2 Enzymes

INTRODUCTION AND HISTORICAL PERSPECTIVE

Perhaps oldest known bio-chemical as well as bio-organic phenomenon is fermentation of juices to alcoholic beverages. Also, this was first chemical transformation catalysed by enzymes contained within living yeast cells. It was discovered in 18th century that fermentation leads to conversion of sugars into carbon dioxide and alcohol.

The 19th century witnessed both the identification of fermentation as a physiological act of yeast cells and introduction of Pasteur's view that life & fermentation are inseparable. By now, extraction of enzymes from bio-cells was known & this discovery like most of scientific discoveries was accidental. In 1897, E. Buchner required a quantity of purified protein for therapeutic purpose. He grounded yeast & sand, filtered the broken cells, and added a large amount of sugar to the filtrate as preservative. To his astonishment sugar was rapidly fermented by the cell free extract.

Harder & Young made an important contribution in 1905 when they showed that **zymase** displayed by Buchner required the addition of heat stable co-factor or co-enzyme. Thus, within a single decade two fundamental discoveries–solublization of zymase activity & introduction of co-enzyme opened the route to the eventual isolation & identification of large number of individual enzymes and co-factors making up the zymase system. Subsequent attempts at the isolation & purification of enzymes proved their worth when J.B. Summer (1928) crystallized **urease** and established the chemical nature of enzyme. Enzymes were shown to be proteins that possess capability of catalysing specific chemical reactions.

In 1930–1940 decade Warburg & his school were successful in crystallizing and purifying a number of respiratory enzymes. Theorell working in Warburg institute reversibly dissociated the **"enzyme"** into a protein part, *i.e.*, **apoenzyme** and a co-factor (prosthetic group). The separated components were inactive. This experiment opened up the possibility of studying the mode of binding of co-factors to proteins (All enzymes are globular proteins). Efforts to break enzymes into their components and reassembling them culminated in 1969 in the first chemical synthesis of enzyme, **ribonuclease,** by two different routes. Along with studies on enzyme structure, investigations of techniques of studying & describing the kinetic parameter of enzyme-catalysed reactions went on at equal pace. The total outcome of these efforts were several theories on detailed mechanism of the catalytic activity of enzymes.

Harder & Young (1905) found that inorganic phosphate as well as co-enzymes

caused stimulation of carbohydrate oxidation in yeast juice. Many of the intermediates in carbohydrate oxidation were soon found to be sugar phosphates and it became, for a time, the practice to assign such compounds the name of its discoverer, e.g., fructose-1, 6-phosphate = Harder and Young ester. However, the fundamental role of phosphate in these processes was known from studies of muscles-biochemistry. Lundsgaard revealed that a phosphogen, phosphocreatin, vanished from contracting muscles in which the carbohydrate metabolism was blocked by iodoacetate. Lohman, Meyerhop, Lipmann and others brought cellular energetics to enzyme level when they showed that the phosphate of certain esters, produced as a result of carbohydrate oxidation, existed in equilibrium with the phosphate of adenosine triphosphate (ATP) and the phosphagens. These compounds were said to contain the **"high energy phosphate bond"** (~P) and were characterized by a very high- $\Delta F°$ (Standard free energy) of hydrolysis. In his review-article Lipmann suggested that such phosphorylated intermediates represent the means by which the cell is able to trap the chemical energy of carbohydrate, energy that is ultimately to be used in payment for the expensive synthetic reactions of life.

Studies on carbohydrate metabolism were not limited to yeast cells, but they were carried out on bacteria, moulds and higher plants also. In muscle cells carbohydrate decomposes to lactic acid. By studies on carbohydrate metabolism it became clear that certain reactions could be grouped into a metabolic cycle.

Investigations on mechanism of enzyme-actions were mainly carried out in the beginning of 20th century and led to two main aspects:

(a) A group of enzymologists reagards enzymes simply as catalytic proteins. Such studies were preceded by the development of elaborate apparatus & techniques for obtaining the enzymes in pure states. Research in this field has provided molecular and kinetic data for a number of crystallized enzymes and has led to general acceptance of **Enzyme-substrate compound theory,** first advanced by Michaelis & Menten in 1913.

(b) On the other side, work was done considering enzymes as a cog in the complicated metabolic machinery of the cell. Such investigations little cared for purity of enzymes; but they worked on biochemical aspect and established metabolic cycles & gave more fundamental understanding of cellular energetics.

CHEMICAL & BIOLOGICAL CATALYSIS

Chemical Catalysis

Catalysts are chemical substances which alter the rate of reaction and the process is known as chemical catalysis. The term catalysis is generally used for the rate enhancement in presence of a foreign substance which itself does not change during the course of reaction & can be recovered as such at the completion of reaction. In certain cases, one of the reaction products itself acts as a catalyst. For example, in the oxidation of oxalic acid by acidified potassium permanganate velocity increases as the reaction progresses. Here rate acceleration is due to the presence of Mn^{2+} ions which are formed during the reaction. This type of phenomenon is known as **auto-catalysis.**

Exact mechanism by which catalyst enhances rate of reaction is not

understood. However, it is believed that catalytic rate acceleration is due to the fact that it provide new alternate low energy pathway for the reaction by lowering energy of transition state [Fig. 2.1].

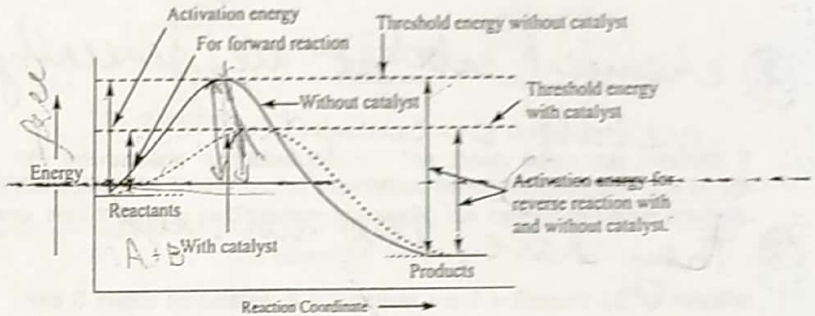


Fig. 2.1 Effect of catalyst on the rate of reaction

As evident form Fig. 2.1, by the addition of catalyst, the activation energy for reverse reaction is lowered by exactly the same amount as for the forward reaction. This explains, why catalyst speeds up both forward and backward reactions to the same extent. Thus, if a catalyst doubles the rate of forward reaction, rate of backward reaction will also be doubled. Hence, catalyst does not alter equilibrium, it only hastens the attainment of equilibrium by speeding up both rates of backward as well as forward reactions to the same extent.

Characteristics of Catalysis: Chemical catalysis is characterized by following aspects:

1. It remains unchanged in regard to amount and composition at the end of reaction. However, it may undergo some physical change.
2. Only a small amount of catalyst is required. For example, one mole of colloidal plantinum can catalyse decomposition of 10^8 litres of hydrogen peroxide. However, in some homogenous catalytic reactions, the rate of reaction is proportional to the concentration of the catalyst. For example, rate of inversion of cane sugar catalysed by hydrogen ions varies with the concentration of hydrogen ions present in the solution.
3. Rate of heterogenously catalysed reactions increases with the surface area of catalyst; this is why solid catalyst is more effective in finely divided state.
4. The catalyst does not alter the position of equilibrium in reversible reaction; it only hastens the attainment of equilibrium. For instance, use of platinum asbestos as catalyst in formation of sulphur trioxide

$$2SO_2 + O_2 \underset{}{\overset{\text{Pt-asbestos}}{\rightleftharpoons}} 2SO_3$$

causes considerable change in the rate of reaction; but does not alter the composition of reactants and product at the equibrium at the given condition of temperature & pressure.

5. Catalyst does not initiate the reaction it only alters reaction rate.
6. Catalyst is specific. For a particular reaction a particular catalyst is required.

For instance, manganese dioxide can catalyse the decomposition of potassium chlorate but not that of potassium perchlorate or potassium nitrate. Thus, mangasere dioxide is specific in its action. Almost all catalysts are specific in action. Substances catalysing biological processes are known as enzymes. Thus, all enzymes are catalysts but all catalysts are not enzymes.

$$2KClO_3 \xrightarrow[\text{(catalyst)}]{MnO_2} 2KCl + 3O_2$$

$$C_6H_{12}O_6 \xrightarrow[\text{(enzyme)}]{\text{Zymase}} 2CH_3CH_2OH + 2CO_2$$

7. Catalysts only alter rates of reactions; they do not initiate them. For example; $KClO_3$ upon heating will always decompose to give potassium chloride and oxygen irrespective to absence or presence of catalyst. However, manganese dioxide will enhance rate of decomposition. Nitrogen & hydrogen always combine to produce ammonia, but presence of catalyst accelerates rate of formation.

But, there are some exceptions. Carbon dioxide and hydrogen give different products in presence of different catalysts as given below:

$$CO + H_2 \xrightarrow{Cu} \underset{\text{Formaldehyde}}{HCHO}$$

$$CO + 2H_2 \xrightarrow{ZnO + Cr_2O_3} \underset{\text{Methanol}}{CH_3OH}$$

$$CO + 3H_2 \xrightarrow{\text{Nickel}} \underset{\text{Methane}}{CH_4} + H_2O$$

8. Catalysts work most effectively at certain specific temperature which is known as optimum temperature for its working or effectiveness.
9. Certain substances mar effectiveness of catalysts; they are known as catalytic poisons. For example, in contact process for the formation of sulphuric acid sulphur trioxide is needed, which is prepared by the combination of sulphur dioxide and oxygen. Rate of its formation is slowed down by the presence of arsenic compounds even if they are present in traces.

Types of Catalysis: Process of catalysis is broadly divided into two types:

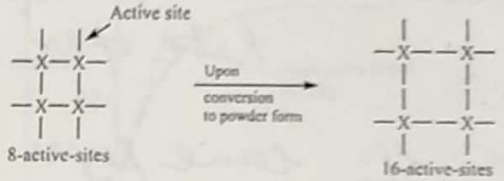
1. Homogenous Catalysis: When both reactants and catalysts are in a single phase.

2. Heterogenous Catalysis: When reactants form one phase and catalyst another (usually solid).

Theories of Catalysis: There are two important theories for action of catalyst:

(A) Active-site theory: According to this theory there are active sites on the surface of catalyst which form unstable bonds with reactants, resulting in strain in the bonds of reactants. Thus old bonds start to break & new bonds start to form. When all the new bonds are completely formed all old bonds break. After the completion of reaction products are released from the surface of catalyst. This theory

explains the catalysis by solids only; because catalysts become more reactive in powder form because of increase in the number of active sites:



(B) Intermediate formation theory: This theory states that reactants & catalysts combine to form a reactive intermediate which, being unstable due to high energy, breaks down to yield products and setting free catalyst for further catalysis.

$$C + S \rightleftharpoons CS^* \rightleftharpoons P + C$$

Here C stands for catalyst; S = Substrate and P = Product; CS^* = Reactive intermediate.

Biological Catalysis

Enzymes are biological catalysts which enhance rate of biochemical reaction, that is, the reactions taking place in organisms. These are generally proteins & cause the reaction to occur even under thermodynamically unfavourable conditions; for example, reactions which generally occur under drastic conditions occur under normal condition of temperature & pressure in animal and plant bodies in their presence. Enzymes participate in bio-chemical reactions without destruction or irreversible modifications during reaction, that is why they are considered biological catalysts. Word enzyme is derived from yeast (in Greek enzyme stands for yeast) & was coined by Kuhne in 1878 to define fermentation of yeast cells. Enzymes differ from catalysts in the sense that they catalyse biological process & undergo change, but are ultimately set free.

In terms of energy changes enzymes do not directly yield products. According to Eyring's **transition state theory** reaction proceeds through **high energy transition state**. Transtition state is reached through kinetic energy of reactants. The energy required is called **activation energy** (E_a) which may be determined from a plot between logarithm of reaction rate versus temperate^{-1}. The transition state (T.S.) is a state of maximum energy and is not any intermediate compound. The concentration of **activated complex** at T.S. determines the rate of chemical reaction. The role of enzymes is that they reduce the energy of activation, thereby allowing the reaction to occur at lower temperature on account of their reversible binding with reactants. The steps involved in enzyme catalysis of a single reaction can be represented as given below:

$$E + S \rightleftharpoons ES^* \rightleftharpoons ES \rightleftharpoons EX^* \rightleftharpoons EP \rightleftharpoons EP^* \rightleftharpoons P + E$$

Where S = Reactant called substrate
P = Product,
ES & EP = enzyme complexes.
EX^* & EP^* = activated complexes at T.S.
Above reaction can be abbreviated as

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

ES = Enzyme substrate complex.

In enzyme catalysed reactions though activation energy is lowered but not energetics of the reaction remain unchanged.

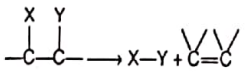
REMARKABLE PROPERTIES OF ENZYMES

1. Specificity

Enzymes are reaction specific, *i.e.*, for a particular reaction a particular enzyme is needed. For example:

(i) Esterase hydrolyses esters only. This is an example of reaction specific enzyme. On the other hand, an enzyme may be substrate specific too. Such enzymes are specific for a particular compound or class of compounds. For instance, urease hydrolyses only urea; phosphatases hydrolyse only phosphate ester.

(ii) Lyases remove group from substrate by mechanism other than hydrolysis, leaving double bonds:



(iii) Ligases link together two compounds. They catalyse reactions forming C—O, C—S, C—N, & C—C bonds.

(iv) Transferases transfer a group (other than hydrogen) between a pair of substrates, S and S′

$$S{-}G + S' \longrightarrow S'{-}G + S$$

(v) Many enzymes exhibit kinetic specificity. For example: esterase; although hydrolyses all esters, but hydrolyses different esters at different rates; pepsin hydrolyses the peptide link, but is most active for those links in which amino group belongs to an aromatic amino acid and the carboxyl group is one of a dicarboxylic amino acid.

(vi) Many enzymes are sterospecific. For instance, maltase hydrolyses α-glycosides but not β-glycosides; whereas emulsion hydrolyses the latter but not the former.

It should be noted that a given enzyme can exhibit more than one of specificities. For example, estereases hydrolyse only esters, may also hydrolyse one enantiomer (of optically active) more rapidly than the other.

2. Catalytic Power

Enzymes catalyse rates of biological processes at extremely faster rates. A chemical reaction in presence of enzymes proceeds hundred to one million times faster. Further, these reactions occur at the body temperature (~ 310 K) and in the physiological pH range (~ 7). They exhibit their activity even when they have been extracted from source.

3. Regulation

In enzyme–substrate reaction the reaction velocity decreases as function of time (Fig. 2.2).

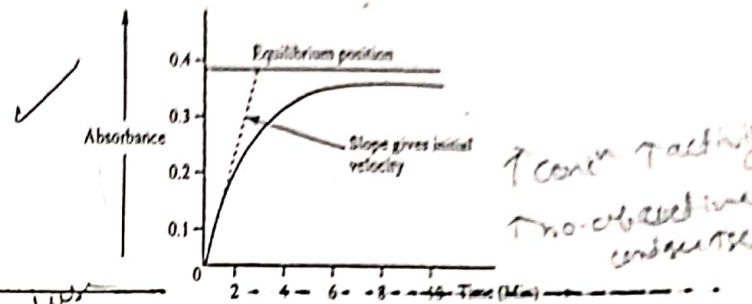


Fig. 2.2 Progress curve of typical enzyme catalysis is reaction

The decline in velocity may be attributed to following reasons:

(i) The approach to an equilibrium & associated influence of the reverse reaction,

(ii) The depletion of substrate level due to the occupancy of activesites.

(iii) The product(s) of reaction may inhibit the enzyme or change it if the medium is not properly buffered,

(iv) The enzymes may undergo some progressive inactivation at the temperature or pH of the reaction due to instability.

For above reasons, enzymes are studied within their initial velocity (V). The initial velocity may be determined from slope of tangent to the curve at the zero (V_0). Concentration of enzyme also alters enzymatic activity. Increase in enzyme concentration increases enzyme activity; because with the increase in the concentration of enzyme the number of active centres increases. Generally, reaction velocity becomes double with doubling the concentration of enzyme.

For most of the enzymatic reactions, if the substrate concentration is initially very high, the rate will first follow a zero-order course and later on will fall down to first-order kinetics. The entire reaction is thus a mixture which cannot be described by a single reaction order. We know that rate is equal to [Product]/time and hence

$$\frac{[\text{Enzyme}]}{\text{time}} = [\text{Product}]$$

This property of enzyme was described for many years as **Enzyme-time-product relationship.** With the given concentration of enzyme a point may be reached when all the substrate molecules get bound to the enzymes & further increase in enzyme does not have any effect on the formation of product (Fig. 2.3)

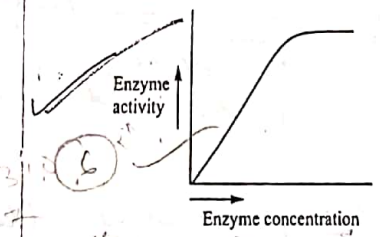


Fig. 2.3 Effect of enzyme concentration on enzyme activity

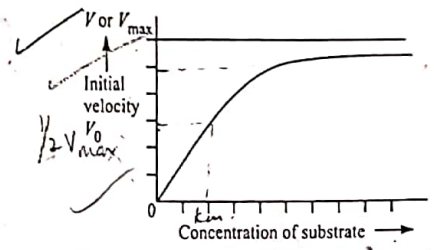


Fig. 2.4 Effect of substrate concentration on reaction rate when enzyme & substrate are incubated for constant time

EXTRACTION AND PURIFICATION OF ENZYMES

In the extraction & purification of enzymes availability and cost of starting material is of prime importance. Concentration of a single enzyme may vary in different tissues. It is important to choose tissue in which enzyme is in high concentration. For this reason yeast, bacteria & fungi have certain advantages as source materials. They have a certain advantage in that these cells may be cultivated under conditions favourable for the production of enzyme in question. There is, however, one disadvantage of difficulty in obtaining large quantities of microbial cells other than yeast.

Once starting material has been selected, a series of steps can be performed over that to effect extraction & isolation of enzyme. A few specific examples of certain methods are discussed below:

1. Sedimentation: If liver tissue is homogenized, as in Potter-Elven hjem apparatus (Fig. 2.8) rather than common blending devices, many of mitochondrial & other particulate cell bodies remain intact. They are easily sedimented out of solution, and with them goes a repertoire of enzymes. Physical separation by sedimentation is of practical utility only in initial phase of separation.

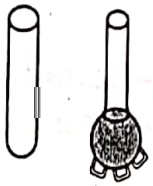


Fig. 2.8
Potter-Elvenhjem homogenizer

2. Extraction: Earlier classification of enzymes was in two main classes (i) soluble or lyoenzyme and (ii) bound or desmoenzyme. This is a poor classification; since desmoenzymes are probably those enzymes for which proper methods of solution have yet not been discovered.

The acetone-powder (from which enzymes can be removed by extraction with buffer) is, by the virtue of its fat-free nature, often the easiest material from which enzymes can be extracted. In any event, a fine-grinding is the first-step. Methods for removing enzymes from micro-organisms include autolysis, lysozyme digestion, grinding, freezing & thawing, sonic disintegration, shaking with solvents, shaking with fine-glass beads, and, finally, explosion by sudden release by pressure.

3. Salt Fractionation: Ammonium sulphate is most useful salt in enzyme fractionation. Its advantages are: high solubility in water (760 g/lt) & a roughly neutral reaction (pH 5 to 6) in concentrated solution. Dixon developed a nomogram chart for the preparation of ammonium sulphate solutions and Kunits gave an equation for calculating the ammonium sulphate to be added to a solution to give the desired final concentration. One disadvantage in use of ammonium sulphate in slightly alkaline solution is that, even at pH 9.3; 50% of ammonium ions are converted into ammonia. The pH of ammonium sulphate solution should be controlled by means of a buffer. However, for crystallization of beef-liver glutamic dehydrogenase sodium sulphate has been used extensively.

4. Solvent Fractionation: Water-miscible solvents like acetone, ethanol, methanol and dioxane help in the isolation of enzymes.

In acetone extraction one should start below 0°C & then proceed towards higher temperatures. Fractionation is done upto highest temperature that will not cause much yield-loss. Because, acetone absorbs strongly in U.V. region, it must be

completely removed by dialysis or by distillation under reduced pressure before the product is subjected to spectral-analysis.

Ethanol has found increasing application in the isolation of enzymes. It has been used to obtain crystalline **lactic dehydrogenase** from rat-liver.

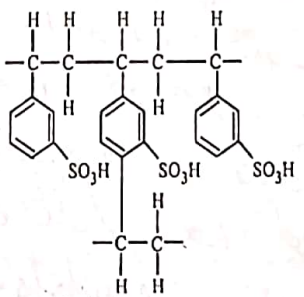
5. Solvent-Metal ion fractionation: An important method for the separation of blood-proteins is by the combination of metal-ions & solvents, particularly Zn^{2+} and ethanol. The zinc-salts of proteins are often more soluble than the sodium and potassium-salts and separate out more easily from the solution. From these, metal ions may be removed by the treatment with citrate, ethylene diamine tetraacetate, or ion-exchange resin.

6. Adsorption: A variety of substances have been used as protein adsorbents. One of the earliest preparations is hydrated aluminium oxide. Calcium phosphate gel has also proved very useful & bentonite has been employed in the isolation of lysozyme.

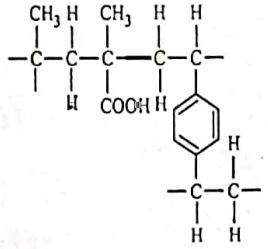
7. Adsorption chromatography: Column chromatography on adsorbents is very effective for the separation of proteins & hence for enzymes also. Anger prepared calcium phosphate gel. This same adsorbent has been critically examined by Swingle & Tiselius for general protein chromatography. Zechmeister has published a review on general subject of enzyme chromatography.

In another approach, using biochemically specific adsorbent, isolation of enzyme depends upon its catalytic specificity rather than on its general properties as protein. For instance, in the isolation of mushroom tyrosinase various adsorbents containing p-azophenol & related groups were prepared from aromatic ethers of cellulose.

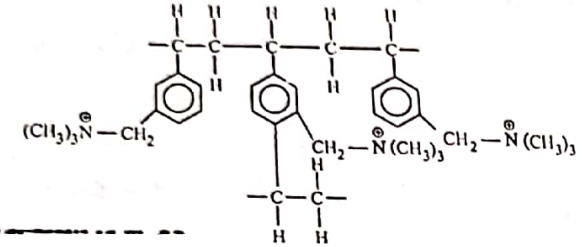
8. Ion-exchange chromatography: The brilliant success of this method is the isolation of low molecular weight compounds such as aminoacids. **Cytochrome C** was purified in 1950, by the passage through a column of **amberite IRC-50** & same method was useful in isolating cytochrome from Ustilago. Ribonuclease & lysozyme have been purified in a similar manner. Ion-exchange chromatography is essentially an electrophoretic separation in which the resin serves as an electrode and gravity as the other. It may yet develop into valuable tool as the chemical industry places more new resins in hands of enzymologists. Structures of some cation-exchangers & anion-exchangers are shown below:



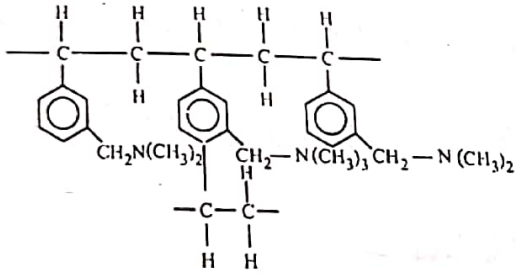
Sulphonic acid cation exchanger



Carboxylic acid cation exchanger



Quartenary amine anion exchanger



Tertiary amine anion exchanger

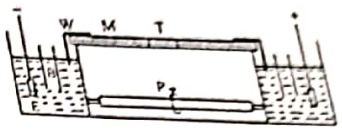
9. Complex Formation: In this procedure **protamine** has been used to a lesser extent as complexing agent. Some enzymes, *e.g.*, **muscle lactic dehydrogenase,** were isolated as the inactive mercury salts. Reactivation was achieved by dialysis against cysteine or potassium cyanide. **Basic lead acetate** has been employed to throw down undesired proteins.

10. Denaturation Reactions: Brief heating from 50°C–70°C, is one of the early steps. This method is sucessful in the crystallization of alcohol dehydrogenase from yeast. Denaturation with trichloroacetic acid is used in the isolation of **cytochrome** C. Shaking with chloroform is a very effective way to remove contaminating proteins.

11. Dialysis: Theorell & Akeson designed electrophoretic cell for separating proteins from salts. A current of air blown over a dialysis bag containing a protein solution is an exceedingly gentle method to effect concentration. As long as evaporation continues, the solution will remain at low temperature.

12. Preparative electrophoresis: Large cells containing upto 100 mL, may be used with special electrodes in the electrophoretic apparatus. Method in which components are completely separated, have been designated as **"Zone electrophoresis."**

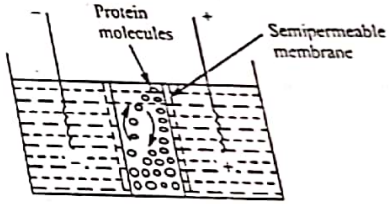
Zone electrophoresis on starch bed or on blocks of sponge rubber has become a very popular tool for protein isolation. A schematic diagram for preparative zone, **electrophoresis cell** is given below:



B = Buffer compartment with baffles
E = Platinum electrodes
W = Filter paper wick
P = Pressure equallizing tube
M = Medium, such as starch, sponge-rubber
T = Trench for sample

This novel technique developed by Kolin is worth mentioning. Here buffer solutions of unequal density are layered over each other to give pH gradient in vertical tube. The sample is introduced & when current is turned on, the various proteins migrate to their isoelectric points & stop. The method is capable of very rapid resolution, since farther the proteins from pI (isoelectric point) the more rapidly they will migrate to isoelectric zone.

The **"electrophoresis-convection"** apparatus of Kirkwood may be applied for separating less complex mixtures. Electrophoresis convection may be regarded as a refinement of the process of **electrodecantation** as illustrated below:



A schematic diagram of the Electrophoresis Convection principle

The pH throughout the apparatus is alkaline to the isoelectric-point of the protein, and the protein has migrated to the anode. This will cause a higher density next to the right side of the membrane, and the protein will settle down on that side, thus giving rise to convection and eventually to a collection of the component at the bottom of the cell.

"Purification (Criteria of Purity)"

The majority of the past and present industrial uses of enzymes have been with crude preparations. However, many of the uses for immobolized enzymes require highly purified forms. Techniques for the large-scale initial isolation and partial purification of enzymes from microbial, plant & animal sources make use mainly of traditional processing steps, scaled-up in some instances directly from the art of the research laboratory. In other cases, newer equipment has been developed, especially for cell disruption and certrifugation. The use of semipermeablle membranes has proved very useful in the initial isolation steps. However, major advances in membrane development are needed for the fractionation of enzyme mixtures by this technique.

Final purification of enzymes is a tedious task, be it in the laboratory or industrial plants, with chromatographic methods in preponderance. Gel permeat

chromatography & affinity chromatography hold very high promise for simplifying the purification of enzymes. The later method consists of contacting a crude enzyme preparation with a solid support to which is attached a reversible inhibitor or some other types of molecule which will selectively & reversibly bind with the enzyme of interest. With the enzymes thus bound to immoblized inhibitors, the support-inhibitor-enzyme complex is separated from the intial crude feed and the purified enzyme eluted from the support-inhibitor portion. The methods like gel-filteration & affinity chromatography as well as other chromatographic methods are appropriate because of reduction in cost of purification of enzymes as well as due to larger supply & varieties of enzymes. However, development needs to be done in scaling up these methods. In purification anaphylactic or precipitation reactions are also useful. In a method antigen-antibody reaction is carried out in a gel such as agar. The compounds are visible as precipitated zone in gel.

FISHER'S LOCK & KEY HYPOTHESIS

Enzymes are highly specific, therefore, the reasonable question is what is their mechanism of action. According to Arrhenius, enzymes catalyse the reaction through the formation of unstable intermediate. Simplest model to explain enzymatic action is Lock & Key model proposed by Fischer. This model assumes that enzyme is rigid three-dimensional body, the surface of which has active-sites which have slots for fitting definite substrates just as a key fits in a particular lock (Fig. 2.9)

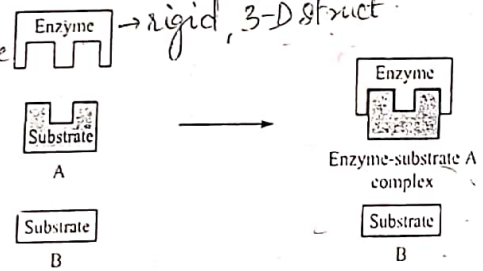


Fig. 2.9 Lock and Key model of enzyme action

An enzyme molecule is very large (consisting of 100 to 200 aminoacid residues); but active-sites, which combine with substrate have definite shape in which substrate can fix, are comparatively small (with few amino acid residues). Amino acids of active sites are located at different places in the chain, whereas, other amino acids, which are not part of active-sites, are located in a definite sequence. This is because of the fact that this sequence allows the whole enzyme molecule to fold in exactly required manner.

An hypothetical example of mechanism of enzyme-action is given in above figure. This is referred to as a **"Lock and Key Mechanism."**

The enzyme enters into a chemical combination with the substrate to form an **enzyme-substrate complex** (Michaelis-Mention hypothesis).

$$E + S \longrightarrow ES$$

The enzyme-substrate complex then breaks down to give the products of reaction. The enzyme is released & can be used over and over again.

$$ES \longrightarrow E + Product$$

The Lock and Key model explains the action of many enzymes. But of other enzymes, there is evidence that this model is too restrictive. Enzyme molecules are in dynamic state, not in static one. There are constant motions within them, so that the active site has some flexibility.

KOSHLAND'S INDUCED FIT HYPOTHESIS

This model was given by Koshland (1966). In the Fischer model, *i.e.*, Lock and Key model, the active-site is presumed to be preshaped to fit the substrate. In the **induced-fit theory**, the substrate induces a conformational change in the enzyme. This aligns amino acid residues or the other groups on the enzyme in the correct spatial orientation for substrate binding & catalysis both. At the same time, the other amino acid residues may get buried in the interior of the enzyme. This is shown in the following Figure. 2.10.

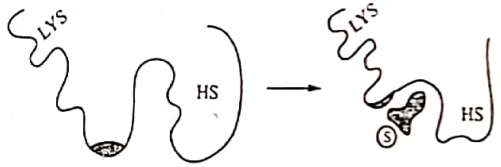


Fig. 2.10 Representation of an induced fit by a conformational change in the protein structure

In this figure, in the absence of substrate, the catalytic and the substrate-binding groups are several bond distance removed form one another. When the substrate approaches there occurs a conformational change in the enzyme protein, aligning the groups correctly for substrate binding and for catalysis. At the same time there also occurs a change in the spatial orientation of the other regions.

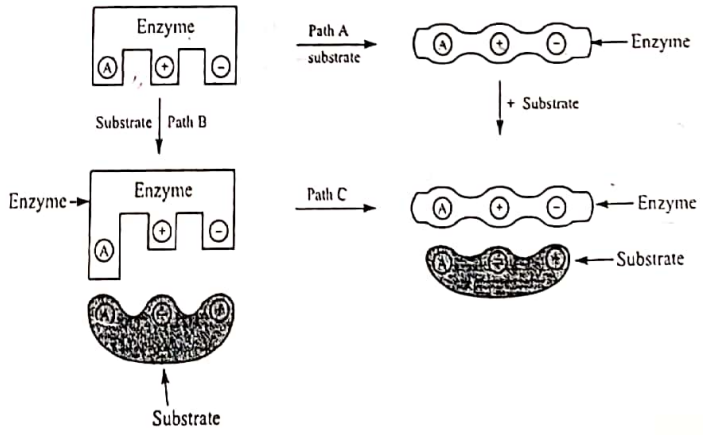


Fig. 2.11 Hypothetical representation of alternate pathways of substrate Induced conformational changes

The main evidence in favour of induced fit model comes from demonstration of conformational changes during substrate binding & catalysis with creatine kinase, phosphoglucomutase, and several other enzymes. Upto this time, the exact sequence of events in a substrate induced conformational change has not been established. There may be several possibilities as shown in Fig. 2.11. Even if one knows the complete primary structure of enzyme, it is not very easy to decide exactly which residues exactly constitute catalytic site.

CONCEPT AND IDENTIFICATION OF ACTIVE SITE BY THE USE OF INHIBITORS

An enzyme has a distinct cavity or cleft in which the substrate is bound. The cleft contains an **active centre** in which the amino acids are grouped together in such a way as to enable them to combine with substrate. The reactive amino acids may lie widely separated in the polypeptide chain. The chain, however, undergoes folding in such a manner that the reactive amino acids come together in active site.

It is believed that when the substrate molecule binds to the active site, its parts are held together in such a way as to cause distortion of chemical bonds, *i.e.*, the bonds are weakened. This distortion of chemical bonds of substrate increases its reactivity, and thus speeds up the rate of reaction. The products of reaction are released because they are less firmly bound. This mechanism is called **strain model of catalysis.**

Inhibitors are compounds which decrease the rate of an enzyme-catalysed reaction. Inhibition is diagramactically shown below.

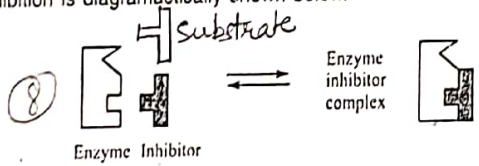


Fig. 2.12 Inhibition

Inhibitors are of two types: Irreversible & reversible inhibitors. Irreversible inhibitors help in the identification of active sites. Actually, irreversible inhibition inovoles the covalent bonding of inhibitors to a functional group at the active site or elsewhere on enzyme. Because progressively declining, irreversible inhibition can't be analysed by **Michaelis-Mention kinetics**; this type of inhibition is frequently used to obtain information regarding the functional amino acids at the active sites of the enzyme too. Irreversible inhibitors include some of the pesticides, *e.g.*, parathion is an active inhibitor of insect acetylcholine sterase upon which normal propagation of nervous impulses relies. Since inhibition is irreversible in which the substrate in the catalytic cycle is converted into a chemically reactive product which remains bound to active site through covalent bonding, the enzyme is rendered permanantly inactive & the identification of active site is done on the basis of its reaction with inhibitor.

AFFINITY LABELLING AND ENZYME MODIFICATION BY SITE DIRECTED MUTAGENESIS

This method & its variants have been introduced for labelling studies and hold greater promise for future due to their versatility. Affinity labelling can be applied to catalytic-regularity & antibody active sites, as it does not depend on having a uniquely active group within this site.

Affinity labelling can be diagramatically represented as follows (Fig. 2.13):

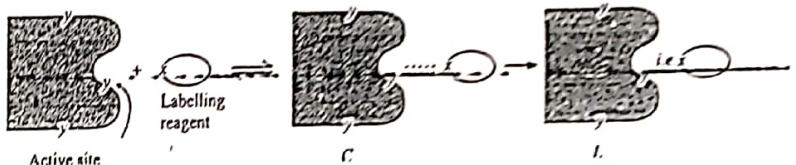
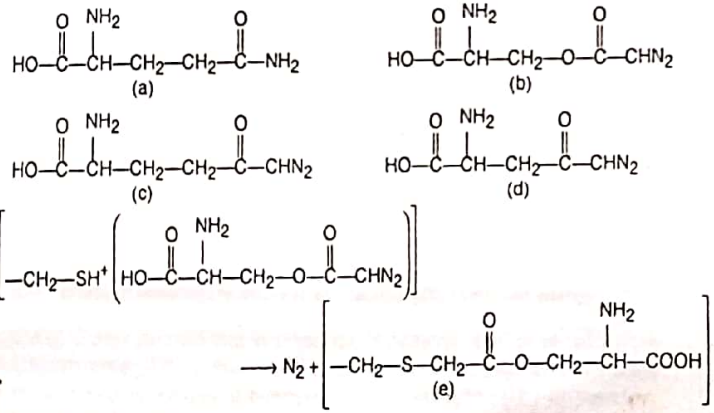


Fig. 2.13 Schematic representation of affinity labelling: After the reversible complex *C* is formed, the irreversible product *L* is produced by reaction between functional group *X* of the labelling reagent and group *Y* of active site

In this method labelling reagent first combines **specifically & reversibly** with the site to form complex **C** by virtue of a suitably small & reactive group **X** on the reagent, it can then react with one or more amino acid residues *Y* in the site to form irreversible covalent bond. Formation of irreversible complex so increases the local concentration of the labelling reagent in the site, as compared to that in concentration in free solution, that reaction with the group *Y* in the site is markedly favoured over reaction with any similar group *Y* outside the site. The group *Y* need not to be an unusually reactive residue of its kind for this to occur. One thing important to mention is that group *X* of reagent should be small so as to be encompassed within the active site of enzyme. Needless to say that this interaction modifies the enzyme by bringing about mutagensis of its active site.

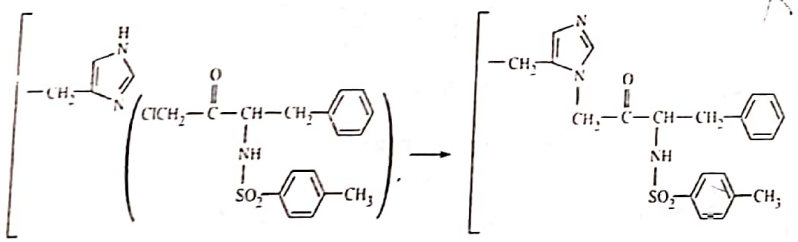
During affinity labelling enzymes modification related chemical principles are apparently involved. An interesting example is catalysis of transfer of amide group of L-glutamine (a) to formylglycinamide ribonucleotide to form formylglycinamidine nucleotide & glutamic acid.



Buchanan and his co-workers found that this enzyme was specifically & irreversibly inhibited by the glutamine antagonists O-diazoacetyl-L-serine (L-azaserine) (b) or 6-diazo-5-oxo-L-norleucine (C) but not by their D-analogs or by the closely similar compound 5-diazo-4-oxo-L-norvaline (d). These striking results suggest that the active compounds first form a specific & reversible complex in the enzyme binding site for L-glutamine, following which the diazoacetyl group (group *X*) reacts with some suitably positioned residue *Y* in the active site. The stereochemical analogy between the diazoacetyl group of these reagents and the amide group of L-glutamine that is transferred in the normal functioning of the enzyme active site strongly suggests that the group *Y* is within the site. Subsequent work by French *et al.* & David et.al showed that this group *Y* is a cysteinyl residue (e). This cysteinyl residue is apparently uniquely reactive, since azaserine does not react with free cysteine, with the highly reactive —SH group in activated papain or with —SH group of serum mercaptaalbumin.

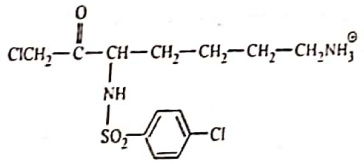
The general idea that active site binding can be used to direct a specific chemical modification was realized and developed independently in a number of Laboratories. The studies of Baker *et al.* were designed to inactivate certain enzymes specifically & irreversibly primarily for chemotherapeutic purposes, rather than to obtain structural information about active sites.

Another important example of the use of affinity labelling (Scheme-I) is the reaction of chymotrypsin with the chloromethylketone derived from *N*-tosyl-L-phenylalanine (TPCK) (a). Here the chloromethylketone is the group *X* attached to substrate-like *N*-tosylphenylalanyl residue. Specific mechanism as depited in the general example (Fig. 2.13) was followed since the native enzyme was modified to the extent of 1 mole per mole of protein, but was not modified in



TPCK
(a)

(b)



TLCK
(c)

Scheme-I

presence of 8 M urea. In other words, an initial reversible binding to active site of native enzyme was implicated. The labelled enzyme was completely inactivated (b). Trypsin, having a different substrate specificity, was not affected by TPCK, but has been shown to be similarly inactivated by its own specific-affinity labelling reagent, the chloromethylketone derived from *N*-tosyl-L-Lysine (TLCK) (c).

The chloromethylketones in each case alkylate histidyl residues that were long inferred to be present in these active sites.

KINETICS OF ENZYME ACTION

Enzyme kinetics can be studied in two parts:

(A) Energy of activation

(B) Steady state enzyme kinetics

(A) Energy of activation: Enzymes catalyse the rate of reaction through the alternate pathway incorporating low energy of activation and thus make the reactions generally occurring under drastic condition possible at normal physiological temperature. Energy of activation is the energy required to reach the reactants to transition state at which new bonds are partially formed & the old bonds are partially broken & is the state of highest energy during the course of reaction, therefore is highly unstable & breaks down to give the lower energy products. Out of all the molecules of reactants only a small fraction contains enough energy to react, *i.e.* the threshold energy. Increase in temperature makes more molecules to aquire energy equivalent to threshold energy (Fig. 2.14). But in living systems the limitation is that reactions take place under almost isothermal conditions as there is very little temperature variation. In fact, enzymes help in the formation of **enzyme-substrate complex** by activating the reactant molecules. Enzyme substrate complex has high energy & makes the reactants with strained bonds, therefore, they react at faster rate. In addition, in **enzyme-substrate complex** reactant molecules are so oriented that their reaction becomes a certainity and not a matter of chance, that is why reaction rate becomes thousands of time faster.

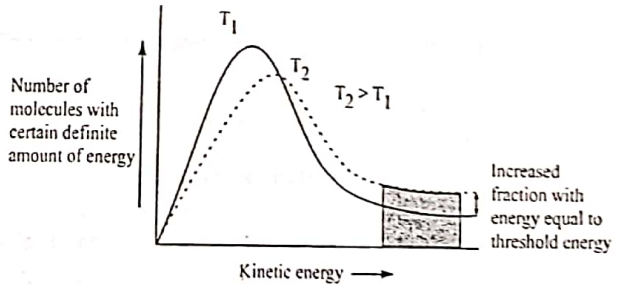


Fig. 2.14 Effect of temperature increase on fraction with threshold energy

Alternatively, it may be said that in presence of enzyme, energy of activation to reach transition state (T.S.) is lowered (Fig. 2.15). However, all over free energy change, under standard conditions of temperature and pressure (ΔG°) of reaction, remains unaltered.

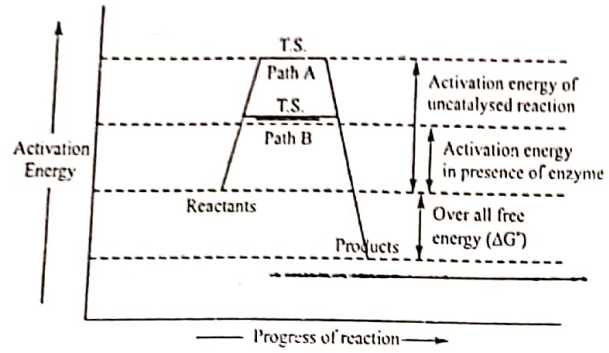


Fig. 2.15 Effect of enzyme on Transition state of a reaction

The enzyme catalyses both forward as well as backward reaction of equilibrium to the same extent. Since ΔG° is not changed, equilibrium constant also remains unchanged, but equilibrium is achieved quickly. Proportion of reactant & product of catalysed as well as uncatalysed reaction also remains same.

(B) Steady-state enzyme kinetics: This theory, proposed by Michaelis and Menten in 1913, is based upon following assumptions:

(i) Enzyme-substrate complex (ES) is in equilibrium with enzyme & substrate

$$E + S \underset{K_{-1}}{\overset{K_1 \text{ fast}}{\rightleftharpoons}} ES$$ ES; E = Enzyme, S = Substrate

(ii) Product-formation is possible only through enzyme-sulbstrate complex:

$$ES \xrightarrow[K_2]{\text{slow}} P + E$$ [P = Product]

Based on these assumptions Michael-Menten equation can be derived as given below:

Let us consider formation of enzyme substrate complex ES.

$$E + S \rightleftharpoons ES$$

$$\frac{d[ES]}{dt} = K_1 [E][S] \quad \ldots (1)$$

It is clear from assumption (i) and (ii) that clear equilibrium is not achieved in the fast process, as Enzyme-substrate complex [ES] in constantly being removed in the slow process. Concentration of enzyme is very much less than concentration of substrates. Therefore, [E] << [S]. Hence, [ES] << [S]. The rate of reaction is given as:

$$r = \frac{d[S]}{dt} = \frac{+ d[P]}{dt} = K_2[ES] \quad \ldots (2)$$

Using steady state approximation for the formation of ES:

$$\frac{d[ES]}{dt} = K_1[E][S] - K_{-1}[ES] - K_2[ES] = 0 \quad \ldots (3)$$

Concentration of free enzymes [E] is not measurable in living process. But total enzyme concentration $[E]_0$ is measurable and can be given by Equation (4).

$$[E]_0 = [E] + [ES] \quad \ldots (4)$$

Here [ES] is bound enzyme concentration, therefore [E] can be given as

$$[E] = [E]_0 - [ES] \quad \ldots (5)$$

Putting this value in Eq. (3), we get

$$\frac{d[ES]}{dt} = K_1([E]_0 - [ES])[S] - K_{-1}[ES] - K_2[ES] = 0 \quad \ldots (6)$$

Upon simplification of above equation & grouping the constants

$$K_1[E]_0[S] = (K_{-1} + K_2 + K_1[S])[ES] \quad (7)$$

or

$$[ES] = \frac{K_1[E_0][S]}{K_{-1} + K_2 + K_1[S]} \quad (8)$$

Upon puttting this value of [ES] in Eq. (2)

$$r = \frac{K_1K_2[E]_0[S]}{K_{-1} + K_2 + K_1[S]} \quad \ldots (9)$$

or

$$r = \frac{K_2[E]_0[S]}{\frac{(K_{-1} + K_2)}{K_1} + [S]} = \frac{K_2[E]_0[S]}{K_m + [S]} \quad \ldots (10)$$

[Upon dividing both numerator and denominetor of eq. (9) by K_1]

Here K_m is **Michaelis constant** & Equation (10) is Michaelis-Menten Equation. Value of K_m is given by the expression:

$$K_m = \frac{(K_{-1} + K_2)}{K_1}$$

This equation correlates the components of enzyme reaction, [S] & [E], to initial & maximum velocity through rate constant (K_m):

$$K_m = \frac{\text{Rate of breakdown of ES}}{\text{Rate of formation of ES}}$$

Michaelis-Menten & Lineweaver-Burk Plots

V or (V_{max}) represents maximum velocity of enzyme reaction, whereas, **Michaelis Constant** (K_m) is the substrate concentration at which enzyme demonstrates 50% of its maximum velocity. Michaelis-Menten equation shows the relation:

$$r = \frac{K_2 [E]_0 [S]}{K_m + [S]} \quad \ldots (11)$$

This equation can be further simplified. When all the enzymes have reacted with substrate the reaction shows maximum velocity (V_{max}). As at that stage no free-enzyme is left $[E]_0 = [ES]$. Therefore, Equation (2) becomes

$$r_{max} \equiv V_{max} = K_2 [E]_0 \quad \ldots (12)$$

Hence, Michaelis-Menten equation (11) can be written as,

$$r = \frac{V_{max}[S]}{(K_m + [S])} \quad \ldots (13)$$

Now, here are two cases:

(i) If $K_m >> [S]$. Then $[S]$ can be neglected from the denominator. Then

$$r = \frac{V_{max}[S]}{K_m} = K[S] \quad \ldots (14)$$

This is a first-order reaction.

(ii) If $[S] >> K_m$. Then, K_m can be neglected from denominator:

$$r = V_{max}[S] = \text{Constant} \quad \ldots (15)$$

This reaction rate follows zero-order kinetics.

If $K_m = [S]$: $r = \frac{1}{2} V_{max}$.

As already stated, Michaelis-Constant is equal to concentration of S at which rate of formation of product is half of maximum rate. K_2 of equation (12) is known as **turnover-number** of enzyme. It is the number of molecules converted in unit-time by one molecule of enzyme. It's value is in between 100–1,000 per second. However, sometimes it may be as large as 10^5 to 10^6/sec. Now question is why kinetics of enzyme-catalysed reaction changes from 1st-order to zero order if the concentration of substrates is increased. Probable reason is that at low concentration most of the active-sites remain unoccupied. But, upon increase in concentration of substrates these active-sites get occupied and cause rate-enhancement. However, at very high substrate concentration reaction-rate becomes constant as all the active-sites remain occupied all the time. **Michaelis-Menten plot** for the kinetics of enzyme catalysed reaction is given below:

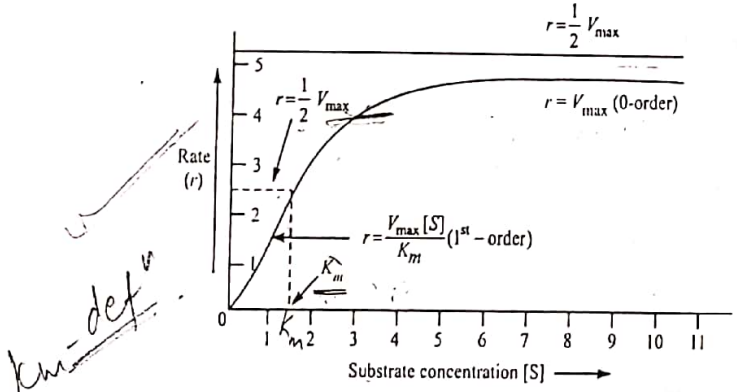


Fig. 2.16 Plot of kinetics of Enzyme-catalysed reaction

Thus, K_m is substrate concentration at which half of the active-sites of enzyme are involved in the formation of substrate enzyme complex. This is characterstic of a particular enzyme. From **Michaelis- Menten equation** it is possible to calculate

the rate of reaction at any substrate concentration if K_m & V are known. Kinetics of enzyme-action is helpful in understanding metabolic pathways. Determination of V_{max} & also K_m directly from the plot of r against $[S]$ is rather difficult. However, Michaelis-Menton equation can be modified to get plots from which V_{max} can be easily determined. Two such plots are **Lineweaver-Burk plot** and **Eadie Hofstee plot.**

Lineweaver-Burk Equation & plot: Michaelis-Menten equation is

$$r = \frac{V_{max}[S]}{(K_m + [S])} \quad \ldots (16)$$

Taking reciprocal of both the sides

$$\frac{1}{r} = \frac{K_m + [S]}{V_{max}[S]} \quad \ldots (17)$$

Upon rearranging it

$$\frac{1}{r} = \frac{K_m}{V_{max}[S]} + \frac{[S]}{V_{max}[S]} \quad \ldots (18)$$

or

$$\frac{1}{r} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad \ldots (19)$$

Equation (19) is **Linewear–Burk equation.** A plot of $\frac{1}{r}$ against $\frac{1}{[S]}$, *i.e.*, **Lineweaver–Burk plot** [Fig. 2.17] is given below:

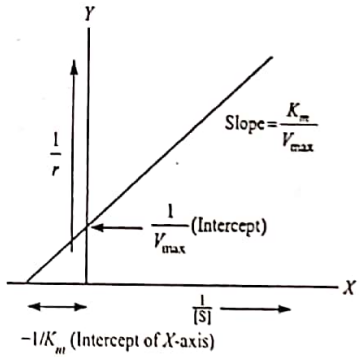


Fig. 2.17 Lineweaver-Burk plot

The Lineweaver–Burk plot though uses the reciprocals of highest reaction rates & concentration of substrates $[S]$, but is a popular method for knowing velocity and Michaelis constant.

Eadie-Hofstee Equation and Plot: Eadie-Hofstee equation (rearranged Michaelis–Menten equation) is:

$$r[S] = \frac{V_{max}}{K_m} - \frac{r}{K_m} \quad \ldots (20)$$

Eadie-Hofstee plot is a plot of $\frac{r}{[S]}$ against r and is a straight line; slope of which gives $-\frac{1}{K_m}$ and intercepts on X-axis & Y-axis give V_{max} & $\frac{V_{max}}{K_m}$, respectively. Thus, from plot (Fig. 2.18) both maximum velocity & Michaelis-Constt. can be determined.

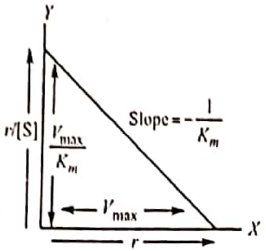


Fig. 2.18 Eadie-Hofstee plot

Variation from expected plot indicates contamination by inhibitors, activators or impurities. For multisubstrate enzyme-catalysed reactions kinetic studies can be made by varying concentration of each substrate in presence of saturating concentration of others. The value of Michaelis constants have been found to be inversely propotional to enzyme activity. High value of K_m indicates that high concentration of substrate is required to get half of maximum rate, *i.e.*, it indicates enzymes have high affinity for substrate.

REVERSIBLE AND IRREVERSIBLE INHIBITION

Poisons act upon living bodies by inhibiting enzymes. For instance, Carbon monoxide causes poisoning by combining with haemoglobin thus making it useless for performing its usual role as carrier of oxygen. Cyanide poisoning is due to its combination with natural substances, particularly with metallic centre of cytochrome. Poisonous effect of arsenates is due to its blocking of enzyme sites in place of phosphates. On the other hand, inhibiting reagents are of utility in pharmacology and medicines. Selective inhibition may be used to tackle cancer-problems. Thus, enzyme inhibition is a phenomenon that needs to be studied. Inhibition can be broadly divided into two catogeries:

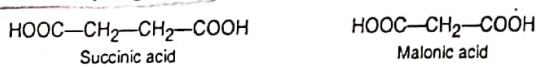
(A) Reversible Inhibition (B) Irreversible Inhibition.

(A) Reversible Inhibition: In reversible inhibition inhibitors are loosely held with the enzymes. In this process free enzymes and inhibitor bound enzymes are in equilibrium with each other. Inhibitors may be removed from enzyme-inhibitor complex by simple methods to furnish active enzymes.

Reversible inhibition can be classified into:

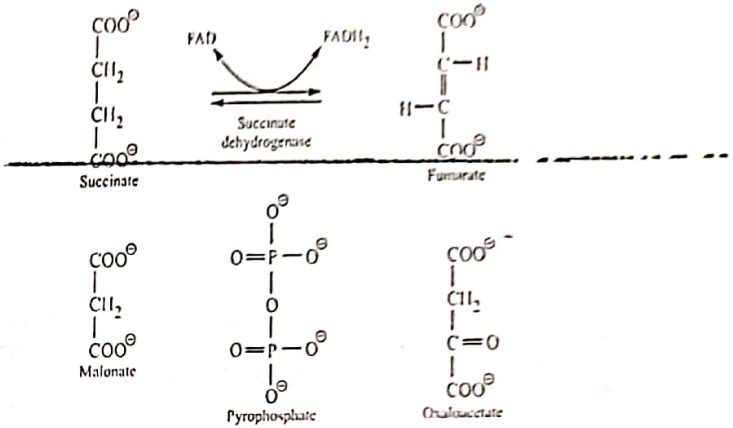
(a) Competitive inhibition (b) Uncompetitive inhibition (c) Noncompetitive inhibition

(a) Competitive Inhibition: Competitive-inhibition depends upon lack of absolute specificity of the chemical reactivity on active site. Here active-site combines more or less loosely with the inhibitor which is structurally related to substrate. A representative example of this class is inhibition by malonic acid of the enzyme succinic dehydrogenase.



Enzyme is not able to distinguish between two dicarboxylic acids, hence both are capable to react with it. The degree of inhibition depends upon malonate

succinate ratio. Upon increasing concentration of succinate it displaces malonate completely form the enzyme. Besides malonate, other inhibitors of this enzyme are pyrophosphate, oxaloacetate & oxalate. Another important example of competitive inhibition is malic dehydrogenase by adenine. Here, inhibitor competes with co-enzyme for active-site on protein.



Competitive inhibitors are not always structure analogues of substrates. For example, salicylate that inhibits 3-phosphoglycerate kinase binds to the site different from active-site. Competitive inhibitos change K_m but not V_{max} of enzyme-catalysed reactions; because number of active-sites remains unaltered. However, larger concentration of substrate is required for the maximum utilization of active-sites, This is why K_m is increased.

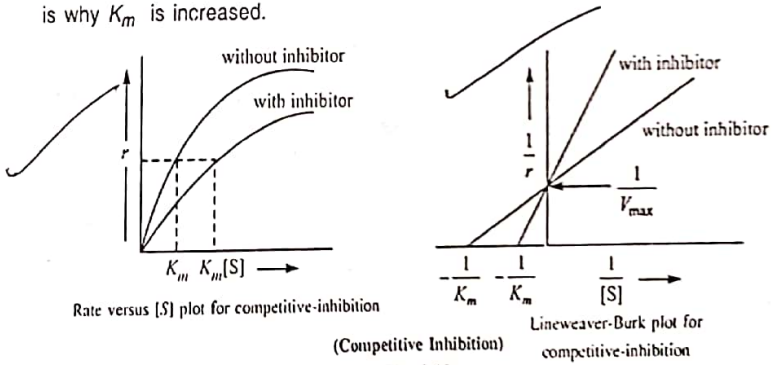


Fig. 2.19

Schematic-diagram for competitive inhibition is given below:

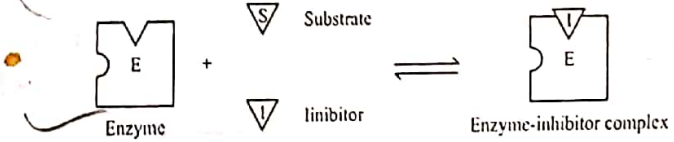


Fig. 2.20

(b) Uncompetitive-Inhibition: Uncompetitive inhibitor binds with already formed **enzyme-substrate complex** & has equal effects both on K_m & V_{max}. This type of inhibition is rare in one-substrate reaction, but causes a type of product-inhibition in reactions with multiple substrates and products. r versus $[S]$ plot and Lineweaver-Burk plot for uncompetitive inhibition are given below:

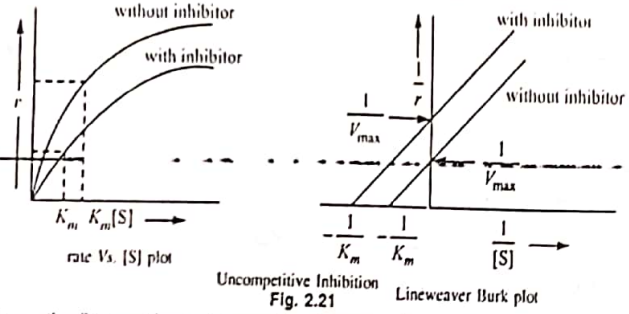


Fig. 2.21

Schematic-diagram for uncompetitive-inhibition is as follows:

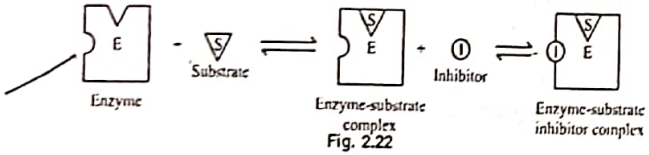


Fig. 2.22

(c) Non-Competitive inhibition: This type of inhibition cannot be reversed by raising substrate concentration [S]. Here, greater concentration of substrate is unable to prevent combination of inhibitor with enzyme. Thus, extent of inhibition is affected by concentration of inhibitor [I] & not by [S]. Non-competitive inhibitors combine with enzyme at a point other than attachment of substrate; but even then they effect active-site. They change only V_{max}; but not K_m. These inhibitors interfere either with formation of enzyme-substrate complex or its breakdown to yield product. Arginase is an enzyme which is competitively and non-competitively inhibited by lysine & monoamino acids, respectively. Non-competitive inhibitors show little structural resemblance with substrate as compared to competitive ones. Heavy metal ions like mercury and lead act as non-competitive inhibitors which bind to strategically positioned sulphydryl groups and modulate enzyme-conformation. Reaction rate (r) versus substrate concentration [S] plot & Lineweaver-Burk plot for non-competitive inhibition are given below:

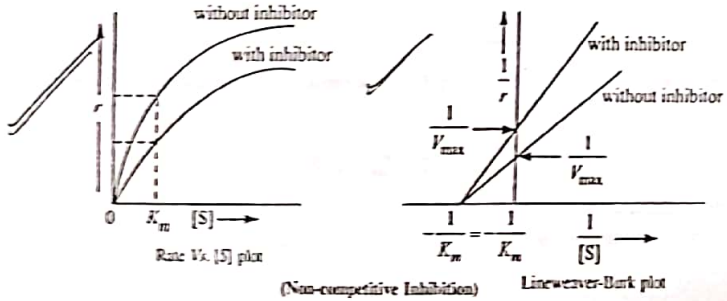
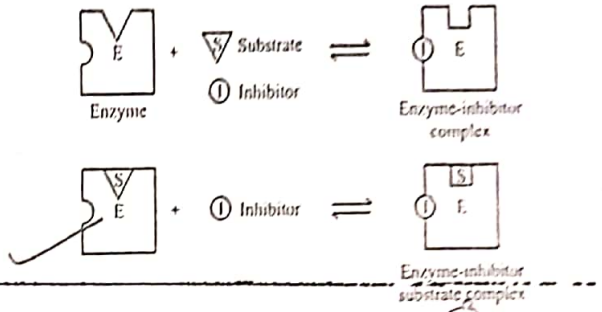


Fig. 2.23

Schematic-diagram for non-competitive inhibition is as follows:



(B) Irreversible-Inhibition: In irreversible inhibition, inhibitor forms covalent bond with functional group at active site or any other centre at enzyme. Irreversible inhibition can not be analysed by Michaelis-Menten kinetics. This type of inhibition gives information about functional amino acids at active site of enzymes. Some pesticides are irreversible inhibitors. For example, parathion is inhibitor of insect acetylcholine sterase upon which normal propagation of nerve impulse depends. Because inhibition is irreversible, inhibitor remains bounded to enzyme active-site by covalent bond, rendening it permanantly inactive. Some inhibitors are active as drugs, on account of irreversible inhibition by them.

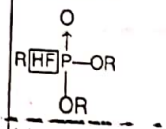
Other Types of Inhibition

Enzymes may be inhibited by any protein denaturant such as urea, trichloroacetic acid, heat, foaming etc. Inhibition by p-chloromercuribenzoate is commonly reversed with cysteine, therefore, it may be concluded that it reacts selectively wth —SH group. Actually, the type of inhibition depends on particular enzyme under investigation. For instance in one enzyme —SH group may be essential for activity, but in another it might be near the point of attachment of substrate.

Chemical Basis of Inhibition

Any reagent that reacts with functional group of protein is potential enzyme inhibitor. Besides chemical modification of protein, any reagent which reacts with or displaces a substrate, coenzyme or required metal ion may inhibit the catalysed reaction. Some representative inhibitiors of enzymes along with mechanism of action are given below:

S. No.	Inhibitor	Sensitive group	Mechanism of action	Representative sensitive enzyme
1.	Iodoacetamide	Sulfydryl (also —NH_2 group)	RS H I CH_2CONH_2 (involves alkylation)	Papin
2.	Iodobenzoate	Sulfydryl	RS H I O C_6H_4COOH RS H (oxidation)	Triose phosphate dehydrogenase
3.	Trivalent arsenicals	Sulfydryl	R—S HCl As-R R—S HCl (mercaptide formation)	Succinoxidase

S. No.	Inhibitor	Sensitive group	Mechanism of action	Representative sensitive enzyme
4.	Chelating agents	Metal	Removal of metal (metal inactivation)	Aspartase
5.	Arsenate	Organic phosphate bond	Competitive acceptor against phosphate (Arsenolysis)	Phosphate transacetylase
6.	Alkoxy halogenophosphate (di-isopropylfluorophosphate)	Serine or imidazole	 (phosphorylation)	Cholinesterase

ALLOSTERIC ENZYMES

When initial velocity of some enzyme catalysed reaction (V_0) is plotted as a function of concentration of substrate [*S*]; curve is not hyperbolic but is sigmodal. Rate of such reaction at a given [*S*] is increased or decreased by the addition of specific substance, *i.e.*, activators or inhibitors (modulators) [Fig. 2.25]. Such enzymes are known as allosteric enzymes. Besides substrate binding sites (active sites), these enzymes possess other sites in which activators & inhibitors may bind and affect catalysis through induced conformational changes in the structure of enzyme (Fig. 2.26).

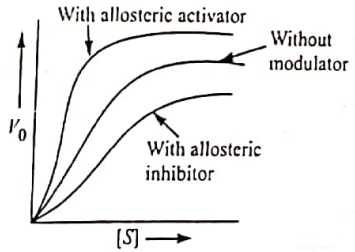


Fig. 2.25 Effect of modulators on enzyme catalysed reaction rates

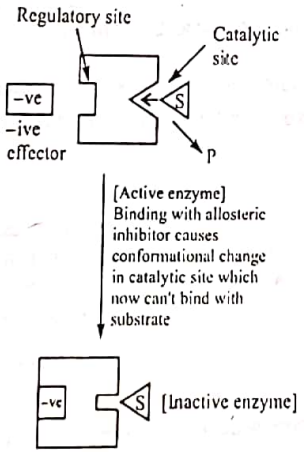


Fig. 2.26 Conformational change in allosteric enzyme

ISOENZYMES

Isoenzymes (also known as isozymes) are oligomeric enzymes which catalyze same reaction but differ in their subunit composition. These differences modify the

rate at which molecular species transform the substrate. Isozymes may be primary or secondary. **Primary isozymes** are produced by multiple gene loci which code for distinct protein molecules or are produced by multiple alleles at a single gene locus. These are also called **alloenzymes**. **Secondary isozymes** are product of post-translational modifications including glycosylation. On account of their different amino acid compositions primary isozymes may be identified on the basis of their different electrophoretic mobility. When enzyme variations are within same species they are known as intra-specific variants. But enzyme variation from different species is called interspecific or phylogenetic variant.

❑❑❑