# UNIT 9 ENZYMES : SPECIFICITY AND MECHANISM OF ACTION

## Structure



## 9.1 INTRODUCTION

You have studied about the structure of cell membranes and the processes of transport across these membranes in the previous units. Membrane proteins act as transporting molecules. The other important function of cellular proteins is to act as enzymes and catalyse biochemical reactions at a rate appropriate to the need of the cell.

In this unit you will study about the nature of enzymes and their functional aspects. Enzymes control the overall metabolic functions of the cell by speeding up the reaction rate. They do not affect the equilibrium constant and remain unaffected at the end of the reaction. The reactants bind to a specific site on the surface of enzyme molecule called active site. Enzymes show specificity for their substrate as well as for the reactions. Various factors such as temperature, pH, concentrations of enzymes and substrate affect the rate of enzyme-catalysed reactions. Some enzymes require additional factors for their normal activity. Allosteric enzymes have more than one active sites which may be located on the same subunit or on different subunits. Inhibitors are the substances that reduce the enzyme activity. Michaelis and Menten derived a mathematical equation to study the relationship between substrate concentration and reaction rate. The curve for enzyme kinetics showing the relationship between these two is a hyperbola. The kinetics of allosteric enzymes show a sigmoidal curve. The detection and estimation of enzymes can be done by using various techniques which can measure their ability to convert the substrate into products.

Various enzyme-catalysed reactions characterise the metabolism of the cell. Regulation of these reactions is achieved by altering the enzyme activity. In the next unit you will read about the regulation of enzyme activity. Before you read this unit, you should refresh your memory about the structure of proteins.

### Objectives

After reading this unit you should be able to:

- describe the role of enzymes in lowering the activation energy and in coupled reactions,
- list the type of enzymes and describe cofactors and discuss the mechanism of enzyme action,
- discuss the effects of temperature, pH and enzyme concentration on the rate of enzyme action,

- draw the curve for Michaelis-Menten equation and Lineweaver-Burk plot to show the enzyme kinetics,
- list the essential features of allosteric enzymes, isoenzymes, inhibitors and assay of enzyme activity.

## 9.2 CHARACTERISTICS OF ENZYMES

Enzymes act as catalysts and influence the rate of reaction in all the living organisms. In fact, essentially all chemical reactions in the living beings are catalysed by enzymes. All the enzymes known till now are proteins with a unique three dimensional structure that provides an active site for binding the other molecules, i.e. substrates to their surface. Each enzyme normally catalyses a few reactions but most often only one type of reaction. They are required in minute quantity/concentration to convert the substrates into products. The enzyme remains unaffected at the end of the reaction. The enzymes molecule is much larger than the molecule of its substrate. The molecular weights of enzymes range from thousands to millions, whereas the molecular weights of substrates are usually in few hundreds. Some enzymes are purely proteins, whereas others require non-protein assistant compounds for their catalytic activity.

### 9.2.1 Activation Energy

All the chemical reactions in a biological system have an energy barrier which prevents reactions from proceeding in an uncontrolled and spontaneous manner. The input of energy required to break this energy barrier or to start a reaction is called the **activation energy**. For example, a mixture of hydrogen and oxygen will not react with each other until they receive enough energy from a heat source to achieve the activation energy. You may have noticed that a tin of petrol or kerosene oil kept exposed to air at room temperature, would not catch fire unless kindled by a spark. This tiny spark is adequate to supply the activation energy for few molecules to react. The amount of energy released as a result of the conversion of the first few molecules of reactant to product is sufficient to activate other molecules. Similarly, chemical reactions taking place in living organisms also require activation energy. In biological systems, enzymes are important components which enhance the chemical reactions by lowering the activation energy and help the reactions to occur at a rate appropriate to the needs of the cell. The enzymes speed up the reactions by lowering activation energy. Enzymes combine with different types of substrates in such a way that they reduce the amount of energy required for a particular reaction (see Fig. 9.1). In the absence of enzymes in your gut, it would take many years instead of few hours for your last meal to be digested. You will read about the mechanism of enzyme actions in the following sections.

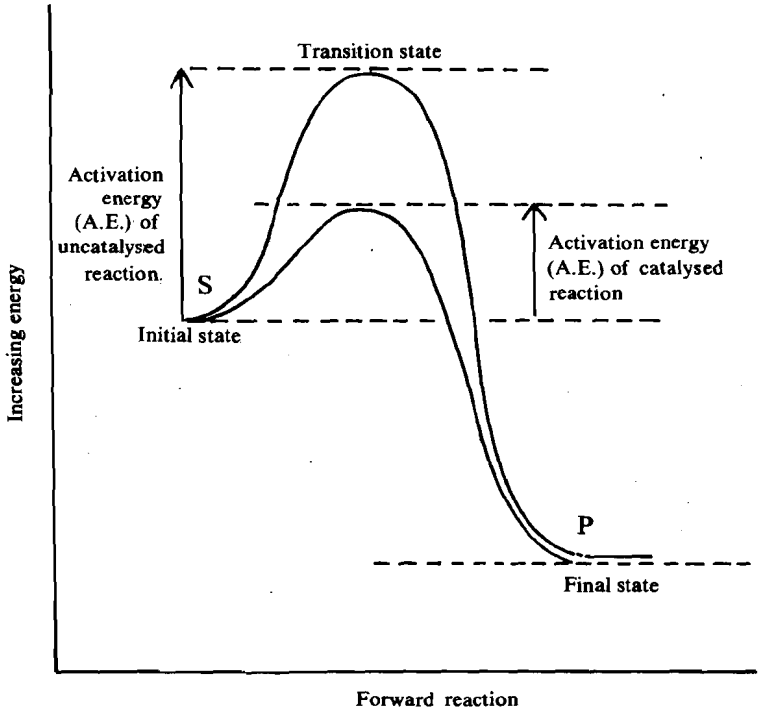
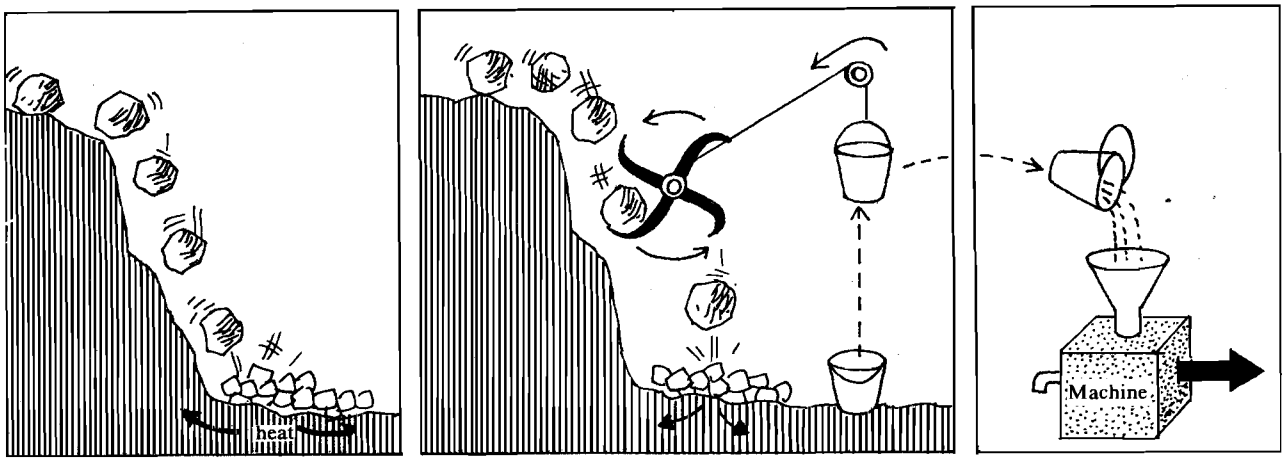



**Fig. 9.1: Lowering of activation energy by an enzyme. The activation energy (A.E.) is the energy barrier which the reactants overcome as they react to form the product. The intermediate transition state of enzyme substrate complex lies at the peak of the activation energy barrier.**

## 9.2.2 Coupled Reaction

The energy available to the cells is obtained by burning of food stuff. The breakdown of these food stuffs is carried out by series of reactions, i.e. catabolic reactions in a complex and controlled way. This energy is utilised by the cell for synthetic processes requiring energy, i.e. anabolic reactions which are endergonic in nature. Thus the catabolic reactions which are exergonic in nature and give energy, are coupled to anabolic chemical reactions. This process of coupling maintains a balance in the living systems.

The coupling of these reactions is an important characteristic of living organism and is carried out with the help of enzyme-directed reaction pathways. This coupling process can be understood by examining a simple analogy where an energy releasing catabolic chemical reaction is represented by stones falling from a hill top (see Fig. 9.2). The kinetic energy of falling stones would normally be wasted in the form of heat generated when they hit the ground (see Fig. 9.2a). A part of this kinetic energy can, however, be trapped and used to drive a paddle wheel that lifts a bucket of water, as shown in Fig. 9.2 b. So we can say that the energy producing reaction of a stone falling is directly coupled to the energy requiring reaction of lifting the bucket of water. A part of the energy of falling stones is now used in lifting a bucket of water and the stones hit the ground with less velocity. So less energy is wasted as heat. The potential energy which is stored in the form of a bucket of water can be used for other purposes such as to drive hydraulic machines (Fig. 9.2 c).




(a) kinetic energy transformed into heat energy only.

(b) part of the kinetic energy is used to lift a bucket of water, and a correspondingly smaller amount is transformed into heat.

(c) The potential Kinetic energy stored in the elevated bucket of water can be used to drive hydraulic machines.

**Fig. 9.2: Schematic diagram of a mechanical model indicating the coupling of reactions. Stones falling from the hill top can be compared with the oxidation of food stuffs (a). Part of the energy of falling stones is conserved in the form of lifted bucket of water which can be compared to the synthesis of ATP(b). The conserved energy can be used for energy requiring reaction such as utilisation of ATP during endergonic reactions (c).**

In the living organisms, enzymes play the role of paddle wheels and generate and trap energy in the form of high energy phosphate bonds of adenosine triphosphate (ATP). The ATP thus formed is utilised as a source of energy for many energy-dependent processes and is described as an energy currency of the cell. ATP is hydrolysed in neutral aqueous solutions accompanied by release of energy. The first cleavage of ATP to produce ADP and inorganic phosphate, releases 7.3 K. cals of energy per molecule (see Fig. 9.3).

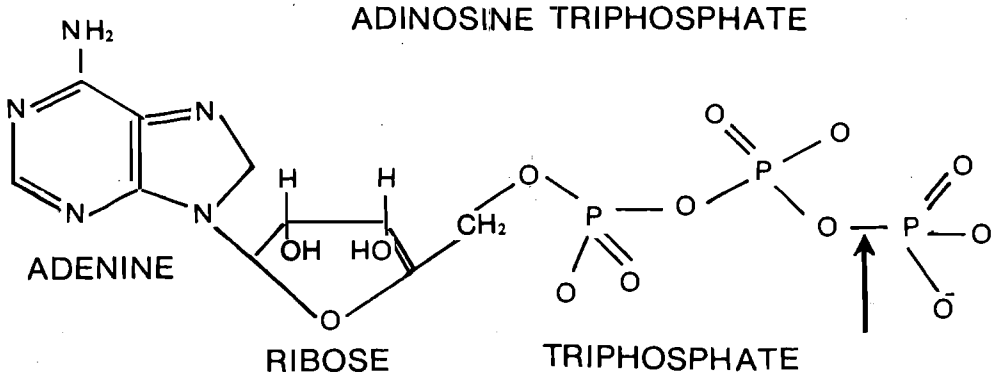



**Fig. 9.3: Structural formula of ATP. The first cleavage indicated by an arrow releases 7.3 K. cals of energy per molecule.**

**SAQ 1**

State if the following statements are True (T) or False (F).

i) Substrate is the reactant of an enzyme catalysed reaction. [ ]

ii) Enzymes are proteins with an ability to catalyse specific reaction. [ ]

iii) All enzyme catalysed reactions produce energy for ATP synthesis. [ ]

iv) Exergonic and endergonic reactions are coupled in all living organisms. [ ]

v) All the enzyme catalysed reactions have higher activation energy. [ ]

## 9.3 TYPES OF ENZYMES

The enzyme commission of the **International Union of Biochemists (IUB)** has classified enzymes into six categories on the basis of their functional specificity. Each category is further sub-divided into particular groups consisting of number of enzymes (Table 9.1). Most of the enzymes end as —'ase'. The first part of the name usually indicates the substrate on which the enzyme acts for example amylase enzyme acts on the substrate amylose or starch. A few enzymes are still called by their old names, e.g., pepsin, trypsin, chymotrypsin etc.

**Table 9.1: Classification of Enzymes (IUB system)**

| No. | Enzyme Category | Type of reaction | Examples |
|---|---|---|---|
| 1. | Oxidoreductases | Oxidation reduction reactions | *Oxidases* : transfer hydrogen to oxygen, e.g. cytochrome oxidase<br>$Cyt\ H_2 + 1/2O_2 \rightleftharpoons Cyt + H_2O$<br>reduced cytochrome — oxidised cytochrome<br>*Dehydrogenases*: transfer hydrogen to a molecule other than oxygen. e.g. lactate dehydrogenase<br>$C_3H_6O_3 + NAD \rightleftharpoons C_3H_4O_3 + NADH_2$<br>lactate — pyruvate<br>This reaction occurs in liver following heavy exercise. |
| 2. | Transferases | Transfer a functionally important group from one molecule to another | *Transaminases* : transfer amino groups, making new amino acids from existing ones<br>e.g. asparatate transaminase<br>aspartate + α–ketoglutarate<br>(amino acid) ⇅ (carboxylic acid)<br>glutamate — oxaloacetate<br>(amino acid) — (carboxylic acid)<br>This occurs in all cells.<br>*Kinases* : transfer phosphate, usually from ATP to another substance<br>e.g. hexokinase<br>ATP + glucose ——> ADP + glucose-6-phosphate<br>This reaction activates glucose prior to its breakdown in respiration. Phosphorylases which add inorganic phosphate without using ATP are also in this category. |
| 3. | Hydrolases | Split molecules in two by the action of water | *All digestive enzymes* fall into this category : pepsin, trypsin etc.<br>e.g. amylase<br>$starch_n + H_2O \rightarrow starch_{n-1}$ + maltose |

| | | | |
|---|---|---|---|
| | | | These reactions occur in the gut and transform large insoluble food molecules into smaller soluble ones which can be absorbed. Similar reactions also occur in the lysosomes of cells.<br><br>*Phosphatases* which remove phosphate groups from organic molecules by hydrolysis also fall in this category. |
| 4. | Lyases | Add or remove groups without involving water | *Carboxylases* : add $CO_2$<br>e.g. ribulose diphosphate carboxylase<br>ribulose diphosphate + $CO_2$ ---> phosphoglyceric acid<br><br>This occurs in the stroma of chloroplasts and is the principal mechanism by which atmospheric $CO_2$ is turned into organic material.<br><br>*Decarboxylases* : remove $CO_2$<br>e.g. pyruvate decarboxylase<br>pyruvic acid ------> acetaldehyde + $CO_2$ released<br>Intermediate step in the conversion of sugars to ethanol during alcoholic fermentation. |
| 5. | Isomerases | Convert one isomer of a compound into a different isomer by redistributing the atoms | *Mutases*<br>e.g. phosphoglucomutase<br>glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate<br>This reaction occurs at early steps in the respiration of sugars. |
| 6. | Ligases | Link together two molecules at the the expense of ATP | *Synthetases*<br>e.g. aminoacyl synthetases<br>Join amino acids to tRNA during protein synthesis. |

**SAQ 2**

According to the IUB system enzymes are classified into six categories. Name these categories and give one example for each.

## 9.4 COFACTORS

As you have read above, all the enzymes are proteinaceous in nature. Most of the enzymes consist of only polypeptide chains. Some enzymes, however, require an additional chemical component for carrying out their catalytic function. These constituents are non-protein and are called cofactors. A complete, catalytically active enzyme together with its cofactor is called a **holoenzyme**. The protein part of the holoenzyme is called **apoenzyme**. The cofactors are of three kinds; prosthetic groups, coenzymes and metal ions.

**Prosthetic groups** are organic compounds and are permanently bound to apoenzymes. For example, in the enzymes peroxidase and catalase, which catalyse the break down of hydrogen peroxide to water and oxygen, heme is the prosthetic group and is a permanent part of the enzyme's active site.

**Coenzymes** are also organic compounds, but their binding with apoenzyme is transient, and occurs only during the catalysis. The same coenzyme molecule may act as cofactor in different enzyme-catalysed reactions. Apart from helping enzymes in their catalytic activity these coenzymes also function as transient carrier, i.e. temporary acceptors for specific atoms or functional groups. Vitamins are essential components of many coenzymes. For example, the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) contain vitamin niacin and are electron carriers. Coenzyme A (CoA) contains vitamin pantothenic acid and carries (transfers) functional groups such as, an acetyl group.

**Metal ions** are required by certain enzymes for their catalytic activity. Metal ions such as $Mg^{2+}$, $Mn^{2+}$ or $Zn^{2+}$ bind with specific side chains at active site and at the same time with the substrate. The binding of metal ions with the substrate helps to break it down into products. For example, zinc is a cofactor for proteolytic enzyme carboxypeptidase. It binds with side chains of amino acid residues in the active site of the enzyme and with the $\alpha$ -carboxyl group of substrate amino acid. It is here that the peptide bonds of substrate are broken by the enzyme. Table 9.2 summarises various type of cofactors.

**Table 9.2: Some cofactors and their related enzymes**

| Cofactor | Enzyme | Reaction |
|---|---|---|
| Prosthetic groups | | |
| Heme | Catalase | $2H_2O_2$---->$2H_2O + O_2$ |
| Heme | Peroxidase | $2H_2O_2$ ---->$2H_2O + O_2$ |
| Coenzymes | | |
| Flavin mononucleotide (FMN) | Some dehydrogenases | Removal of hydrogen atoms |
| Thiamine pyrophosphate (TPP) | Some decarboxylases | Removal of $CO_2$ |
| Metal ions | | |
| $Zn^{2+}$ | Carboxypeptidase | Hydrolysis of proteins |
| $Cu^{2+}$ | Ascorbic acid oxidase | Ascorbate $\rightleftharpoons$ dehydroascorbate |
| $Mg^{2+}$ | Hexokinase | Glucose + ATP ---> glucose phosphate |

**SAQ 3**

Differentiate between coenzymes and prosthetic group. Answer briefly in the space provided.

.....

.....

.....

.....

## 9.5 MECHANISM OF ENZYME ACTION

In spite of the excellent information that has accumulated over the last several years on the physical, chemical and structural aspects of enzymes, much remains to be understood about their mechanism of action. Significant information on the mechanisms by which ribonuclease, chymotrypsin, lysozyme and some other enzyme catalysed reaction is, however, available.

The general mechanism of enzyme action can be viewed as having three stages. The enzyme first binds to the substrate followed by the formation of enzyme substrate complex (ES) which finally alters the substrate and forms the product. The events in enzyme catalysed reaction are represented by the equation.

binding catalysis Release

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

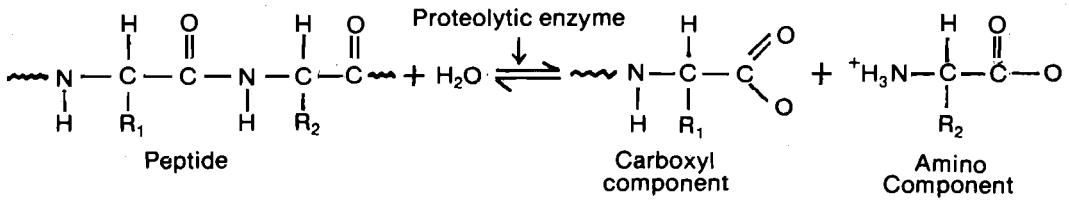
where E is enzyme S is substrate
P is product ES is enzyme substrate complex

As you know an enzyme speeds up a reaction by lowering its activation energy. Enzymes have specific regions on their surface to which the substrate binds and catalysis takes place. This region is called as the active site of the enzyme. The enzyme recognises and binds the substrate to its active site and forms an **activated enzyme substrate complex**. The enzyme acts upon the specific bonds within the substrate resulting in the formation of the product. Thus the enzymes lower the energy barrier and allow the organisms to carry out reactions faster.

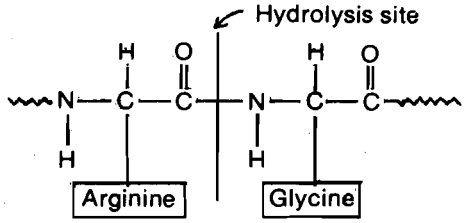
An enzyme lowers the energy barrier, i.e. activation energy of both the forward and reverse reactions to the same extent. It does not alter a reaction's equilibrium state but merely increases the speed with which the reaction approaches equilibrium. Furthermore, since a catalyst such as, an enzyme is not permanently changed by participation in a reaction, it comes out exactly as it started, and is ready to catalyse the reaction again. One of the factors responsible for enzyme action is their specificity about which you will read in the following subsection.

## 9.5.1 Enzyme Specificity

One of the most significant features of enzymes is their specificity with respect to the nature of the reaction they catalyse and also in the actual reactant, i.e. substrate molecule. For example, proteolytic enzymes catalyse the reactions involving proteins, whereas urease and succinic dehydrogenase act exclusively on urea and succinate respectively. Enzyme specificity varies even within a particular group of enzymes. Let us take the example of proteolytic enzymes. The reaction catalysed by these enzymes is the hydrolysis of peptide bond (Fig.9.4a). Subtilisin cleaves any peptide bond joining the amino acids of the substrate, whereas enzyme thrombin catalyses the hydrolysis of peptide bonds only between arginine and glycine (Fig. 9.4 b). Still some other proteolytic enzymes have different degree of specificity, for example trypsin breaks peptide bonds only on the carboxyl side, i.e. C-terminal of amino acids lysine and arginine (Fig. 9.4 c). You have read about the N-terminal and C-terminal of amino acids in Unit 5. The specificity differences are due to the biological functions of various proteolytic enzymes. Since bacteria use any protein as a source of carbon and nitrogen, subtilisin enzyme found in them can act upon any type of proteins. Enzymes trypsin and chymotrypsin have limited specificity as they break down the proteins into 5 to 20 amino acids long fragments in the mammalian digestive system.




(a)

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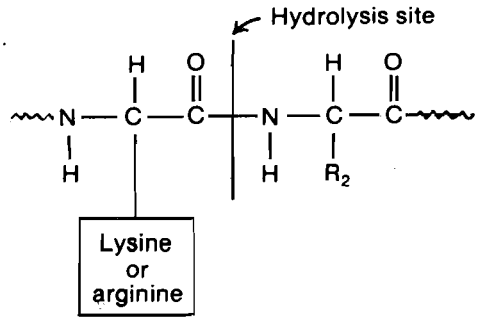


(c)

**Fig. 9.4: a) Proteolytic enzymes carry out hydrolysis of peptide bonds; b) Specificity of enzyme thrombin; c) Specificity of enzyme trypsin.**

Thrombin participates in blood clotting and shows absolute specificity as it splits only a single peptide bond in fibrinogen releasing the fibrin monomer which helps in the formation of fibrin clot. The least specific group of enzymes is hydrolases enzymes.

Some enzymes exhibit stereospecificity acting exclusively on different stereoisomers of the same compound e.g., the kidney D-amino acid-oxidase acts on D-isomers of amino acids. However, a small group of enzymes such as racemases catalyse the interconversion of D and L forms. Some enzymes exhibit geometrical specificity acting only on cis or trans isomers. Cis and trans isomers are geometrical isomers and have different arrangement of their constituent atoms and groups with respect to the double bonds joining the two carbon atoms. For example, maleic acid and fumaric acid have the same molecular formula but differ in the arrangement of the group round the C = C bond. As shown in Fig.9.5 Maleic acid, with both the carboxylic group on the same side, is the cis form of fumaric acid, the trans isomer in which these groups are on the opposite side.

Geometrical (cis-trans) isomers

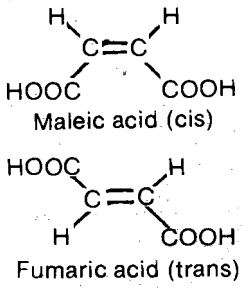


**Fig 9.5: Maleic acid and fumaric acid are geometrical, cis-trans isomers.**

In view of the specificity exhibited by enzymes in binding of the substrates, Emil Fischer in 1984 put forward the **lock and key hypothesis**. According to this hypothesis the substrate molecules fit into the active sites of the enzymes like a key fits into the lock. This results in the formation of transient ES-complexes which breaks down into enzyme and product (Fig 9.6 a). The enzyme substrate complex can be isolated from the enzymes that work slowly thus giving the direct evidence for the formation of these ES-complexes.

Although the lock and key model accounts for much of the enzyme substrate specificity, certain observations about enzyme behaviour cannot be explained by this model. For example, some times compounds other than the actual substrate bind to the enzyme and fail to form the reaction products. In the 1960's Daniel Koshland proposed the **induced-fit hypothesis** according to which the active site of the enzyme does not initially exist in a shape complementary to the substrate but is induced by the substrate to assume the complementary shape to bind the substrate molecule. Active site of an enzyme is thus regarded flexible in nature (see Fig. 9.6 b). However, a flexible active site is not necessary for all enzyme catalysed reactions as some are adequately explained by the lock and key model.

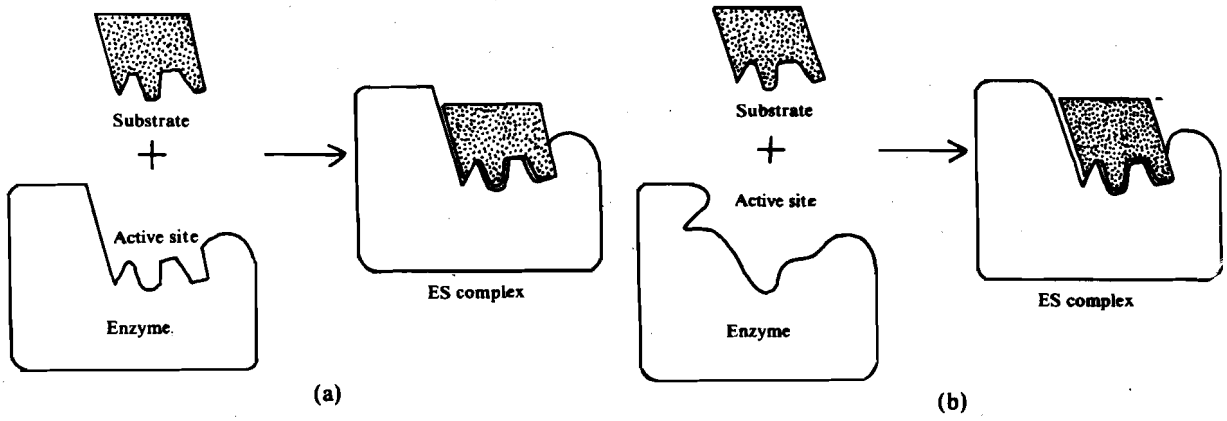


**Fig. 9.6:a) Lock and key model: the active site of the enzyme is complementary in shape to that of the substrate. Substrate fits in the active site like a key fits in the lock.**

**b) Induced fit model: the enzyme changes its shape upon binding the substrate. The active site acquires complementary shape to that of substrate only after the substrate is bound.**

## 9.5.2 Molecular Basis of Enzyme Action

The molecular basis of enzyme action depends on its three dimensional structure. The making and breaking of chemical bonds by an enzyme in a reaction are preceded by the formation of an enzyme substrate complex. As you have read earlier, the substrate is bound to a specific region of enzyme called active site. The properties and positions of side chains of the enzymes exposed at the active site determine which substrates will bind to it. The specific amino acid side chains at the active site are not necessarily close to each other in the linear protein chain. Folding of the protein chain, however, brings these groups together. For instance, negatively charged side chain groups, like those of asparatate and glutamate, can be forced together, due to the folding of the enzyme protein molecules in spite of their tendency to repulse each other. This leads to an increase in affinity for the positively charged

groups of the substrate. Similarly, some amino acid side chains forced together as a result of folding of peptide chains, may interact through hydrogen bonding, for example in trypsin a normally non-reactive serine molecule ($CH_2OH$) becomes highly reactive by acquiring two negative charges (Fig. 9.7).

When an enzyme is denatured, the native three dimensional structure is disrupted. Amino acid side chains of the active site unfold and separate from the site. This prevents their participation in a chemical reaction thus inactivating the enzyme. Enzyme inactivation is also caused even when only a functional side group is displaced or substituted in the enzyme.

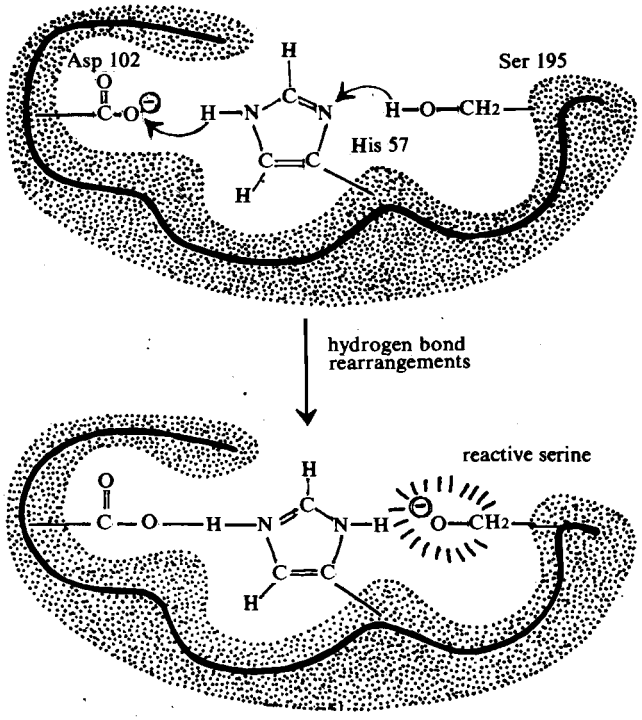


**Fig. 9.7: Rearrangements of hydrogen bond makes serine molecule highly reactive in the active site of the enzyme.**

Although enzymes differ widely in structure, specificity and mode of catalysis, various mechanisms operate at the active site of enzymes, each contributing to the lowering of activation energy. These mechanisms can be generalised as follows:

- The binding of reacting molecules with each other brings them in close proximity, thus increasing the chance of a reaction.
- The substrates are bound to the enzyme in such a manner that the formation of several types of temporary non-covalent bonds between them force a redistribution of charges within substrates. This redistribution imposes a strain on specific covalent bonds in the substrates resulting in the breaking of the bond.
- Hydrophobic amino acids eliminate water molecules from the active site to create a water free zone so that nonpolar groups react more easily.
- Acidic and basic amino acids in the active site of the enzyme transfer the protons to and from the reactants for catalysis of reaction. This is known as acid-base catalysis.
- Enzymes react with the substrate to form transient, high energy unstable covalently linked enzymes substrate-complex which undergoes further reactions to form products.

Although these factors are believed to contribute to the acceleration of reaction rate by various enzyme, the exact mechanism involved is yet to be known.

**SAQ 4**

Complete the following statements with appropriate words.

i) The substrate binds on the ..... of the enzyme and forms .....

ii) Enzymes do not alter the ..... of the reactions.

iii) ..... acts upon any peptide bond between the amino acids.

iv) Thrombin has ..... specificity.

v) According to ..... active site assumes the complementary shape after the ..... is bound.

vi) ..... of the polypeptide chains bring the side chains together in the active site region.

vii) ..... amino acids create a water free zone for ..... reactants.

viii) Transfer of protons to and from the reactants is ..... catalysis.

## 9.6 FACTORS AFFECTING THE RATE OF ENZYME ACTION

Enzymes are capable of enhancing the reaction rate enormously, sometimes a million times compared to uncatalysed reactions. The rates of enzyme catalysed reactions are expressed as the "**turnover number**". The turnover number is the number of substrate molecules transformed into product in unit time by a single enzyme molecule or by single catalytic site, and depends upon the enzyme concentration. So if the enzyme concentration is known, the turnover number can be calculated.

$$\text{Turnover number} = \frac{\text{moles of product formed per unit time}}{\text{moles of enzymes}}$$

Turnover numbers of several enzymes are listed in Table 9.3. The highest turnover number is of carbonic anhydrase which converts $3.6 \times 10^6$ $CO_2$ molecules to $H_2CO_3$ per minute. International Union of Biochemistry has now recommended a new unit for reaction rates called Katal (Kat). One Kat is the amount of enzyme that converts one mole of substrate into product per second.

**Table 9.3: Turnover Number of Some Enzymes**

| Enzyme | Turnover Number |
|---|---|
| Carbonic anhydrase | $3.6 \times 10^6$ |
| Acetylcholinesterase | $1.5 \times 10^6$ |
| Urease | $1.0 \times 10^6$ |
| Amylase | $1.0 \times 10^5$ |
| Lactic dehydrogenase | $6.0 \times 10^4$ |
| Chymotrypsin | $6.0 \times 10^3$ |
| Lysozyme | $3.0 \times 10^1$ |

Rates of enzyme catalysed reactions depend on several factors like temperature, pH, concentration of enzyme and substrate etc.

### 9.6.1 Effect of Temperature

Within certain limits temperature influences the enzyme catalysed reactions in the same way as it affects ordinary uncatalysed chemical reactions. As the temperature increases, the rate of a chemical reaction increases owing to an increase in the number of activated molecules. But when the temperature rises above a certain limit, it destroys the tertiary structure of the enzyme causing the loss of its activity. Similarly low temperature, such as freezing temperatures, generally inactivate the enzyme. It, therefore, follows the rule that for every enzyme under a given set of conditions, there is a temperature at which the activity of the enzyme is at its maximum. This is known as optimum temperature (see Fig. 9.8). Most enzymes are denatured above 50°C. Optimum temperature for mammals and birds is about 35°- 40°C whereas in plants and in other animals it is between 20°- 35°C. Some procaryotes and certain fishes found in Antartica region are exceptional in that they are adapted to life in hot spring and at freezing cold temperatures. They have optimum temperature at over 80°C and below freezing point respectively.

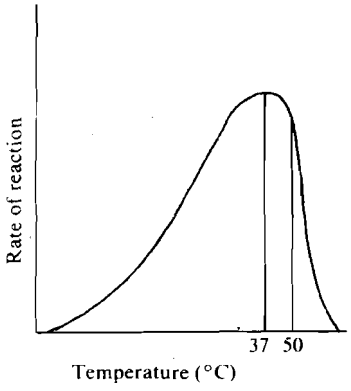


**Fig. 9.8: Effect of temperature on the rate of enzyme catalysed reactions. Optimum temperature varies for different enzymes and different species.**

## 9.6.2 Effect of pH

The hydrogen ion concentration or the pH has a marked influence on the rate of enzyme reaction. Characteristically, each enzyme has a pH value at which the reaction rate is optimum usually within the pH range 7.0 ± 1.5. This is known as optimum pH at which a certain enzyme causes a reaction to progress most rapidly. On each side of this pH value the reaction is decreased and at certain pH values on both sides an enzyme may be inactivated or even denatured. Therefore, while studying enzymes, buffers are used to keep the enzyme at an optimum pH.

While majority of enzymes function optimally around neutral pH values, some enzymes like pepsin in stomach has an unusually low optimum pH. This is an adaptation to the acidic conditions of stomach which has pH ranging from 1.5 to 2.5 due to the presence of HCl in the gastric juice (see Fig. 9.9). Pancreatic enzymes have their optimum pH in highly alkaline range.

Buffers are the combination of substances that resist changes in pH when acid or bases are added. It consists of a weak acid and its conjugate base or a weak base and its conjugate acid, for example acetic acid ($CH_3COOH$) and acetate ($CH_3COO^-$) can serve as a buffer when pH of the solution has to be adjusted around pH 1.

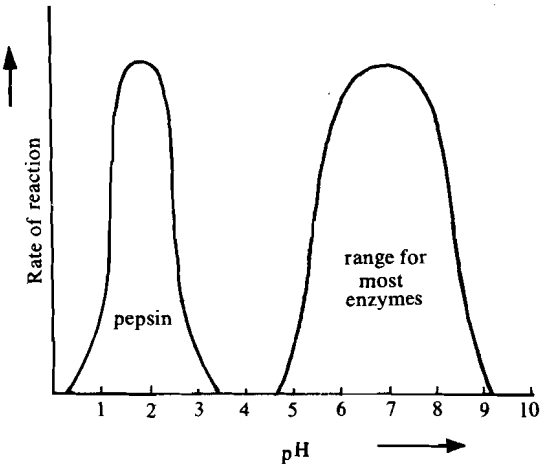


**Fig. 9.9: Effect of pH on the rate of enzyme catalysed reactions. Proteolytic enzyme pepsin has low optimum pH.**

## 9.6.3 Effect of Enzyme Concentration

As you know, Enzymes increase the reaction rate through catalysis. Under constant conditions of temperature, pH and substrate concentration, the velocity of the reaction is directly proportional to the amount of enzymes present, i.e. the reaction rate is increased with the increase in enzyme concentration. However, the equilibrium constant for the reaction is not affected by the presence of enzymes. A chemical reaction in the cell can be started or stopped by making or destroying a particular enzyme. As you have read, turnover number is a useful parameter to study the effect of enzyme concentration. Before we proceed further try the following SAQ.

**SAQ 5**

Match the statements in column I with those in column II.

| Column I | | Column II | |
|---|---|---|---|
| i) | Temperature influences enzyme catalysed reactions [ ] | a) | It is active only in acidic medium. |
| ii) | Mammals and birds also have optimum temperature. [ ] | b) | Enzyme concentration has its affect on reaction rate. |
| iii) | Enzyme pepsin acts on peptide bonds in the stomach. [ ] | c) | Enzymes are inactivated at freezing temperatures. |
| iv) | Reactions rate is directly proportional to the amount of enzyme. [ ] | d) | It is between 35°C to 40°C. |

Enzymes not only speed up the reactions, they also have a marked effect on the kinetics of the reaction. Substrate concentration plays an important role in enzyme kinetics about which you will read in the following section.

## 9.7 ENZYME KINETICS

The concentration of substrate also influences the rate of enzyme catalysed reaction. At low substrate concentrations, the number of substrate molecules are too few to occupy all the active sites of the enzyme molecules, and the initial velocity of the reaction is directly proportional to the substrate concentration. With the increase in substrate concentration more and more active sites are occupied by many more molecules of substrate. A stage will finally be reached when the substrate concentration is high enough to occupy all the active sites of the enzymes. At this saturation point the maximum velocity of the reaction is attained and is called **Vmax**. This maximal velocity is not affected by further increase in substrate concentration. The curve of the rate of enzyme reaction against substrate concentration shows the shape of a rectangular hyperbola for enzymes composed of single polypeptide chain with one active site (see Fig. 9.10). At a particular concentration of substrate where half the enzyme molecules are saturated with substrate molecules, the velocity (V) of the reaction is half the maximal velocity (**Vmax/2**). This substrate concentration at half the maximal velocity is termed as **Km** or **Michaelis constant**.

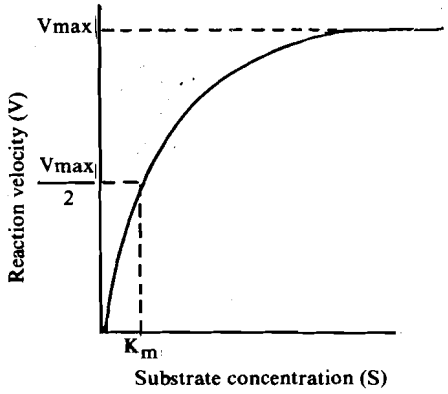


**Fig. 9.10: Relationship between reaction rate (v) and substrate concentration (s). The initial velocity of the reaction increases with the substrate concentration. At high substrate concentration v becomes independent of s. Michaelis constant Km is equal to substrate concentration when reaction rate is half its maximum value (Vmax/2). All such curves are hyperbola.**

In 1913 Leonard Michaelis and Maud Menten proposed a simple yet convincing model to explain the Kinetics of enzyme reaction. According to this model, enzyme (E) combines reversibly with substrate (S) to form the enzyme-substrate complex (ES). The ES subsequently breaks down to E and to product (P). This is represented by the following equation :

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + P \qquad 9.1$$

$K_1$ represents the rate constant for the formation of ES. $K_2$ and $K_3$ represent the rate constants for the dissociation of ES to E + S and E + P respectively.

Soon after the enzyme is mixed with substrate, the concentration of ES builds up and remains unchanged as long as S is in large excess. When the rate of formation of ES is equal to its breakdown, the ES concentration will be constant and the reaction system is said to be in a **steady state**. Putting this mathematically,

$$K_1[E][S] = K_2[ES] + K_3[EP]$$

$$\text{or } \frac{K_2 + K_3}{K_1} = \frac{[E][S]}{[ES] + [EP]} \qquad 9.2$$

At any substrate concentration some of the enzyme molecules are in the form of ES while E represents the free enzyme. Total enzyme molecules are therefore equal to free enzyme molecules plus enzyme molecules in the substrate complex form, i.e.

$$E\ (Total) = E + ES$$

The concentration of free enzyme or that of ES as enzyme substrate complex is difficult to determine experimentally. Equation 9.2 can, however, be, converted to a more convenient form assuming that

i) at any given substrate concentration, velocity (V) of an enzyme catalysed reaction is proportional to the concentration of ES, and

ii) maximum velocity attainable by the enzyme at saturating substrate concentrations (Vmax) is proportional to the total enzyme concentration.

Based on these assumptions, Michaelis-Menten derived the following equation from equation 9.2.

$$V = \frac{V_{max}\,[S]}{K_m + [S]} \qquad 9.3$$

Km is the Michaelis-Menten constant and is equal to $\frac{K_2 + K_3}{K_1}$

As said earlier, Km can be also defined as the substrate concentration where enzyme exhibits half of the maximum reaction velocity, i.e. when S = Km, then V = Vmax/2. Thus Km is a measure of the degree of binding or affinity of the enzyme for the substrate. Small values of Km denote high affinity while larger Km values indicate weak affinity. An enzyme has a characteristic Km value for a given substrate.

As shown in Fig. 9.10, Km value can be determined experimentally by determining the rates of reaction at various substrate concentrations, using a fixed amount of enzyme. However, most enzymes do not readily attain maximum velocity and continue to show small increases in velocity even at very high substrate concentration. For a more convenient calculation of Km, Lineweaver and Burk introduced a different form of Michaelis-Menten equation by taking reciprocal of both sides of the equation 9.3

$$\frac{1}{V} = \frac{K_m}{V_{max}\,[s]} + \frac{1}{V_{max}}$$

By plotting reciprocal of velocity (1/V) against the reciprocal of substrate concentration (1/S) one gets a straight line from which Km and Vmax can be easily calculated (Fig. 9.11)

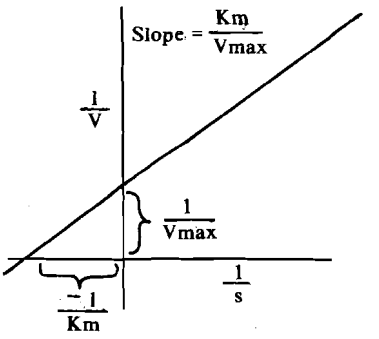


**Fig. 9.11: Lineweaver-Burk plot showing the relationship between reciprocal of reaction rate (1/V) and reciprocal of substrate concentration (1/S). The plot is a straight line and holds true for enzymes obeying Michaelis-Menten relationship.**

**SAQ 6**

Explain briefly the relationship between substrate concentration and Michaelis constant.

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## 9.8 ALLOSTERIC ENZYMES

Some enzymes may have two functionally different binding sites. One of the site, the active site, binds the substrate and catalyses the reaction. The other type of site, known as allosteric or **regulatory site** binds another molecule which is called **effector** or **modulator**. Such enzymes are known as allosteric enzymes. These enzymes are oligomeric in nature, i.e. they have more than one subunit. The active site and allosteric site may be located on the same subunit or on different subunits of the enzyme and the conformational changes caused by binding are transmitted between subunits. Effector molecules are of two types: positive effectors, i.e. **activators** that enhance enzyme activity and negative effectors or **inhibitors** which inhibit enzyme activity. Binding of effectors causes conformational changes in the allosteric enzymes which influence their catalytic activity (see Fig. 9.12). We can also say that allosteric enzyme action is regulated by effector molecules. You will read about the functions of effectors in Unit 10. These effectors molecules can bind at the same allosteric site or at different allosteric sites.

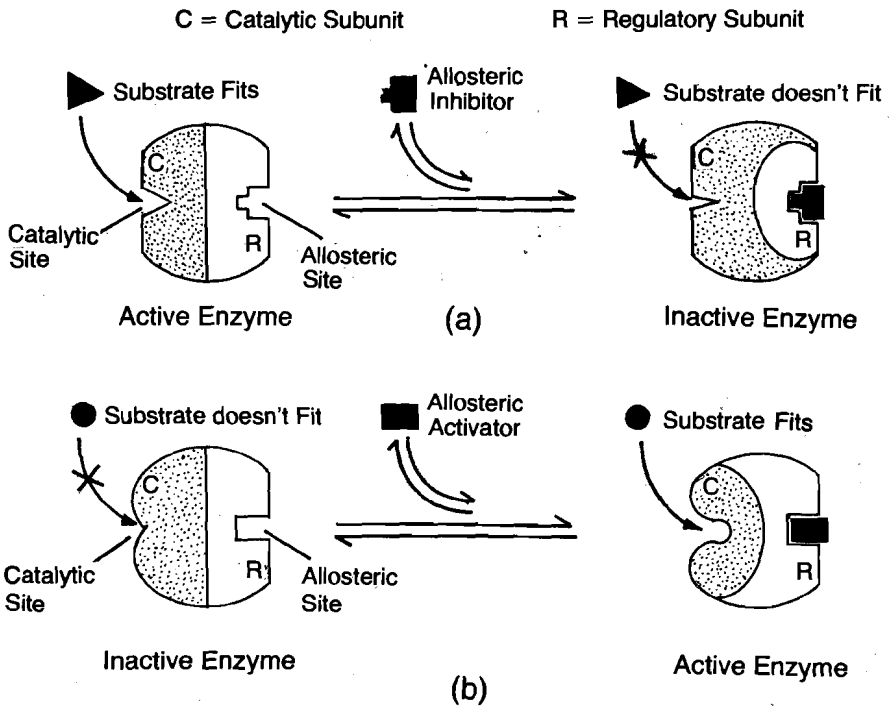


**Fig. 9.12: Schematic diagram showing the functioning of allosteric enzymes.**

Binding of a substrate to one active site may influence subsequent bindings to other active sites of an enzyme with more than one active sites. This behaviour of enzymes is known as **cooperativity**. When binding of the first substrate molecule promotes binding of subsequent substrate molecules, it is called **positive cooperativity. Negative cooperativity** is when, after the binding of the first substrate molecule subsequent substrate binding occurs less readily. Allosteric enzymes and enzymes that show cooperativity do not obey Michaelis-Menten Kinetics. Such enzymes which are composed of more than one subunit and more than one active site show a sigmoidal curve when reaction velocity is plotted against substrate concentration as shown in Fig. 9.13.

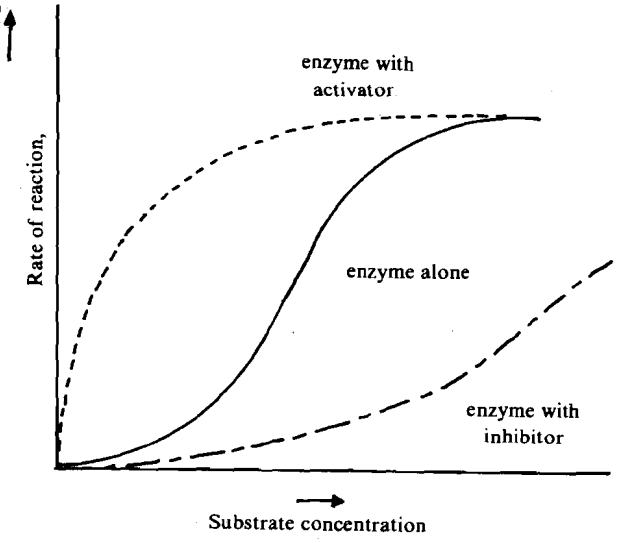


**Fig. 9.13: Allosteric enzymes show sigmoidal curve when reaction rate is plotted against substrate concentration. The effects of allosteric activator and inhibitor are also shown.**

Many allosteric enzymes function at the beginning of the metabolic sequence pathway and are inhibited whenever the end product of the reaction pathway accumulates. Such inhibition is described as the **feedback inhibition**. Several important metabolic pathways are regulated by feedback inhibition both in procaryotes and eucaryotes. You will read more about it in Unit 10.

## 9.9 ISOENZYMES

Several enzymes exist in their multiple forms termed **isoenzymes** or **isozymes**. These isoenzymes are not isomers. All isoenzyme forms of a given enzyme catalyse the same reaction but are chemically distinct molecules. They may differ in amino acid composition, amino acid sequence, charge, molecular weight, etc. They usually differ in one or more kinetic properties such as Km, Vmax for their substrate.

Isoenzymes are found in all vertebrates, insects, plants and unicellular organisms. Different tissues may contain different isoenzymes which may differ in their affinity for the substrates. One of the first and best known example of universally occurring isoenzymes is lactate dehydrogenase that exists in five different forms : $M_4$, $M_3H$, $M_2H_2$, $MH_3$ and $H_4$. Each of these isozymes is a tetramer made up of four polypeptide subunits. These subunits are of two types and occur in different combinations in the isozymes. These two types of subunits are named as M and H types depending upon their location; M in muscles and H in heart (Fig. 9.14).

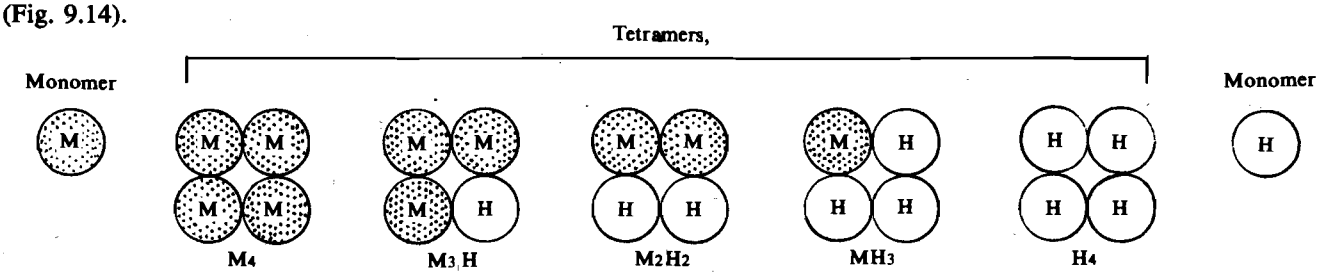


**Fig. 9.14: Lactate dehydrogenase enzyme is a tetramer, has five isozymic forms. Each tetramer has M and/or H types of monomers in different numbers.**

All the forms of lactate dehydrogenase isoenzymes do not necessarily occur in equal proportion in all the cells as $M_4$ dominates in muscles and $H_4$ is predominantly present in the heart. Other isoenzyme forms include oxidases, dehydrogenases. Several regulatory enzymes also exist in isoenzyme forms. The molecular difference of isoenzyme molecules is due to the difference in their locations such as malate dehydrogenase, which occurs in different forms in cytosol and in mitochondria. Analysis of isozymes is frequently used in medical diagnosis.

## 9.10 INHIBITORS

Enzyme activity can be inhibited by certain chemical substances called inhibitors. Enzyme inhibitors have provided information about substrate specificity of enzymes, the nature of functional groups at active site and the mechanisms of the catalytic activity. Some drugs considered to be useful in medicine function by inhibiting certain enzymes, for example, the inhibition of bacterial enzymes which affects the bacterial metabolism and, therefore, their growth and multiplication.

Enzyme inhibitors are of two types : Irreversible and reversible.

### i) Irreversible inhibitors

These inhibitors bind covalently to the enzymes causing the permanent loss of their catalytic activity by altering or destroying a functional group on the enzyme molecules. Arsenic, lead, mercury and various insecticides are all irreversible inhibitors. Even at low concentration of these the enzymes are precipitated.

### ii) Reversible inhibitors

These inhibitors bind non-covalently to enzymes and their effects can be reversed. Important information on the structure of the active sites of various enzymes have been obtained with the help of reversible inhibitors. The two main types of reversible inhibitors are competitive and non-competitive inhibitors.

**a) Competitive inhibitors**: Competitive inhibitors compete with the real substrate for binding to the active site of the enzyme because they resemble the substrate molecules. The catalytic ability of the enzyme is not affected by this binding but the ability of the substrate to bind to the enzyme is reduced. However, these inhibitors cannot be transformed into products. Competitive inhibition is reversed simply by increasing the substrate concentration. For example, succinate dehydrogenase, which catalyses the removal of 2 hydrogen atoms from succinate, is inhibited by malonate which resembles

succinate structurally. However, malonate is not dehydrogenated by the enzyme succinate dehydrogenase. It simply occupies the active site. The inhibition is reversed by increasing the succinate concentration.

b) **Non-competitive inhibitors**: These inhibitors bind at a site on the enzyme other than the catalytic sites, i.e. substrate binding site, altering the conformation of the enzyme molecule and leading to the distortion and inactivation of the catalytic site. Non-competitive inhibitors bind reversibly to both the free enzyme and the ES complex to form the inactive complexes. Addition of more substrate will not restore the previous rate of reaction in contrast to competitive inhibitors. Important non-competitive inhibitors are the naturally occurring metabolic intermediates for example L - threonine dehydratase is inhibited by L - isoleucine. Competitive and non-competitive inhibitors are not necessarily harmful. Both are used extensively by cells for metabolic regulation.

**SAQ 7**

a) Fill in the blanks with appropriate words.

i) Allosteric enzymes are ..... proteins.

ii) Effectors induce..... changes in allosteric enzymes.

iii) Multiple forms of an enzyme are known as .....

iv) The ..... of the isoenzymes may occur in different combinations.

b) Differentiate between competitive and non-competitive inhibitors. Answer briefly in the space provided.

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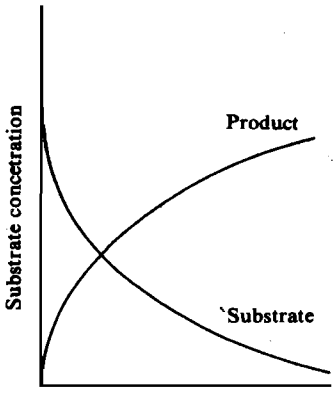


**Fig. 9.15: Concentrations of Substrate and product vary during the course of enzyme-catalysed reactions. As the reaction proceeds, substrate concentration decreases and product concentration increases.**

## 9.11 ASSAY OF ENZYME ACTIVITY

All enzymes so far known are proteins, yet the specific tests for protein can not be used for the detection and/or quantification of enzymes. Evidently such tests cannot make distinction between enzymes and non-enzymes proteins and between various enzymes. The amount of enzymes in a given solution or tissue extract can be conveniently measured or assayed quantitatively by using techniques that can measure their ability to convert the substrate into product.

One can detect the presence of an enzyme by using a specific quantification procedure either for the substrate or for product. Under optimal conditions the velocity of enzyme catalysed reaction is directly proportional to the concentration of the enzyme. One can therefore determine the concentration of enzyme from the velocity of the disappearance of the substrate or that of the formation of product under optimal conditions. As shown in Fig. 9.15 during the enzyme action the concentration of substrate decreases, while that of product increases. This quantitative estimation of enzyme activity is called as the assay of an enzyme. Activity of enzyme is represented in enzyme units.

By international convention one unit of enzyme activity is defined as that amount which transforms one micro mole substrate to product in one minute under optimal conditions. Enzyme assays are carried out at their optimal pH, temperature and with a near saturating concentration of substrates.

Concentration of different substrates or products can be determined using methods such as colorimetric, spectrophotometric, fluorometric or isotopic labelling procedures. The choice of the procedure is determined by the nature of the substrate or product. For example, spectrophotometric assays are used when either the substrate or product absorbs light of some specific wavelength. Highly sensitive assay procedures are usually based on fluorescence or radioactive measurements.

In many instances substrates are modified by introducing specific groups for the estimation of their products. Such modification of the substrate should not, however, result in decrease

in the rate of its transformation to product by the enzyme. For instance the introduction of p-nitrophenyl phosphate compound in substrate makes the substrate useful for the assay of phosphatases. The product of enzyme action is an ionised form having p-nitrophenylate ion which is of yellow colour and the intensity of colour indicates the activity of the enzyme.

We finish this unit on characteristics and mechanism of enzymes. We will continue with the regulation of enzyme activity in the next unit.

## 9.12 SUMMARY

In this unit you have studied that:

- enzymes are proteins that catalyse the biochemical reactions by lowering the activation energy. Enzymes increase the rate of the reactions but do not alter the equilibrium point,
- enzymes facilitate the coupling of energy releasing exergonic reactions to energy requiring endergonic reactions,
- enzymes are highly specific with respect to the nature of reaction as well as to their substrate. Enzymes substrate specificity has been explained by lock and key and induced fit hypothesis,
- reactions by the enzymes are facilitated on the basis of proximity and orientation of side chains of the substrate and enzyme, acid-base catalysis by the side chain of charged amino acids of active site and by covalent interactions between enzymes and substrates,
- most enzyme catalysed reactions follow a hyperbolic substrate saturation curve which is explained by the Michaelis-Menten equation for enzyme Kinetics. Substrate concentration at half the maximum velocity of reaction rate is called Michaelis-Menten constant, Km,
- changes in the concentration of enzymes, substrates, pH and temperature influence the rate of enzyme catalysed reaction,
- some enzymes require additional factors called cofactors for their catalytic activity. Prosthetic groups are organic cofactors and are permanently bound to the enzymes. Coenzymes are also organic compounds but their association is transient. Some enzymes require metal ions for their activity,
- allosteric enzymes are oligomeric and have more than one active site where the allosteric effector molecules bind and influence enzyme activity. Influence of one substrate binding on subsequent substrate bindings is known as cooperativity. The graph of kinetics of allosteric enzymes and enzyme cooperativity are sigmoidal.
- enzyme activity is also regulated by inhibitors. Inhibitor actions can be reversible or irreversible. Reversible inhibitors may be competitive or non-competitive,
- enzyme activity can be estimated by different procedures such as spectophotometric, fluorometric and isotopic labelling methods.

## 9.13 TERMINAL QUESTIONS

1) What do you understand by activation energy?

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2) Discuss briefly the variations in the degree of enzyme specificity.

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3) Discuss briefly the Michaelis-Menten Kinetics for enzyme action.

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4) Explain briefly the functioning of allosteric enzymes.

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## 9.14 ANSWERS

**Self-assessment Questions**

1) i) T, ii) T, iii) F, iv) T, v) F

2) i) Oxidoreductases-Cytochrome oxidase
   ii) Transferases-hexokinase
   iii) Hydrolases-amylase
   iv) Lyases-Decarboxylase
   v) Isomerases-Phosphoglucomutase
   vi) Ligases-Aminoacyl synthetases

3) Coenzymes are the organic cofactors which are transiently bound to enzymes for their catalytic activity.

4) i) active site, enzyme-substrate complex
   ii) equilibrium state
   iii) subtilisin
   iv) absolute
   v) induced fit hypothesis, substrate
   vi) folding
   vii) hydrophobic, non-polar
   viii) acid-base

5) i) c, ii) d, iii) a, iv) b

6) Michaelis constant Km is equal to the substrate concentration, when reaction rate is half its maximum value i.e. $\frac{Vmax}{2}$ At this stage half the enzyme molecules are saturated with substrate molecules.

7) a) i) oligomeric, ii) conformational, iii) isozymes
   iv) subunits

   b) Competitive inhibitors resemble the substrate and compete for the active site. These do not affect the catalytic ability of an enzyme. Non-competitive inhibitors do not resemble the substrate and reduce the catalytic properties of the enzymes.

8) Calometric, spectrophotometric, fluorometric and isotopic procedures are used for enzyme assay. Enzyme assays are carried out at their optimum pH, temperature and saturating concentration of the substrate.

**Terminal Questions**

1) The energy required to start a reaction is called the activation energy. In biological systems activation energy is lowered with the help of enzymes.

2) Enzymes exhibit specificity for the substrate as well for the reactions they catalyse. There is also variation in specificity within the particular groups of enzymes. Example of proteolytic enzymes can be given.

3) The initial reaction rate is directly proportional to the substrate concentration. At higher substrate concentration all enzymes are saturated and maximum velocity of the reaction is achieved. The relation between substrate concentration and reaction rate is shown by an hyperbola curve. The equation derived by Michaelis-Menten for one enzyme one substrate reactions is

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

4) Allosteric enzymes are regulated by other molecules called as effectors. These effectors bind to the allosteric or regulatory site of the enzyme and may act as activator or inhibitor of enzyme action.