs universally (in animals, plants as well as microbes) or confined to a particular Kingdom.
- Working with microbial and animal enzymes is easier compared to plant enzymes since plants are generally rich in phenolics, which on exposure with air get converted into quinones and quinones bind with enzyme protein and makes it in active.
- On the other hand, it is easier to get a plant tissue provided plants are grown in plenty in the surrounding compared to get animal tissue or a pure microbe
- For animal tissue, either one will have to sacrifice the animal in the laboratory or will have to bring the tissue from a slaughterhouse. In case of microbes, one will have to grow microbe in pure form on a suitable growth medium under aseptic conditions after getting inoculum of the microbe.

Since almost all the enzymes (with few exceptions) are heat labile and not much stable at room temperature, the entire process of enzyme isolation, purification is carried out at 0-4°C using a cold room.

The component of the homogenization technique like pestle and mortar, bowl of the Waring blender should also be in chilled condition. While homogenizing in a pestle and mortar, it should be surrounded by the ice flakes. In case of Waring blender bowl, many people also wrap a cloth wet with chilled water.

Distilled water is used as isolating (homogenizing) medium, but generally a buffer of a suitable ionic concentration and pH is preferred in order to maintain enzyme activity Every enzyme is stable in a particular pH range only.

Techniques used for enzyme isolation

Once a promising source material has been identified the next step is to extract the protein from this source. The objective in extracting proteins is to get them from the site where they occur in the tissue, into solution where they can be more easily manipulated and separated out. Most tissue proteins occur within cells, and possibly within organelles in the cells, and in these cases it is necessary to break open the cells and organelles, to release their protein contents. The methods chosen to disrupt the cells and organelles should be such that the proteins themselves are minimally damaged.

Osmotic shock

It is a sudden change in the solute concentration around a cell, causing a rapid change in the movement of water across its cell membrane. Water will tend to flow into the cells and organelles by osmosis, promoting their lysis and release of their proteins. To further promote the disruption of cell membranes, a low concentration of organic solvent, e.g. 2% n-butanol, is often added to the extraction buffer.

Pestle and mortar

Pestle and mortar is a moderate technique for tissue homogenization. Mechanical breakdown occurs during the process. Sometimes, grinding is done in the presence of purified sand or glass beads for aberration. Pestle and mortar is considered to be a moderate grinding technique and rupturing of the cell organelles does not occur if isotonic grinding medium without detergent is used.

Blenders

Waring blender (commonly called as mixie) is comparatively harsh technique of grinding the tissue compared to pestle and mortar and is mostly used for homogenizing the harder tissues (generally the plant tissues). If the worker is interested in isolating intact cell organelles, then Waring blender is not a preferred technique. Waring blender is first operated at low speed forfew seconds and then at medium speed(s) for few seconds before bringing it at high speed. Time of grinding at various speeds is decided according to the nature of the tissue being ground. If homogenization has to be done for a little

longer time, then it is generally done after few seconds interval after every minute of grinding at high speed to avoid heating during operation of the Waring blender.

Ultra- Sonicator

This technique of rupturing the cells is generally used for microbial/ bacterial cells. Ultra-sonicator generates low as well as high wavelength ultrasonic waves. For the purpose, a suitable probe depending on the volume of the homogenizing medium is selected and connected with the ultra-sonicator. The container having cells and homogenizing (isolating) medium is put in chilled condition by covering the container with ice. There is much generation of heat during ultra**-**sonication, therefore, ultrasonic waves are thrown in the sample after few seconds interval**,** every 10 to 15 seconds ultrasonication.

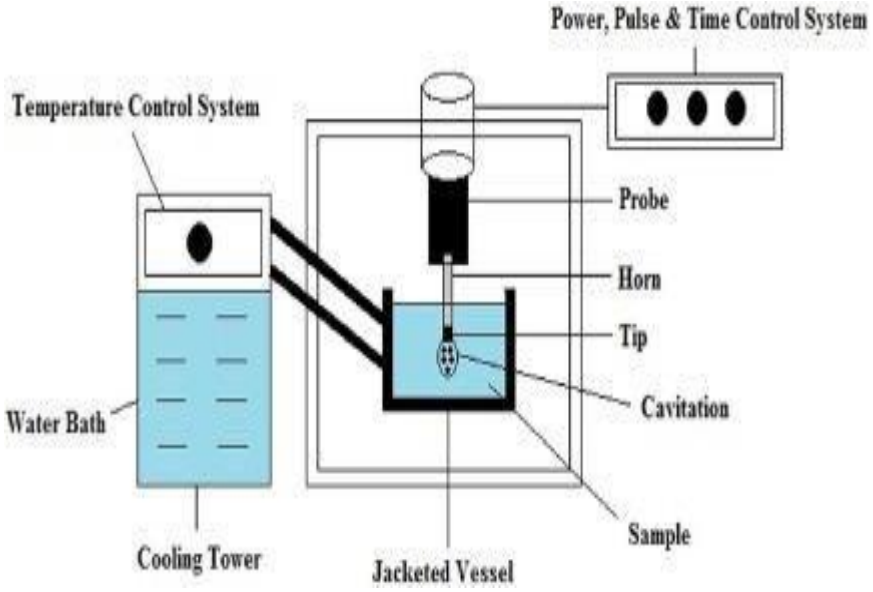


Fig 1 Ultra- Sonicator

Vir-Tis homogenizer

This is considered to be a mild technique and generally used for homogenization of soft tissues such as animal tissues. Here a motorized pestle with teeth like aberrations is used. With Vir-Tis homogenizer, generally no rupturing of cell organelles occurs during grinding provided isotonic medium with no detergent is used.

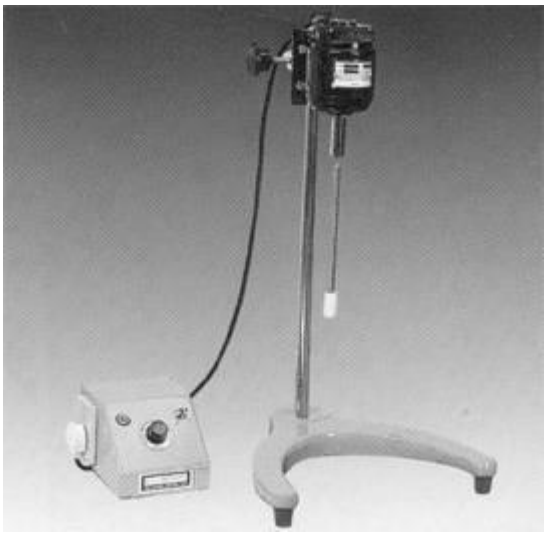


Fig 2 Vir-Tis homogenizer

Potter Elvejm homogenizer

This is also a mild technique and is used for homogenization of soft animal tissues. Potter Elvejm Homogenizer is a simple equipment having a pestle like glass rod with teeth like aberrations on its tip. There are down aberrations in the tube too on which teeth of the rod are fitted during up and down process of the rod. Up and down process of the pestle is done manually by hand or by mechanical device.

Lytic enzymes

Cell wall and cell membrane lytic enzymes like cellulase, pectinase, xylanase**,** pectin methyl esterase, lysozyme etc. can be used for rupturing the cells. Enzymes being costly are not commonly used for making cell free preparation for isolation of enzymes. In plant tissue culture, lytic enzymes are used to prepare protoplast.

Freeze-Thaw

With certain susceptible microbes and eukaryotic cells, repeated freezing and thawing results in extensive membrane lesions with release of periplasmic and intracellular proteins.

Acetone powder

Drying with acetone is a good method for rupturing the cell membrane. Using acetone, powder of the tissue may be prepared which may be stored in a Deep freezer for a long time. It forms a convenient starting material from which the enzyme may be extracted

with the isolating medium, whenever required. However, one has to take much precautions of low temperature (generally –20oC), otherwise, acetone may denature the enzyme protein.

Isolation of enzymes from sub-cellular organelles requires rupturing of the organelle. Generally for the purpose, organelle is isolated in intact form thus removing the contaminating proteins of the cytoplasm and other cell organelles. Afterwards, cell organelle is ruptured in the presence of a suitable detergent like tween, teepol, digitonin etc.

Methods of enzyme purification

The purification of a particular enzyme involves removal of other substances (proteins as well as non-proteins) present in the preparation.

Purification of an enzyme protein is generally a multi-step process exploiting a range of biophysical and biochemical characteristics such as

- Its relative concentration in the source
- Solubility
- Charge
- Size (molecular weight)
- Hydrophobicity/ hydrophilicity of the target protein.

In general, design of the purification technique/ protocol should be focused on

- High recovery
- Highly purified enzyme protein
- Reproducibility of the methods
- Economical use of the chemicals (reagents) and
- Shorter time for complete purification.

Proteins are relatively labile and get denatured at high temperatures and variation in pH. Each protein has its own physico-chemical characteristics. The techniques selected for enzyme purification should be moderate and native conformation of the enzyme protein should not change as a result of purification. During purification, degree of purity and percent of recovery should be checked after each step of purification.

In general, it has been observed that enzymes are more unstable in dilute solutions. Therefore, while designing the purification procedure, initially emphasis is given on concentrating the protein concentration in the sample rather than purification. After concentration, emphasis is given to purification (removal of unwanted proteins) and lesser loss of enzyme activity of the targeted enzyme.

Commonly, the first step in enzyme purification is based on fractionation of proteins on the basis of solubility of proteins in aqueous solutions of salts or organic solvents.

Fractionation of the proteins on the basis of solubility in aqueous solutions of salts or organic solvents

The solubility of a protein is the result of polar interactions with the aqueous solvent, ionic interactions with salts and repulsive electrostatic interactions between alike charged molecules. The properties of water may be changed by changing the ionic concentration and pH. The addition of miscible organic solvents, other inert solutes and polymers with temperature variation can be manipulated to cause selective precipitation. Isoelectric precipitation may also be used since protein is least soluble when net charge on it is zero.

Salting out

Generally, salting out of proteins using ammonium sulfate is used as the first step in the enzyme purification. However, other salts like sodium sulfate may also be used but ammonium sulfate is most common.

A large number of water molecules bind with the salt reducing the amount of water available to interact with the protein molecules. Precipitation of proteins using salt also removes non-protein impurities present in the enzyme homogenate (crude extract). At a particular concentration of the salt, unbound water will keep the protein in the soluble form. Generally, solid salt is added in the range of 0 to 30%, 30 to 60% and 60 to 90 % with continuous gentle stirring keeping the pH constant (near neutrality or slightly alkaline by the addition of dilute ammonia dropwise). Care is taken that no local precipitation of protein occurs.

On the other hand, care is taken that no denaturation of protein should occur due to stirring. Stirring is done either with the help of a glass rod (if volume is not too much) or with the help of a motorized mechanical stirrer. After addition of the salt, the suspension is stored for few hours in cold condition for complete precipitation. The precipitate is collected by centrifugation in cold condition and thereafter the precipitate is dissolved in suitable medium (which may be same as the isolating medium or may be little different).

Protein fractionation using organic solvents

Generally, acetone is used for fractionation of the enzyme protein. While using acetone, extreme care is to be taken otherwise acetone will denature the enzyme protein. Chilled acetone at –20oC is used and continuous stirring is done to avoid denaturation of the proteins. Like salt fractionation, here also fractionation of proteins is done by using different concentrations of acetone like 10%, 20%, 30% and so on. The suspension is stored for few hours before centrifugation at –20oC to collect the precipitate. The precipitate is dissolved in suitable medium.

Protein fractionation using nonionic polymers

Nonionic polymers can be used for precipitation of enzyme proteins. Polyethylene glycol (PEG) is commercially available in different molecular weight ranges like 6000, 40,000, 360,000. Lower molecular weight PEG is more soluble in aqueous solvents compared to high molecular weight, therefore, PEG of 6000 molecular weight is preferable. Different ranges of PEG (like 0-3%, 3-10%, 10-20%, 20-30%, 30-40%, 40-50%) are used for fractionation of proteins.

Protein fractionation by heat treatment

In many cases, the enzyme protein to be purified is fairly stable at temperature like 55^{o}C or 60^{o}C. At this temperature, many unwanted proteins get denatured and precipitated out with little or no loss of enzyme activity. The protein solution is kept for 5 to 10 minutes at this temperature and afterwards immediately chilled by keeping in ice. In few cases of allosteric enzymes, the enzyme is stable at high temperature in the presence of an effector molecule. However, this method is not in much use since there is always

a possibility of getting the conformation of the enzyme protein changed at higher temperature.

Three-phase partitioning (TPP)

Three-phase partitioning (TPP) is a method in which proteins are salted out from a solution containing a mixture of water and t-butanol. t-Butanol is infinitely miscible with water but upon addition of sufficient ammonium sulfate the solution splits into two phases, an underlying aqueous phase and an overlying t-butanol phase. If protein was present in the initial solution, three phases would be formed, protein being precipitated in a third phase between the aqueous and t-butanol phases.

The amount and type of protein precipitated is dependent upon the ammonium sulfate concentration, as in conventional salting out. Unlike in conventional salting out, however, the protein precipitate is largely dehydrated and has a low salt content.

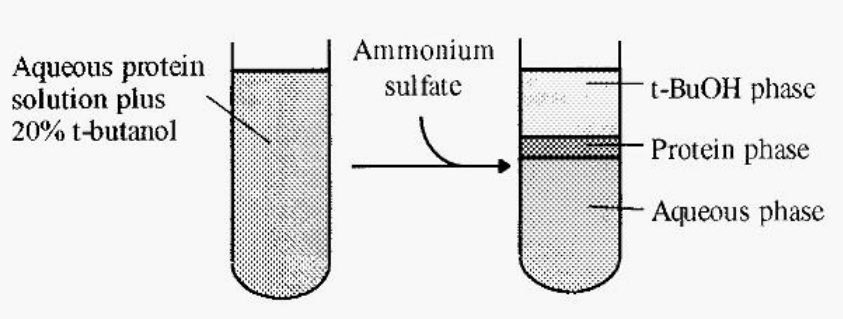


Fig 3 Three-phase partitioning.

Dialysis

Dialysis is typically used to desalt protein solutions, or to effect a buffer exchange, i.e. to get the protein from one buffer solution into another (Note that “desalting” and “buffer exchange” are really the same process).

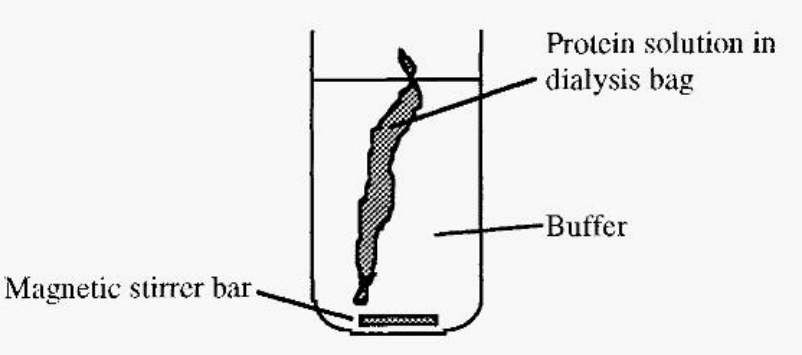


Fig 4 Dialysis using a visking dialysis bag.

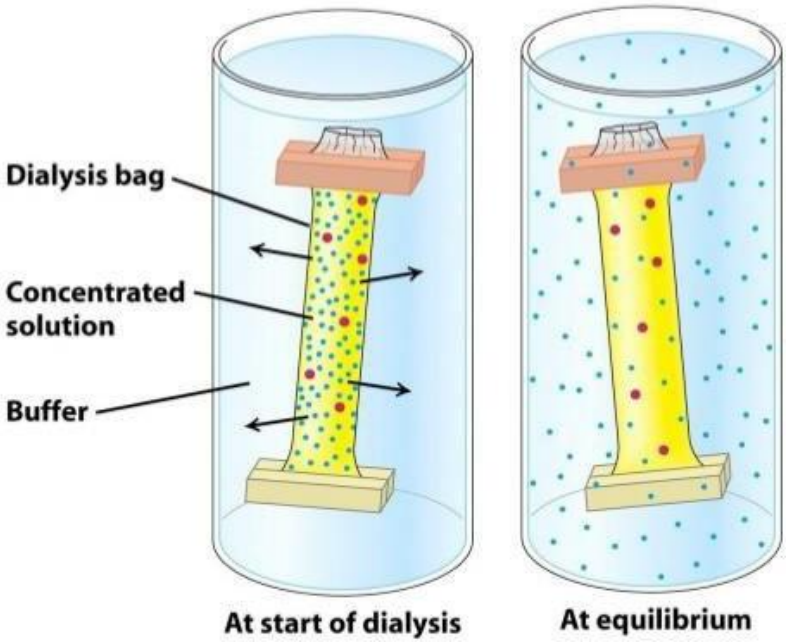


Fig 5 Dialysis

Dialysis can be done in various ways, but in the laboratory it is most commonly done using “Visking” tubing. This is a cellulosic material reconstituted into tubular form, dried, and supplied in rolls. A length can be cut from the roll, hydrated by immersion in water for several minutes, and clamped or knotted at one end to form a sealed “dialysis bag”. The protein is introduced into this bag and the open end is sealed by clamping or knotting. The dialysis bag is immersed in a large volume of distilled water or buffer for several hours at 4°C to effect exchange of the permeable ions and molecules, the dialysis solution being changed at intervals (every few hours).

During dialysis, water enters the dialysis bag due to the osmotic pressure of the protein solution. For this reason a dialysis bag must not be filled, but a potential space must be left to accommodate the increasing volume of the protein solution. Note that if the dialysis bag is sealed with knots, the knot should be tightened by pulling only on the outside, not on the bag side of the knot, to avoid stretching the bag and thus distorting the pores.

Ultrafiltration

Ultrafiltration is a technique related to dialysis, and can also be used to desalt protein solutions, effect buffer exchange, or concentrate protein solutions. It is more expensive than dialysis, however, as special equipment and membranes are required.

In this technique, pressure is applied to the solution to cause a bulk flow of water and dissolved low molecular weight solutes, through the membrane, while high molecular weight solutes are retained.

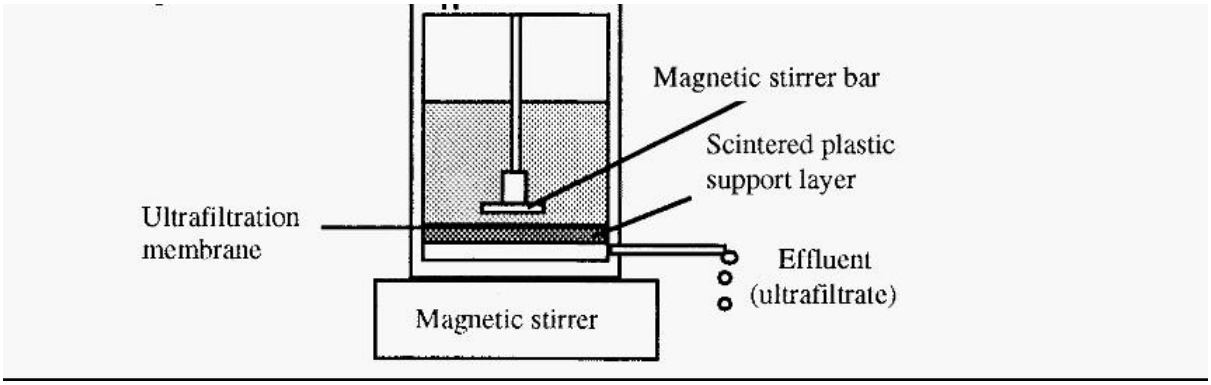


Fig 6 An ultrafiltration cell.

Chromatographic separation of the enzyme proteins

The word “chromatography” means “writing with colour” and refers to the early observations on the separation of dyes by paper chromatography. All chromatographic separations depend upon the differential partition of solutes between two phases, a mobile phase and a stationary phase. Such partition between two phases is described by the so-called partition coefficient or distribution coefficient.

The distribution coefficient (Kd) is a constant and can be defined as

$$K_d = \frac{\text{concentration of solute in A}}{\text{concentration of solute in B}}$$

For enzyme purification, commonly used chromatography techniques are:

(i) Ion exchange chromatography

(ii) Adsorption chromatography

(iii) Gel filtration chromatography and

(iv) Affinity chromatography.

In general, the procedure of carrying the work is same in all types of chromatography. First, the enzyme protein sample to be purified is applied onto the pre-equilibrated column and thereafter, the sample from the column is eluted with buffer with a series of steps of different solute concentrations, with a gradient of solute or with a specific ligand for the desired enzyme protein. The effluent eluted out from the column is collected as a series of fractions using a fraction collector, tested for enzyme activity and protein.

Ion exchange chromatography

- The basic principle involved in ion exchange chromatography is binding of charged proteins on the ion exchanger by electrostatic attraction (ionic bonds) between charged groups on the proteins and opposite charges on the exchanger.
- Unbound proteins are removed from the column by washing with the same medium used for pre-equilibrium. Bound proteins are eluted by passing buffer of higher ionic strength (using salts like sodium or potassium chloride) or by using buffer of different pH.
- Two types of ion exchangers are in common use for separation of enzymes: Anion exchangers and cation exchangers.
- The most commonly used anion exchanger is diethyl amino ethyl cellulose (DEAE cellulose). Some other are amino ethyl cellulose (AE cellulose), triethyl

amino ethyl cellulose (TEAE cellulose) and guanido ethyl cellulose (GE cellulose).

- The most commonly used cation exchanger is carboxy methyl cellulose (CM cellulose). The other examples of cation exchangers are phospho cellulose (P cellulose) and sulfo ethyl cellulose (SE cellulose). These exchangers have cellulose matrix, which is considered to be inert. The other matrices used in exchangers are Sephadex and Sepharose.

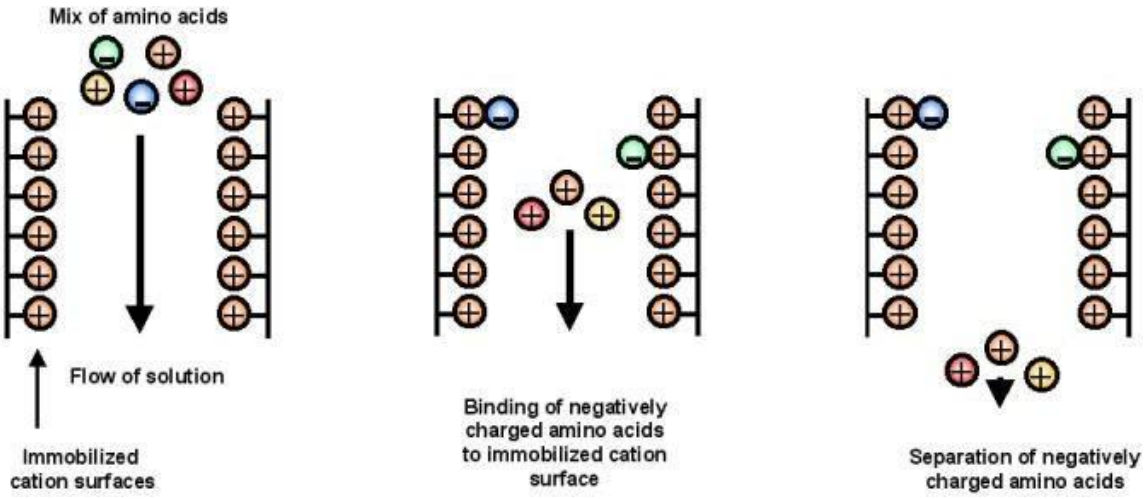


Fig 7 Ion exchange chromatography (Anion exchange)

1. Equilibration of the ion exchanger in a buffer in such a way that the molecule(s) of interest will bind in a desirable way.
2. a) Application of the sample. Solute molecules carrying the appropriate charge are bound reversibly to the gel.
   b) Unbound substances are washed out with the starting buffer.
3. Elution with a gradient of e.g. NaCl. This gradually increases the ionic strength and the molecules are eluted. The solute molecules are released from the column in the order of the strengths of their binding i.e. the weakly bound molecules elute first.
4. Substances that are very tightly bound are washed out with a concentrated salt solution and the column is regenerated to the starting conditions.

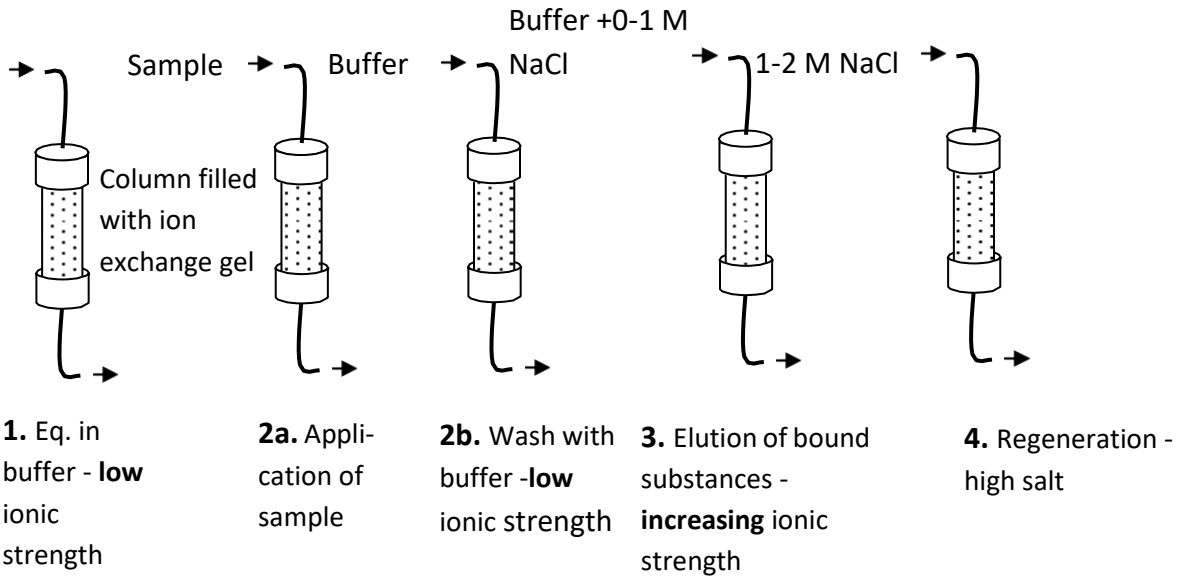


Fig 8 Ion Exchange chromatography

Adsorption chromatography

The basic principle in this type of chromatography is binding of the proteins on the matrix by physical adsorption on the surface of insoluble matrix (through weaker bonds like hydrogen, van der Waals bonds). Afterwards, proteins are eluted from the column matrix by using a suitable elution buffer either having change in ionic concentration or pH. The commonly used matrices in adsorption chromatography are: (i) calcium phosphate gel; (ii) alumina gel and (iii) hydroxylapatite gel.

In this type of chromatography, gel to protein ratio is important for physical adsorption. It is preferable to carry a trial experiment in centrifuge tubes. A constant amount of the gel is put in each tube and different amounts of protein sample are added in each tube so that ratio of 0.1 to 2.0 in different tubes be obtained. After addition of sample, it is mixed with the gel and allowed to bind for few minutes. Afterwards, tubes are centrifuged and enzyme activity is determined in different tubes supernatants. If enzyme activity is present in a supernatant, it means binding of the enzyme protein (of interest) on the gel did not occur. From this trial experiment, one can determine, what will be the optimum gel to protein ratio so that enzyme protein of interest gets adsorped on the gel surface. Afterwards, accordingly, size of the packed gel in the column be decided. For elution, generally either buffer of high ionic strength or buffer with salt like NaCl or KCl is used.

The gels used in adsorption chromatography are commercially available. The gels may also be prepared in the laboratory. It is found that older gels are more effective in separation compared to newly prepared gel. In the laboratory, calcium phosphate gel is prepared by addition of sodium tri phosphate to a diluted solution of calcium chloride and pH is adjusted to 7.4. A precipitate of calcium phosphate formed is washed to remove excess ions.

Alumina gel is prepared by the addition of a hot solution of aluminum ammonium sulfate to a solution containing ammonium sulfate, ammonia and water at 60^{o}C. The solution is cooled, the precipitate of alumina formed is washed with water to remove excess ions. Hydroxylapatite gel is prepared by addition of calcium chloride and di sodium hydrogen phosphate to a solution of one molar sodium chloride. The precipitate of hydroxylapatite formed is treated with alkali and heated to boiling for about 40 to 50 minutes. Afterwards, it is cooled and washed with water to remove excess ions.

Gel filtration (Molecular sieve) chromatography

The basic principle is based on the size and shape of the proteins. Here, gel particles have sponge like porous matrix as a structure with controlled dimension. The gel particles are swollen and equilibrated with appropriate medium and afterwards is packed in the chromatography column. Gel particles are spherical in shape. The molecules (proteins) to be separated enter in the porous matrix of the gel particles and too large molecules are not entered in the porous matrix and are eluted out from the column. Every gel is characterized by exclusion limit that means the proteins of more than that molecular weight will not enter in the matrix and eluted out as such (without separation). Void volume is considered as the space between the gel particles in the packed column. It is determined by passing blue dextran, which has very high molecular weight. Molecules with masses below the exclusion limit of the gel are eluted from the column in order of their molecular mass (weight) with the largest eluting first. Larger molecules have lesser of the interior volume of the gel available to them than the smaller molecules.

The commonly used gel filtration gels are of dextran, agarose, polyacrylamide. These gels are having registered trade names of the manufacturers. For example, dextran gels having registered trade name' Sephadex' are in much common use. Gel filtration chromatography (with matrix having much lesser exclusion limit such as Sephadex G-25) is also used for desalting purpose. Since in Gel filtration chromatography, separation is based on molecular weight (if shape of all the molecules is same), this chromatography has been commonly used for determination of molecular weight of proteins.

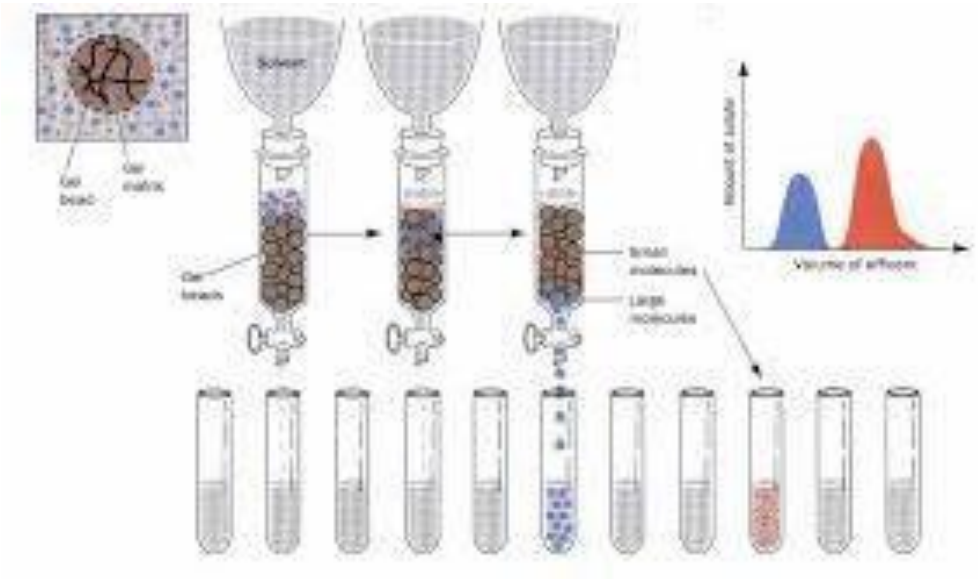


Fig 9 Gel filtration chromatography

Advantages of Gel Filtration

Can handle biomolecules that are sensitive to changes in pH, concentration of metal ions or harsh environmental conditions.

Separations can be performed in the presence of essential ions, detergents, urea guanidine hydrochloride at high or low ionic strength. At 37 °C or in the cold room according to the requirements of the experiment.

Common terms in size exclusion chromatography

1. The total volume (Vt): the sum of the volume of the gel matrix, the volume inside the gel matrix, and the volume outside the matrix. The total volume is also, in most cases, equal to the amount of the eluent required to elute

a substance through the column, when the substance is small enough to completely penetrate the pores of the gel.

2. Inner volume (Vi): the volume of the eluent inside the gel matrix. The volume inside the beads.
3. Void volume (Vo): the volume of eluent outside the gel matrix. This is the volume required to elute a substance so large that it cannot penetrate the pores at all. Such a substance is said to be completely excluded, such as dextran blue 2000.
4. Elution volume (Ve): the volume of eluent required to elute any given substance.
5. Gel volume (Vg): the volume of dry gel.

Affinity chromatography

The basic principle involves bio-specific interaction of the enzyme protein of interest with an immobilized ligand, which may be substrate, analogue of the substrate, inhibitor, activator. Inert materials like agarose, polyacrylamide, glass beads, cellulose etc have been used as supporting medium (matrix). The ligand is attached so that its enzyme interaction function is not impaired. Subsequently, elution is done by treatment resulting in dissociation of the desired enzyme ligand complex. Nowadays, affinity matrices (ligand immobilized with the matrix) are commercially available.

Immuno-affinity chromatography is also an affinity chromatography where antibody of the protein is used as ligand. The basic principle of antigen antibody interaction in this chromatography is applied. Although it is a good technique for purification of a protein, it is not in common use for enzymes since generally enzyme gets inactivated after binding with the antibody.

Besides, another affinity chromatography called Dye Affinity Chromatography is also used for enzyme purification. Specific dye bound matrices are available commercially which are used in Dye Affinity Chromatography. One such popular dye matrix is Green A. Binding affinity of the dye is ranging from 1 to 15 mg protein per ml gel. In dye affinity chromatography too, elution is done by using higher ionic strength (presence of salt in the elution medium).

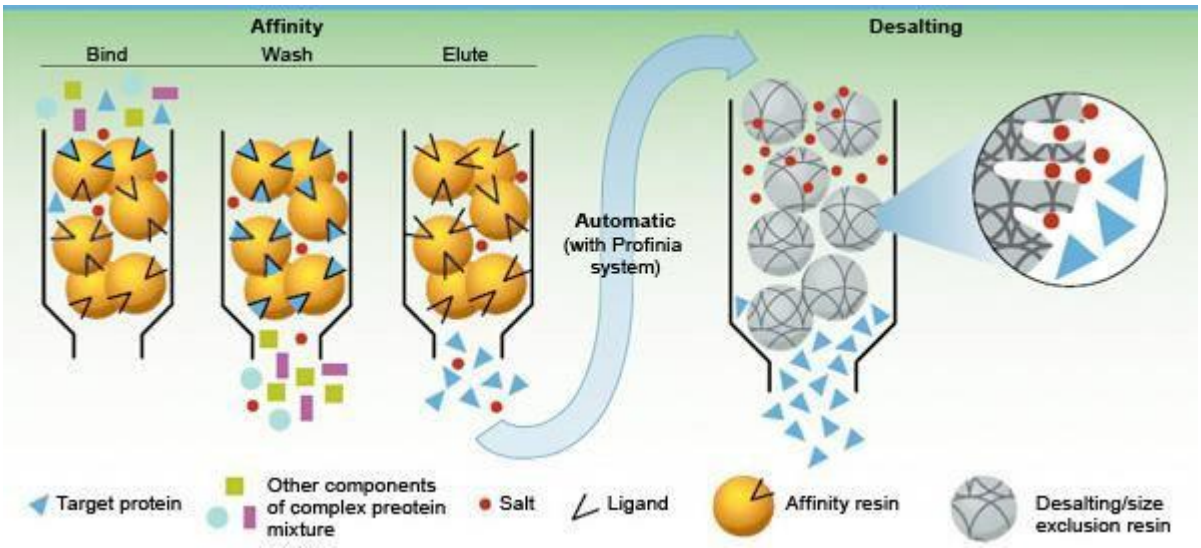


Fig 10 Affinity Chromatography

Turnover number

In enzymology, turnover number (also termed k_{cat}) is defined as the maximum number of chemical conversions of substrate molecules per second that a single catalytic site will execute for a given enzyme concentration $[E]_T$. It can be calculated from the maximum reaction rate $V_{max}$and catalyst site concentration $[E]_T$as follows:

$$k_{cat} = \frac{V_{max}}{[E]_T}$$

For example, carbonic anhydrase has a turnover number of 400,000 to 600,000 s^{-1}, which means that each carbonic anhydrase molecule can produce up to 600,000 molecules of product (bicarbonate ions) per second.

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – V – Enzyme Technology – SBTA1401

Application of enzymes in medicine.

Enzyme	Principle Sources of Enzyme in blood	Clinical applications
Alanine aminotransferase	Liver	Hepatic parenchymal diseases
Alkaline phosphetase	Liver, bone, intestinal mucosa, placenta	Bone diseases, hepatobiliry diseases
Amylase	Salivary glands, pancreae	Pancreatic diseases
Aspartate aminotrasferase	Liver, skeletal muscle, heart erythrocytes	Hepatic parenchymal disease, muscle disease
Cholinesterase	Liver	Organophosphorus insecticide poisoning, hepatic parenchymal disease
Creatine kinase	Skeletal muscle, heart	Muscle diseases(M.I.)
γ-glutamyl transferase	Liver	Hepatobiliary diseases, marker of alcohol abuse
Lactate dehydrogenase	Heart, liver, skeletal muscle, erythroctes, platelets, lymph nodes	Hemolysis, hepatic parenchymal diseases, tumor marker
lipase	Pancreas	Pancreatic diseases
5'-nucleotidase	Liver	Hepatbiliary diseases
Trypsin	pancreas	Pancreatic diseases

Lactate Dehydrogenase

Catalyses the reversible inter-conversion of lactate and pyruvate. The enzyme is widely distributed in the body, with high concentrations in cells of cardiac and skeletal muscle, liver, kidney, brain and erythrocytes: measurement of plasma total LD activity is therefore a nonspecific marker of cell damage.

LD has a molecular weight of 134 kDa and is composed of four peptide chains of two types: M (or A) and H (or B)

The subunit compositions of the five isoenzymes are listed below in order of their decreasing anodal mobility in an alkaline medium.

LD-1 (HHHH; H4) = migrates fastest towards the

anodeLD-2 (HHHM; H3M)

LD-3 (HHMM; H2M2)

LD-4 (HMMM; HM3)

LD-5 (MMMM; M4)

Clinical significance

- Normal range of total LDH: 180-360 U/L= 3.1-6.1 μkat/L
- It is increased in plasma in myocardial infarction, acute leukemias, generalized carcinomatosis and in acute hepatitis. Estimation of its isoenzymes in more useful in clinching diagnosis between hepatic disease and myocardial infarction.
- LDH1 fraction predominates in cells of cardiac muscle, erythrocytes and kidneys.
- LDH5 is the most abundant form in the liver and in skeletal muscle. Whereas in many conditions there is an increase in all fractions, the finding of certain patterns is of diagnostic value.
- Predominant elevation of LDH1 and LDH5. (LDH1 greater than LDH5 occurs after myocardial infarction, in megaloblastic anaemia and after renal infarction.
- Predominant elevation of LDH2 and LDH3 occurs in acute leukaemia: LDH3 is the main isoenzyme elevated due to malignancy of many tissues.
- Elevation of LDH5 occurs after damage to the liver or skeletal muscle.

Aspartate Transaminase

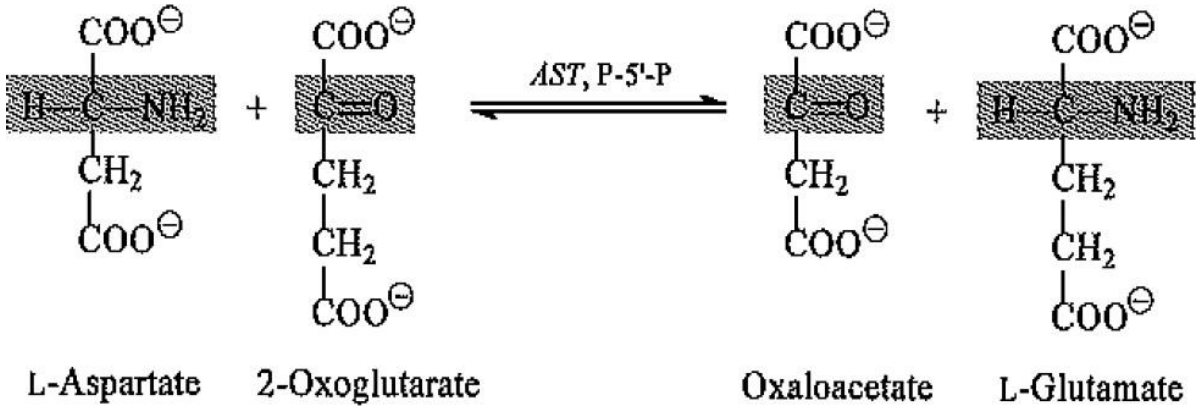
Clinical Significance

Normal values of AST:

male: <35 U/L = <0.60 µkat/L

Female: <31 U/L = <0.53 µkat/L

AST (glutamate oxaloacetate transaminase. GOT) is present in high concentrations incells of cardiac and skeletal muscle, liver, kidney and erythrocytes. Damage to any of these tissues may increase plasma AST levels. Half- life = 17 hours.



CAUSES OF RAISED PLASMA AST ACTIVITIES

- Artefactual.-Due to in vitro release from erythrocytes if there is haemolysis or ifseparation of plasma from cells is delayed.
- Physiological-During the neonatal period (about 1.5 times the upper adult reference limit).
- Marked increase (10 to 100 times the upper adult reference limit):
    - Circulatory failure with 'shock' and hypoxia:
    - Myocardial infarction
    - Acute viral or toxic hepatitis.

Alanine Transaminase

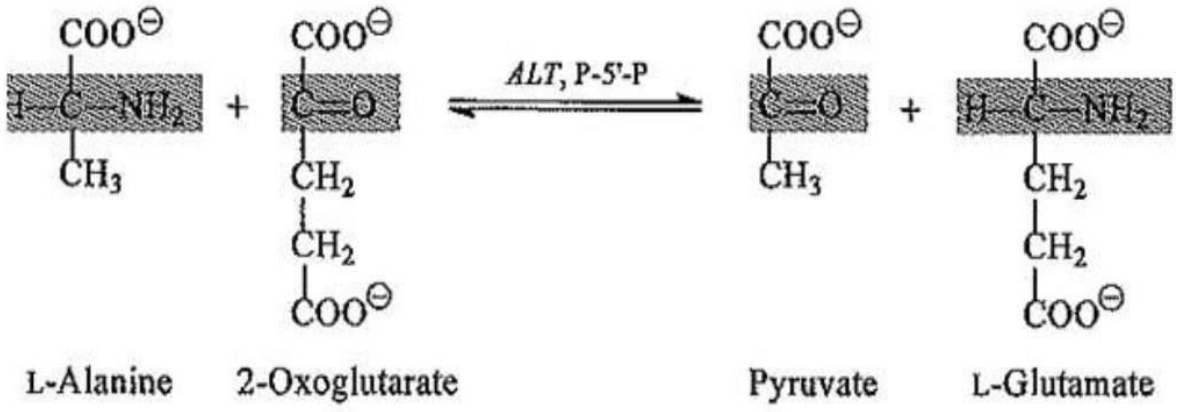
Clinical Significance

Normal values of ALT:

male: <45 U/L = <0.77 µkat/L

Female: <34 U/L = <0.58 µkat/L

ALT (glutamate pyruvate transaminase. GPT) is present in high concentrations in liver and to a lesser extent, in skeletal muscle, kidney and heart. Half- life = 47 hours In liver damage, both enzymes are increased but ALT increases more. In myocardial infarction AST is increased with little or no increase in ALT.



CAUSES OF RAISED PLASMA ALT ACTIVITIES

- Marked increase (10 to 100 times the upper limit of the adult reference range

  circulatory failure with 'shock' and hypoxia:

  Acute viral or toxic hepatitis.

- Moderate increase:

  Cirrhosis (may be normal or up to twice the upper adult reference limit): infectious

mononucleosis (due to liver involvement):

Liver congestion secondary to congestive cardiac failure:

cholestatic jaundice (up to 10 times the upper reference limit in adults); surgery orextensive trauma and skeletal muscle disease (much less affected than AST)

Alkaline phosphatase

Clinical Significance

The alkaline phosphatases are a group of enzymes that hydrolyse organic phosphates at high pH. They are present in most tissues but are in particularly high concentration in the osteoblasts of bone and the cells of the hepatobiliary tract, intestinal wall, renal tubules and placenta. The exact metabolic function of ALP is unknown but it is probably important for calcification of bone.

In adults plasma ALP is derived mainly from bone and liver in approximately equal proportions: the proportion due to the bone fraction is increased when there is increased osteoblastic activity that may be physiological.

Causes of raised Plasma ALP activity

- Physiological: There is a gradual increase in the proportion of liver ALP with

  age:In the elderly the plasma bone isoenzyme activity may increase slightly.

- Bone disease

  Rickets and osteomalacia

  Secondary hyperparathyroidism .

- Liver disease-.
- Malignancy.

  Bone or liver involvement or direct tumor production.

Table: Reference intervals for alkaline phosphatase activities in serum

Sex	Age (years)	Reference Interval (U/L)
Male/Female	4–15	54–369
Males	20–50	53–128
	≥60	56–119
Females	20–50	42–98
	≥60	53–141

Acid Phospbatase

Acid phosphatase is present in lysosomes, which are organelles present in all cells with the possible exception of erythrocytes. Extralysosomal ACPs are also present in many cells:

a. prostate,

b. bone (osteoclasts),

c. spleen

d. platelets

e. erythrocytes.

Normal range - 1.5-4.5 U/L= 0.03-0.08 μkat/L

Elevated ACP levels found in

a. Paget disease

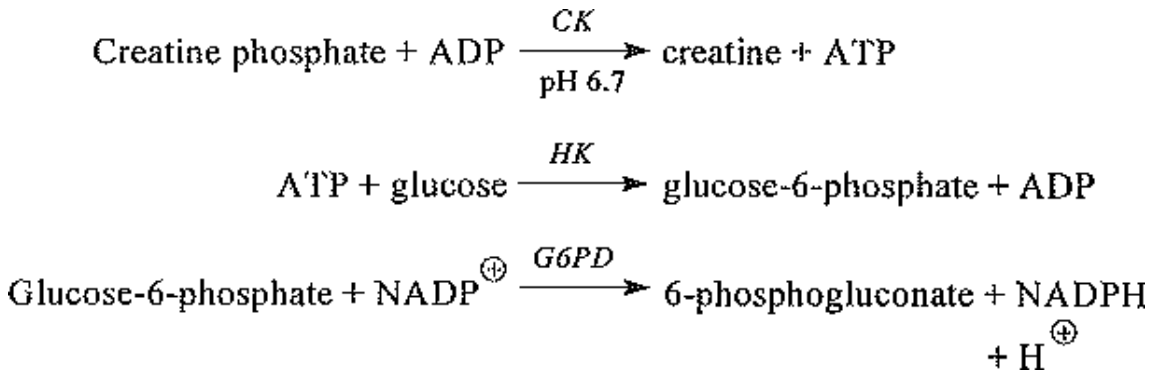
b. Hyperparathyroidism with skeletal involvement

c. Presence of malignant invasion of bones by cancers

The only nonbone condition in which elevated activities of TR-ACP are found in serum is Gaucher disease of the spleen, a lysosome storage disease.

Creatine Kinase

CK is most abundant in cells of cardiac and skeletal muscle and in brain, but also occurs in other tissues such as smooth muscle.



Normal range for total CK:

Male : 46-171 U/L= 0.78-2.90 µkat/L

Female: 34-145 U/L= 0.58-2.47 µkat/L

Serum CK activity is greatly elevated in all types of muscular dystrophy. In progressive muscular dystrophy (particularly Duchenne sex-linked muscular dystrophy), enzyme activity in serum is highest in infancy and childhood (7-10 years of age) and may increase long before the disease is clinically apparent. Serum CK activity characteristically falls as patients get older and as the mass functioning muscle diminishes with the progression of the disease. About 50%-80% of the asymptomatic female carriers of Duchenne dystrophy show threefold to six-fold increase of CK activity. Quite high values of Ck are noted in viral myositis, polymyositis and similar muscle disease. However in neurogenic muscle disease,such as:

a. Myasthenia gravis

b. Multiple sclerosis

c. Polimyeltis

d. Parkinsonism

ISOENZYMES OF CK

CK consists of two protein subunits, M (for muscle) and B (for brain), which combine to form three isoenzymes. BB (CK-1), MB (CK-2) and MM (CK-3). CK-MM is the

predominant isoenzyme in skeletal and cardiac muscle and is detectable in the plasma of normal subjects.

CK-MB accounts for about 35 per cent of the total CK activity in cardiac muscle and less than five per cent in skeletal muscle: its plasma activity is always high after myocardial infarction. It may be detectable in the plasma of patients with a variety of other disorders in whom the total CK activity is raised, but this accounts for less than six per cent of the total.

CK-BB is present in high concentrations in the brain and in the smooth muscle of the gastrointestinal and genital tracts. Although they have also been reported after brain damage and in association with malignant tumours of the bronchus, prostate and breast, measurement is not of proven value for diagnosing these conditions. In malignant disease plasma total CK activity is usually normal.

Other Clinical correlations

1. Niemann-Pick disease: Acid Sphingomyelinase Deficiency

- **Sphingomyelin**, a ubiquitous component of cell membranes, especially neuronalv membranes, is normally degraded within lysosomes by the enzyme **sphingomyelinase**.
- In patients with **Niemann-Pick disease**, inherited deficiency of this enzyme causes spingomyelin to **accumulate in lysosomes** of the brain, bone marrow, and other organs.
- Enlargement of the lysosomes interferes with their normal function, leading to cell death and consequent **neuropathy.**
- Symptoms include **failure to thrive** and **death** in early childhood as well as **learning disorders** in those who survive the postnatal period.

2. Homocysteinuria: Cystathionine β-synthase Deficiency

1. **Cystathionine β-synthase** catalyzes conversion of homocysteine to cystathionine, a critical **precursor of cysteine**.

2. Deficiency of this enzyme leads to the most common form of **homocystinuria**, a pediatric disorder characterized by **accumulation of homocysteine** and reduced activity of several sulfotransferase reactions that require this compound or its derivatives as substrate.

3. Accumulation of homocysteine and reduced transsulfation of various compounds leads to abnormalities in connective tissue structures that cause altered blood vessel wall structure, loss of skeletal bone density (**osteoporosis**), **dislocated optic lens**

(ectopia lentis), and increased risk of **blood clots**.

3. Enzyme Replacement Therapy for Inborn Errors of Metabolism

- **Lysosomal enzyme deficiencies**, which frequently result in disease due to **accumulation of the substrate** for the missing enzyme, are suitable targets for **enzyme replacement therapy (ERT).**
- In ERT, **intravenously administered enzymes** are taken up directly by the affected cells through a receptor-mediated mechanism.
- ERT provides temporary relief of symptoms but must be given repeatedly and is not a permanent cure

BIOSENSOR

Biosensors were first reported in 1960s. They are a class of devices that have found a widespread use, ranging from the detection of gas molecules to the real time tracking of chemical signals in biological cells.

A biosensor is an analytical device which converts a biological response into an electrical signal. The term 'biosensor' is often used to cover sensor devices used in order to determine the concentration of substances and other parameters of biological interest even where they do not utilise a biological system directly.

In general a sensor comprises an active sensing element and a signal transducer and produces an electrical, optical, thermal or magnetic output signal. The sensing element

is responsible for the selective detection of the analyte and the transducer converts a chemical event into an appropriate signal that can be used with or without amplification.

The sensing element consists of a biological material (protein, cell receptor, antibody, enzyme). The sensor is used to monitor biological processes or for the recognition of biomolecules. The sensing of the biomaterial can be made invitro or invivo:

Invitro biosensing

The sample solution (blood, urine) is dropped at the biosensor and the output signal gives information on the composition of the solution. For example: measurements of blood glucose concentrations.

Invivo biosensing

Dynamic systems aiming for instance to measure the rate of uptake of relevant species or to estimate the spatial distribution of the concentration of an analyte in a living organism. For example: portable sensing devices for continuous monitoring of parameters of blood glucose, carbón dioxide, pH, neurochemical analysis.

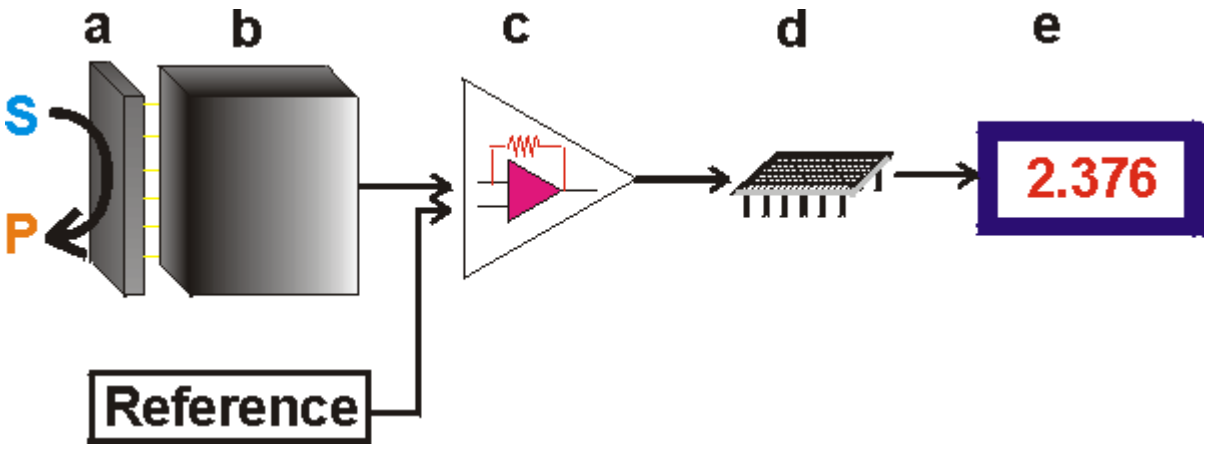


Fig 1 Biosensor

a. The biocatalyst
b. Transducer
c. Amplifier

d. Processer
e. Display

Potential Applications of Biosensors

- Agricultural, horticultural and veterinary analysis
- Pollution, water, and microbial contamination analysis
- Clinical diagnosis and biomedical applications
- Fermentation analysis and control
- Industrial gases and liquids
- Mining and toxic gases
- Explosives and military arena
- Flavors, essences and pheromones

The enzyme biosensors can be classified based on the measuring electrical parameters as :

(1) Conductimetric

The measured parameter is the electrical conductance/resistance of the solution. When ectrochemical reactions produce ions or electrons the overall conductivity/resistivity of the solution changes. This change is measured and calibrated to a proper scale.

(2) Amperometric

This high sensitivity biosensor can detect electroactive species present in biological test samples. Since the biological test samples may not be intrinsically electro-active, enzymes needed to catalyze production of radio-active species. In this case, the measured parameter is current.

(3) Potentiometric

In this type of sensors the measured parameter is oxidation/reduction potential (of an electrochemical reaction). The working principle of that when a ramp voltage is applied to an electrode in solution the current flow occurs because of electrochemical reaction.

The voltage at which these reactions occurs indicate a particular reaction and particular species

GLUCOSE ENZYME ELECTRODE

The first glucose enzyme electrode relied on a thin layer of the enzyme GOx (glucose oxidase) entrapped over an oxygen electrode via a semipermeable dialysis membrane. Note that the entire field of biosensors can trace its origin to this original glucose enzyme electrode, the first bioelectrode. Measurements were made based on the monitoring of the oxygen consumed by the enzyme catalyzed reaction

$$\text{glucose} + \text{oxygen} \xrightarrow{\text{GOx}} \text{gluconic acid} + H2O2$$

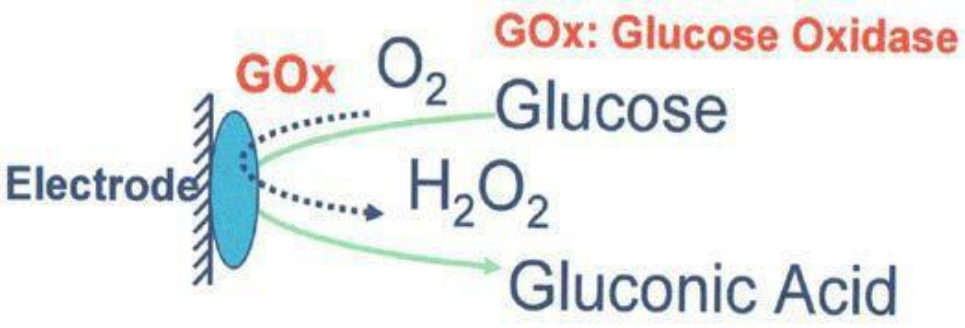


Fig 2 Glucose biosensor

A negative potential was applied to a platinum (Pt) cathode for reductive detection of the oxygen consumption: $O_2 + 4H^+ + 4\ e^- \rightarrow 2H_2O$

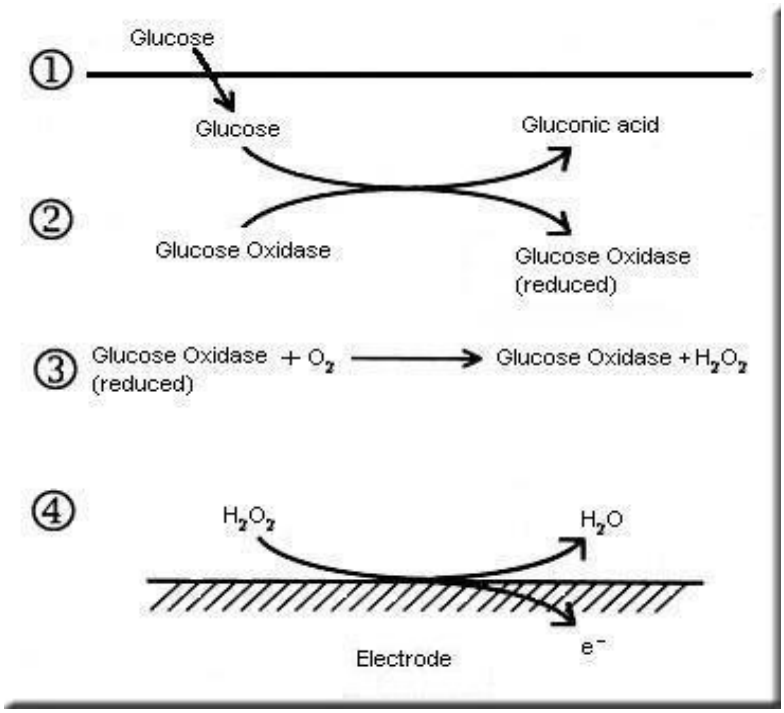


Fig 3 Working principle of glucose biosensor

ENZYME ELECTRODES

Potentiometric electrodes for the analysis of molecules of biochemical importance can be constructed in a fashion similar to that used for gas-sensing electrodes. The most common class of potentiometric biosensors are the so-called enzyme electrodes**,** in which an enzyme is trapped or immobilized at the surface of an ion-selective electrode.

Potentiometric biosensors have also been designed around other biologically active species, including antibodies, bacterial particles, tissue, and hormone receptors.

Enzyme electrodes are devices in which the analyte is either a substrate (also called reactant) or a product of the enzyme reaction, detected potentiometrically or amperometrically

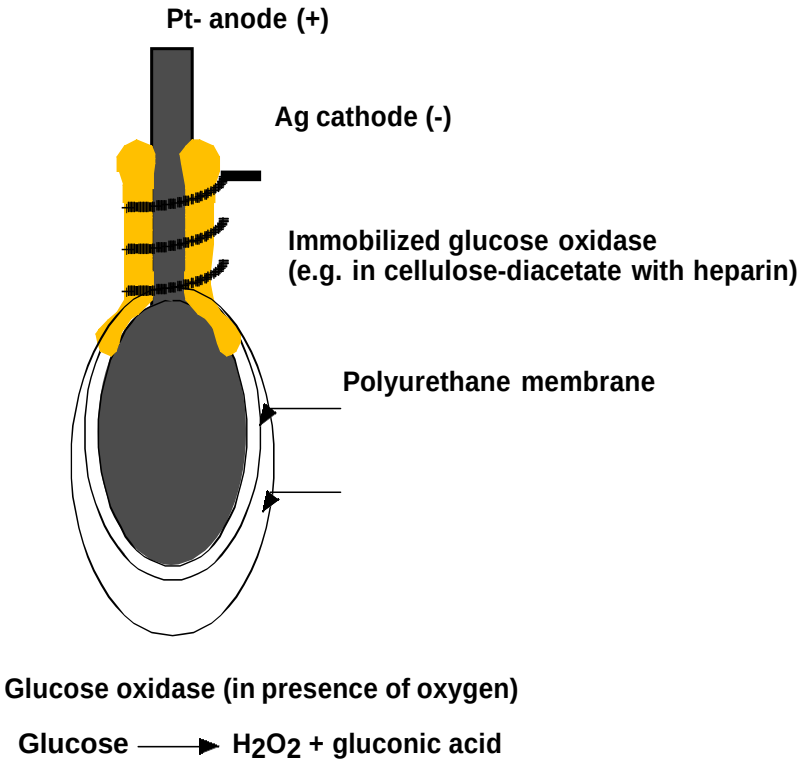


Fig 4 Enzyme Electrode

Example : glucose sensor substrate (glucose) diffuses through a membrane to the enzyme layer where glucose is converted

Both oxygen (which is being consumed) and H_2O_2 (which is being produced) can be measured electrochemically (in an amperometric technique), or the local pH change can be monitored (in a potentiometric measurement)

Other Enzyme Electrodes

- Cholesterol electrodes

- Lactate electrodes

- Penicillin electrodes

Cholesterol electrodes

In clinical diagnosis, cholesterol is an important indicator in human blood for hypertension, myocardial infarction and arteriosclerosis. The normal range of total cholesterol in blood plasma, of which one-third is free cholesterol and two-thirds are cholesterol ester, is 3.96 ± 0.8 mM (153 ± 31 mg/100 mL)

An amperometric biosensor with poly(3-thiopheneacetic acid) as a matrix has been fabricated for linking cholesterol oxidase, in order to carry out the detection of cholesterol. In addition, ferricinium ion (Fc^+) was added as a mediator in the sensing system to prevent the formation of H_2O_2.

Cholesterol + ChO_{ox} → Cholestenone + ChO_{red}

ChO_{red} + $2Fc^+$ → ChO_{ox} + 2Fc

2Fc → $2Fc^+$ + 2e−

Where ChO_{ox} and ChO_{red} represent the oxidized state and the reduced state of cholesterol oxidase, respectively. As the reactions involved are in series, the cholesterol concentration can be determined indirectly by sensing the current contributed from the electrochemical oxidation of Fc.

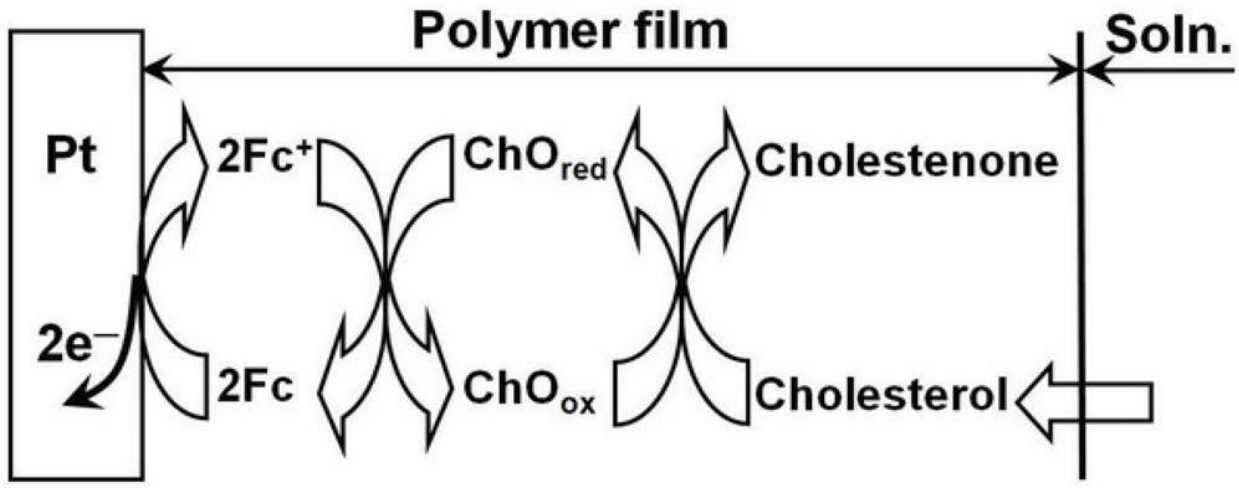


Fig 5 Electrocatalytic oxidation of cholesterol on the enzyme electrode

Lactate electrode

Lactate is the key metabolite of anaerobic glycolytic pathway and its accurate determination is in growing demand in many fields such as clinical and sport medicine and the fermentation industry. The detection is based on the principle that lactate is converted to pyruvate and hydrogen peroxide by lactate oxidase. The enzyme was immobilized onto a PB modified film polarized at adequate potentials for measuring the generated hydrogen peroxide.

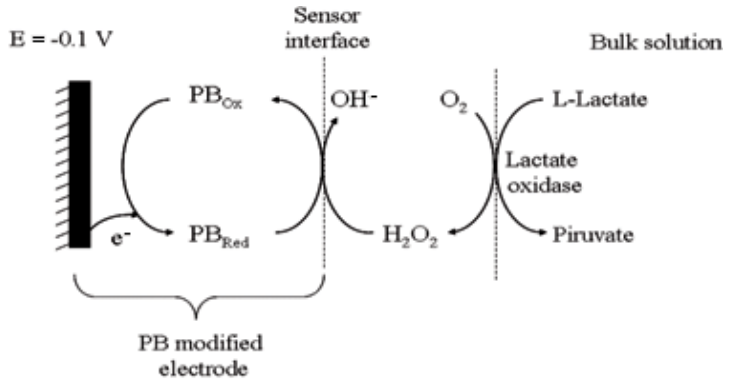


Fig 6 Lactate electrode

Penicillin electrode

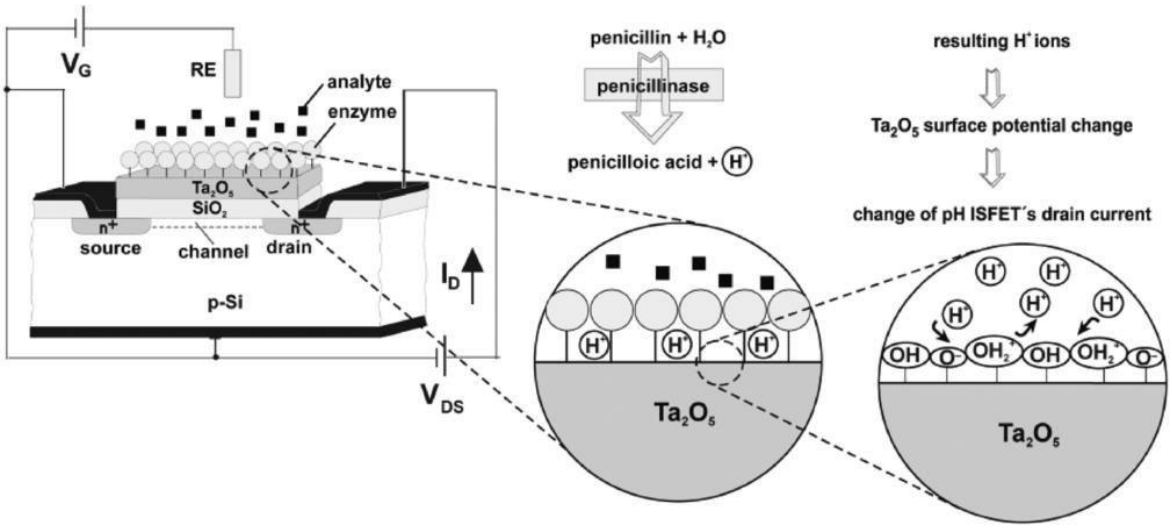


Fig 7 Penicillin electrode

Used to measure the amount of penicillin present by using a penicillinase enzyme electrode. The enzyme was immobilized with tantalum pentoxide (Ta_2O_5).