Chapter I

Introduction to Enzymes
1.01 General Features of Enzymes:

Enzymes are biological catalysts\textsuperscript{1,2} and possess all the features which a usual chemical catalyst possesses\textsuperscript{3} \textit{i.e.} are required in small amounts, remain unchanged after the cycle of catalysis, enhance the rates of forward as well as backward reactions by the same factor leaving the position of equilibrium unchanged. In addition to these features of a usual chemical catalyst, enzymes have some special features in which they differ from the usual catalyst. They have enormous catalytic efficiency. An enzyme enhances the rate of a chemical reaction generally by a factor greater than $10^6$. The enzymes are specific in the choice of their substrates and also in the choice of the reactions which they catalyse. Moreover, enzymes work under milder conditions and the activities of the enzymes are regulated\textsuperscript{1}. Enzymes are the key to the functioning of biological systems and any abnormality in their functions may lead to some abnormality in the function of the biological system\textsuperscript{4}.

Till 1970, it has been common belief that only protein molecules catalyse biological reactions and hence all enzymes were proteins\textsuperscript{5}. Two independent research groups, one led by Prof. S. Altaman and the other led by Prof. T. R. Ceach have shown\textsuperscript{6} that RNA molecules also catalyse biological reactions and are enzymes. The term Ribozyme has been used for RNA enzymes\textsuperscript{6}. Though the number of non-protein enzymes is very small at present, it is hoped that it will increase with time and other proteinous molecules will come to the list of enzymes.
The term Abzyme has been used to represent a class of enzymes developed by utilizing the specificity of binding of antibody with antigen. A description of Abzyme is given by Strayer and is not attempted here.

1.02 Enzyme Nomenclature:

In order to remove ambiguity in the nomenclature of enzymes, the International Enzyme Commission has recommended a procedure for the classification of the enzymes. According to recommendations, each enzyme is assigned a recommended name, usually short and appropriate for everyday use, a systematic name, which identifies the reaction it catalyses and a classification number which is used where accurate and unambiguous identification of an enzyme is required. An example is given by the enzyme catalyzing the reaction.

\[
\text{Enzyme: } \text{ATP + Creatine} \rightleftharpoons \text{ADP + Phosphocreatine}
\]

The recommended name of this enzyme is creatine kinase and the systematic name based on the reaction catalysed by this enzyme is ATP: Creatine phosphotransferase. Its classification number is [E. C. 2.7.3.2] where E. C. stands for enzyme commission. The first digit (2) stands for the class name (transferases). All the enzymes have been classified in six classes and each class has been assigned a number starting from 1 to 6. The second digit (7) stands for the sub-class (phosphotransferases), the third digit
(3) stands for the sub sub-class (phosphotransferases with a nitrogenous group as acceptor) and the forth digit (2) designates creatine kinase.

1.03 Enzyme Units:

Activity of an enzyme is measured in terms of enzyme unit (IU) which is defined as the amount of enzyme which catalyses the transformation of 1 μ mole of the substrate per minute under standard assay conditions. The standard assay conditions refer to specified substrate concentration, temperature, pH, ionic strength and any other parameter which influences the activity. Recent enzyme unit is katal which is defined as the amount of enzyme which catalyses the transformation of one mole the substrate per second under standard assay conditions. One katal is equivalent to $6 \times 10^7$ IU. The purity of an enzyme preparation is expressed in terms of specific activity which is defined as the units of enzyme per unit mass. In terms of international enzyme unit, specific activity is defined as enzyme units per mg whereas in terms of katal, specific activity is defined as the number of katal per kg of the enzyme.

1.04 Purification and Characterization of Enzymes:

Though the applied aspects of enzymology could be studied using impure or partially pure enzymes, studies on the fundamental aspects of an enzyme need purification of the enzyme to homogeneity and its characterisation. A large number of enzymes have been purified and there is extensive literature on enzyme purification and characterization. The general steps involved in the purifications of enzymes are:
1.04(i) Making of an Extract:

If the enzyme is not present in a biological fluid, it has to be extracted using a suitable solvent. Sometimes cell disintegration may be required using ultrasonic disintegrator or other cell disintegrating devices.\textsuperscript{11}

1.04 (ii). Concentration of the Enzyme:

Generally enzymes are present in the form of dilute solution\textsuperscript{5} from which it needs to be concentrated before purification is attempted. Three methods have generally been used for concentrating enzymes from the initially diluted solutions of enzymes. The first one is precipitation of enzyme, using ammonium sulphate or other precipitating reagents\textsuperscript{11}. The second one is ultra filtration using membranes with suitable molecular weight cut off values. The ultra filtration membranes and the experimental devices are commercially available. The third is freeze drying. In addition to these, some other methods like removal of the solvent by sucrose have also been used.

1.04(iii) Fractionation on Column Chromatography:

The most commonly used fractionating columns are those packed with ion exchange materials or gel filtration columns. Most of the text\textsuperscript{11-14} books on enzyme purification have discussed the procedures involved in details and hence this point is not discussed here.
1.04(iv) Other Advanced Techniques\textsuperscript{11}: In order to cut short the time required for the purification of the enzymes, many companies have developed prepacked HPLC columns which in combination with HPLC equipment have made the purification procedures of enzymes quite convenient. The only difficulty with HPLC is its price and the maintenance which individual research worker can not afford.

A typical enzyme purification table is given below to illustrate the different steps involved in enzyme purification.
**Purification chart for acid phosphatase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (IU)</th>
<th>Specific activity (unit/mg)</th>
<th>Purification Yield (fold) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Crude extract</td>
<td>2165</td>
<td>1,70,400</td>
<td>18005</td>
<td>0.01050</td>
<td>1</td>
</tr>
<tr>
<td>(ii) Ethanol precipitate</td>
<td>1060</td>
<td>20,900</td>
<td>1340</td>
<td>0.06638</td>
<td>6</td>
</tr>
<tr>
<td>(iii) DEAE Sephadelx</td>
<td>123</td>
<td>1,784</td>
<td>706</td>
<td>0.396</td>
<td>38</td>
</tr>
<tr>
<td>(iv) Biogel-110</td>
<td>145</td>
<td>788</td>
<td>491</td>
<td>0.623</td>
<td>59</td>
</tr>
<tr>
<td>(v) DEAE Cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatase-I</td>
<td>85</td>
<td>94</td>
<td>171</td>
<td>1.83</td>
<td>174</td>
</tr>
<tr>
<td>Phosphatase-II</td>
<td>95</td>
<td>117</td>
<td>217</td>
<td>1.86</td>
<td>177</td>
</tr>
<tr>
<td>(vi) Hydroxyapatite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatase-I</td>
<td>32</td>
<td>10.0</td>
<td>99</td>
<td>8.97</td>
<td>855</td>
</tr>
<tr>
<td>Phosphatase-II</td>
<td>40</td>
<td>9.4</td>
<td>134</td>
<td>14.3</td>
<td>1360</td>
</tr>
</tbody>
</table>
SDS polyacrylamide gel electrophoresis\textsuperscript{16}, analytical gel filtration chromatography\textsuperscript{14} and isoelectric focusing\textsuperscript{17} in gels have been used for checking the homogeneity of enzyme preparations. The SDS polyacrylamide gel electrophoresis and analytical gel filtration are used for checking homogeneity in molecular weight whereas isoelectric focusing is used for checking the homogeneity of charge on enzyme molecules.

1.05 General Features of Structures of Enzymes:

Since most of the enzymes are proteins, the structures of enzymes are not different from the structures of proteins. Amino acids are the basic structural unit of protein\textsuperscript{18}. The carboxylic group of one amino acid combines with amino group of another amino acids to form a peptide bond. The geometry of peptide bond is shown in Figure 1.05.1 (a). The important features of the peptide bond are:

(i) It has a partial double bond character. The bond length is 1.31 Å which is between a C–N single bond (1.49 Å) and C= N double bond (1.27 Å). Due to partial double bond character, free rotation about peptide bond is not possible whereas rotations about C\textsubscript{\alpha}–C and C\textsubscript{\alpha}–N are possible and are represented by \(\psi\) and \(\phi\) respectively.

(ii) The carbonyl oxygen is almost always trans- to the hydrogen attached to peptide nitrogen.

(iii) Carbonyl carbon, carbonyl oxygen, peptide nitrogen, hydrogen attached
to peptide nitrogen and \( C_\alpha \)-carbons on both sides of peptide bond are in a plane.

Proteins are formed by repetition of the peptide bond but the essential features of the peptide bond mentioned above are maintained throughout the structures of proteins.

The determination of three dimensional structures of proteins has indicated following type of repetitive structural units\(^{18-20}\) which constitute the structures of proteins.

1.05.1 \( \alpha \)-Helix:

The \( \alpha \)-helix is a rod like structure. The tightly coiled polypeptide chain forms the inner part of the rod and the side chains extended outward in a helical array. The \( \alpha \)-helix is stabilised by hydrogen bonding between the N–H and C=O groups of the main chain. The C=O group of each amino acid residue is hydrogen bonded to N–H group that is situated four residues ahead in the linear sequence. In this way all main chain N–H and C=O groups are hydrogen bonded. Each residue is related to the next one by a translation of 1.5 Å along the helix axis and a rotation of 100°. This gives 3.6 amino acid residues per turn of the helix. The pitch of the \( \alpha \)-helix is 5.4 Å, the product of the translation (1.5 Å) and the number of residues per turn (3.6 Å). The screw sense of the helix can be right handed (clock-wise) or left handed (counter clock-wise).
The α-helix contents of proteins are highly variable. In some, the α-helix is major structural motif while others may be devoid of the α-helical structural unit. The α-helical structural unit is shown in Figure 1.05.2 (b).

1.05.2 β-Pleated Sheet:

A second type of commonly occurring structural unit is β-pleated sheet which is shown in Figure 1.05.3 (c). Sheets are formed when two or more almost fully extended polypeptide chains are brought together side by side so that regular hydrogen bonds can form between the polypeptide backbone amide N−H and carbonyl oxygen group of the adjacent chain. The β-pleated sheet can occur in two different arrangements. The chain arranged with the same N to C polypeptide sense are called parallel β-sheets, whereas the chains with opposite N to C sense are called antiparallel β-sheets. The β-pleated sheet differs from the α-helix in the sense that it is a sheet rather than a rod. A polypeptide chain in the β-pleated sheet is extended rather than being coiled as in the α-helix. The axial distance between adjacent amino acids is 3.5 Å in β-sheets in contrast with 1.5 Å in the α-helix. The β-pleated sheets are stabilised by hydrogen bonds between N−H and C=O groups in different polypeptide chains, whereas in the α-helix the hydrogen bonds are between N−H and C=O groups in the same polypeptide chain.

1.05.3 Collagen Helix:

A third periodic structural unit found in the proteins is collagen helix, which has triple strands bind around each other to form super
helical cable as shown in Figure 1.01(d). The rise per residue in this super helix is 2.9 Å and the number of residues per turn is nearly 3.3. The three strands are hydrogen bonding to each other. The hydrogen donors are the peptide N–H groups of glycine residues and the hydrogen acceptors are the peptide C=O groups of residues on the other chain. The direction of hydrogen bond is perpendicular to the long axis of the rod. The hydroxyl groups of hydroxy proline residues and the bridging water molecules also participate in hydrogen bonding, which stabilises the triple helix.

1.05.4 β–Bends:

In order to fold a polypeptide chain to a compact globular form, there must be some way to change the direction of the polypeptide chain. Analysis of the three dimensional structures of numerous proteins have revealed that many of these chains reversals are accomplished by a common structural element called β-bend. The essence of this turn is that the C=O group of residues (n) of a polypeptide is hydrogen bonded to the N–H group of the residues (n +3). The structure of β-bend is shown in Figure 1.05.4 (e). β-Bends are also called β-turns, reverse turns or hair-pin bends.

Examination of a large number of known three dimensional protein structures has shown that the various amino acids have tendencies to form different structural unit i.e. glutamic acid, methionine and alanine seems to be the strongest α- helix former and valine, isoleucine and tyrosine are most probable β-sheet formers. Proline, glycine, asparagine, aspartic acid and serine are most probable β-bend formers.
Fig. 1.05.1 — Structural elements of proteins

(a) Geometry of peptide bond,
(b) Geometry of $\alpha$-helix.
Fig. 1.05.2-Continued…(c) ß-sheets
Single polypeptide chain of tropocollagen

Three stranded tropocollagen molecule (2.80 \times 1.4 \text{ nm})

Fig 1.05.3-4 Continud... (d) Collegen helix$^{21}$
1.05.5 Levels of Structures of Proteins:

There are four levels of structures of proteins. The simple sequence of amino acid residues and the location of disulfide bonds are termed the primary structure of protein. It is worth mentioning that the sequence of amino acid residues determines the three dimensional structure of a protein. The secondary structure refers to the special arrangement of amino acid residues that are near to one another in the linear sequence. The α-helix, β-pleated sheet, collagen helix and β-bend are elements of secondary structure. The tertiary structure refers to the spatial arrangement of amino acid residues that are far apart in the linear sequence. Proteins containing more than one polypeptide chains have an additional level of structural organisation. Each polypeptide chain is called a subunit. The term quarternary structure is used to represent the spatial arrangement of such subunits with respect to each other. In addition to the above mentioned four levels of structures, recent studies on protein conformation has revealed two additional levels of structures. The supersecondary structure refers to the clusters of secondary structure. For example, a β-strand separated from another β-strand by an α-helix is found in many proteins, this motif is called a βαβ unit. The supersecondary structure is regarded as intermediate between the secondary structure and tertiary structure.

Some polypeptide chains fold into two or more compact regions that may be joined by a flexible segment of polypeptide chain. Such compact regions are called domains.
1.06 The Conformation of a Polypeptidechain:

The conformation of a polypeptide chain is defined by the location of three sets of atoms that are linked together namely, the α-carbon (cα), carboxyl carbon (c1), and amide nitrogen atoms. Their positions can be defined by the angles of rotation about the bonds connecting the three atoms; ϕ, ψ, and ω as shown in the fig. (fig.1.05.1). This figure illustrates a fully extended segment of a chain with a residue I and parts of the following residue i+1 and preceding residue i-1. It may be noted that stearic clashes restrict the number of conformations available to the polypeptide chains. The conformational space that is accessible is represented in a two dimensional plot of ϕ against ψ, known as the Ramachandran diagram named after its inventor, Prof. G. N. Ramachandran18.

1.07 Three dimensional structures of protein:

X-ray crystallography has been the single most important technique in the investigation of the structures of enzymes and it has provided the experimental basis of our present knowledge of the structures of enzymes. The technique requires single crystals of enzyme molecules. During the recent years techniques for obtaining protein single crystals have developed to a stage where more and more enzyme molecules are getting crystallized in the form of single protein crystals and more and more structures of enzymes are being reported.
1.08 The Active Site of an Enzyme:

The enzyme catalysis takes place in a small portion of the enzyme molecule. The portion of enzyme molecule where chemical catalysis takes place is called active site of the enzyme\(^1,3\). The active site of the enzyme is a intricate three dimensional structure, where amino acid residues involved in chemical catalysis are arranged in a particular way to facilitate the enzyme catalysis\(^1\). The active site of an enzyme occupies relatively small volume compared to the total volume of the molecule\(^1\). One of the objectives of the determination of the three dimensional structures of the enzymes is to locate the active sites of the enzymes.

1.09 Co-enzyme and Co-factors:

Some enzymes require the presence of one or more non-protein components which are called co-factors\(^21,22\). The cofactor may be metal ion or an organic molecule called a co-enzyme. The catalytically active enzyme co-factor complex is called holoenzyme. When the co-factor is removed from the protein, the remaining protein is inactive and is called an apoenzyme. The enzymes containing metal ions bound to it are called metalloenzymes.

1.10 Enzyme Kinetics:

There are two aspects of enzyme kinetics\(^23-28\).
110.1 The Presteady State Enzyme Kinetics or Transient State Kinetics:

When a solution of enzyme is mixed with substrate solution, enzyme molecules may go through the formation of one or more enzyme intermediates on a fast time scale. This initial phase of enzyme kinetics is called presteady state enzyme kinetics. The continuous flow method\textsuperscript{26}, stopped flow method\textsuperscript{26} and the rapid quenching techniques\textsuperscript{26} have been used for studying presteady state kinetics of enzymes. Since presteady state kinetics has not been used in the research work presented in this thesis, detailed description of the presteady state enzyme kinetics has not been given here.

1.10.2 Steady State Enzyme Kinetics:

It is this aspect of the enzyme kinetics, which has been extensively studied\textsuperscript{28}. The steady state kinetics\textsuperscript{26} of enzymes refers to the steady state rate of formation of the product after the transient phase of enzyme kinetics is over. A simple mathematical treatment of the steady state velocity of the one-substrate enzyme reaction scheme as shown below was given by Michaelis and Menten\textsuperscript{29}.

\[
E + S \xrightleftharpoons[k_1]{k_2} E + P
\]

The Michaelis-Menten treatment is based on following assumptions:
(i) The concentration of the enzyme is very small in comparison to the concentration of the substrate \([S]\) so that the concentration \([S]\) does not deplete significantly as a result of enzyme catalysis during the initial phase. Generally the substrate \([S]\) is taken in \(m\) \(M\) range, whereas the enzyme concentration is in \(\mu\) \(M\) or \(n\) \(M\) range.

(ii) Initially the concentration of the product is effectively zero so that the reverse reaction is insignificant.

(iii) The enzyme substrate complex \([ES]\) is in equilibrium with the free enzyme concentration \([E]\) and the substrate concentration \([S]\).

However, Briggs and Haldane\(^30\) have shown that the equilibrium assumption is not that essential and even the steady state with respect to the enzyme substrate complex \([ES]\) leads to the same mathematical expression for the steady state velocity of the enzyme catalysed reaction. Since the steady state approximation has wider application, it has been widely used for deriving the steady state velocity of the enzyme catalysed reaction\(^28\).

The final expression for the steady state velocity of an enzyme catalysed reaction is given by

\[
V = \frac{V_{\text{max}} [S]}{K_m + [S]} \quad \text{(1.10.2)}
\]

where \(v\) is steady state velocity and \([S]\) is substrate concentration.
\[ \text{V}_{\text{max}} = K_2 [E]_0 \]  
\[ (1.10.3) \]

where \([E]_0\) is total enzyme concentration. \(K_m\) is called Michaelis constant and is given by

\[ K_m = \frac{k_1 + k_2}{k_1} \]  
\[ (1.10.4) \]

It follows from equation (1.08.2) that

(i) When the substrate concentration \([S]\) is small in comparison to \(K_m\)

\[ v = \frac{V_{\text{max}} [S]}{K_m} \]  
\[ (1.10.5) \]

which suggests that the steady state velocity of the enzyme catalysed reaction is directly proportional to the substrate concentration \([S]\) at the low substrate concentration.

(ii) When the substrate concentration \([S]\) is much greater than \(K_m\)

\[ v = V_{\text{max}} \]  
\[ (1.10.6) \]

Thus, at the higher substrate concentrations \([S]\), the steady state velocity of the enzyme catalysed reaction is independent of the substrate concentration \([S]\).

(iii) When \(K_m = [S]\)

\[ v = \frac{V_{\text{max}}}{2} \]  
\[ (1.10.7) \]
Thus, when $[S]$ is equal to $K_m$, the steady state velocity is half of its maximum value.

1.10.3 Determination of $K_m$ and $V_{max}$:

Taking the reciprocal of the both sides of equation 1.08.2

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1.10.8)$$

It is obvious from equation (1.08.8) that the plot of $1/v$ versus $1/[S]$ will give a straight line with an intercept of $1/V_{max}$ and a slope of $K_m/V_{max}$. The reciprocal of the intercept gives $V_{max}$ and division of the slope by the intercept gives $K_m$. The plot of $1/v$ versus $1/[S]$ is called a Lineweaver-Burk plot or double reciprocal plot and has widely been used for the determination of $K_m$ and $k_{cat}$.

1.10.4 Physical Significance of $K_m$:

It has already been shown in equation (1.08.7) that when $K_m = [S]$, the steady state velocity $v = V_{max}/2$ indicating that $K_m$ is equivalent to that substrate concentration at which half of the active sites of the enzyme are occupied by the substrate.

If $k_1$ is much greater than $k_2$, it follows from equation (1.08.4) that

$$K_m = \frac{k_{-1}}{k_1} \quad (1.10.9)$$
Since $k_{-1}/k_1$ is equal to the equilibrium constant for the dissociation of ES complex,

$$K_m = K_{ES} \quad \text{(1.10.10)}$$

In other words $K_m$ is equal to dissociation constant of the ES complex if $k_2$ is much smaller than $k_{-1}$. When the condition $k_{-1} > k_2$ is met; $K_m$ is the measure of the strength of binding in the ES complex. The high $K_m$ value indicates weak binding of the substrate to the enzyme, whereas the low $K_m$ value indicates strong binding.

1.10.5 Physical Significance of $V_{\text{max}}$:

It is obvious from equation (1.08.3) that if the value of $V_{\text{max}}$ is known, the catalytic rate constant $k_2$ can be determined by substituting the value of the enzyme concentration $[E]_0$. The catalytic rate constant is also called turnover number of an enzyme and is equal to the number of substrate molecules converted into products by an enzyme molecule in a unit time when the enzyme is fully saturated with the substrate $[S]$. Higher the value of $k_2$, more efficient is the enzyme.

1.10.6 Kinetic Perfection in Enzymatic Catalysis:

It follows from equation (1.08.2) that if the substrate concentration $[S] \ll K_m$, the steady state velocity $v$ of the enzyme catalysed reaction is given by

$$v = \frac{k_2}{K_m} \frac{[E]}{[S]} \quad \text{(1.10.11)}$$
When \([S] \ll K_m\), the concentration of the free enzyme \([E]\) is nearly equal to the total concentration of enzyme \([E]_0\) and equation (1.08.11) reduces to

\[
v = \frac{k_2}{K_m} [S] [E]_0 \quad \text{(1.10.12)}
\]

Thus, when \([S] \ll K_m\), the enzymatic velocity depends on the value of \(k_2/K_m\) and \([S]\). Equation (1.08.12) is also valid for enzymes having more complex reaction pathways than the scheme shown in equation (1.08.1). Their maximal catalytic rate denoted by \(k_{cat}\) depends on several rate constants, rather than on \(k_2\) alone. The general form of equation (1.08.12) can be written as

\[
v = \frac{k_{cat}}{K_m} [S] [E]_0 \quad \text{(1.10.13)}
\]

The value of \(k_{cat}/K_m\) for the scheme (1.08.1) is given by

\[
\frac{k_{cat}}{K_m} = \frac{k_2 k_1}{k_1 + k_2} < k_1 \quad \text{(1.10.14)}
\]

The \(k_{cat}/K_m\) is an apparent second order rate constant that refers to the properties and reactions of the free enzyme and the free substrate. The \(k_{cat}/K_m\) is also called specificity constant because discrimination between two competing substrates is determined by the ratio \(k_{cat}/K_m\) and not by the value of \(K_m\) alone. The enzymes for which \(k_{cat}/K_m\) values are of the order of the second order rate constant for the diffusion controlled reactions i.e. \(10^8-10^9\) L Mole\(^{-1}\)s\(^{-1}\) are considered as evolved to the kinetic perfection.
1.10.7 Multi-Substrate Enzyme Kinetics:

The kinetic equations derived so far are based on the enzyme that reacts with a single substrate. However, many of the principles developed for the single substrate enzyme system may be extended to multi substrate systems. The general solution of the equation for such system is complicated and is not discussed extensively here. The steady state kinetics of such system is thoroughly discussed by Cornish–Bouden\textsuperscript{23}, Segel\textsuperscript{24}, Fromn\textsuperscript{31}, Wong\textsuperscript{32}, Cleland\textsuperscript{33} and Dalziel\textsuperscript{34}.

1.10.8 Graphical Representation of the Steady State Kinetic Data:

It has already been shown that Michaelis–Menten equation (1.08.2) can be transformed into a linear form (1.08.7) for analysing the data graphically and for determining the value of $K_m$ and $V_{\text{max}}$. The plot of $1/[v]$ vs $1/[S]$ is a straight line for an enzyme obeying Michaelis–Menten equation. Such plots have commonly been used in the steady state kinetics and are termed Lineweaver–Burk plots\textsuperscript{35} or double reciprocal plots. The disadvantage of this plotting procedure is that it compresses the data points at higher substrate concentration into a small region and emphasises the points at lower concentrations. It has the advantage that it is easy to read the values of $[v]$ against $[S]$.

Another common plotting procedure is by Eadie and Hofste\textsuperscript{36,37} which is based on the equation,

$$v = V_{\text{max}} - K_m \frac{v}{[S]} \quad \text{(1.10.15)}$$
The Eadie–Hofstee plot does not compress the data points corresponding to the higher substrate concentrations and is considered more accurate and superior to the Line weaver–Burk plot. However, it suffers from the disadvantage that the values of $v$ cannot be determined rapidly at a given value of $[S]$. Lineweaver–Burk plots are still most commonly used plots in the steady state kinetic analysis of the enzyme.

1.10.9 Deviations from Michaelis–Menten Kinetics:

Though most of the enzymes obey Michaelis–Menten kinetics in a limited range of substrate concentration, deviations from Michaelis–Menten kinetics in the extended range of the substrate concentration is a rule rather than exception. The Lineweaver–Burk plots or Eadie-Hofstee plots done in the extended range of substrate concentrations generally exhibit non-linearity. The non-linear steady state enzyme kinetics has been analysed in case of many enzymes by Bradsley and Coworkers. There are number of factors which lead to non-linear enzyme kinetics. If the substrates of the enzyme binds to the enzyme at a site, other than the one at which products are released, the substrate may modify the activity of the enzyme and lead to non-linearity in Lineweaver–Burk plots or Eadie–Hofstee plots. For the detailed analysis of non-linear kinetics one is referred to the research work published by Bardsley and Coworkers.

1.11 Enzyme Inhibitors:

The rate of the enzyme catalysed reaction may be altered in a specific manner by compounds other than the substrates. Such molecules, which
decrease the rate of enzyme catalysed reaction, are called inhibitors. The studies on the effect of such agent are of practical importance due to following reasons:

(i) Inhibition and activation of the enzymes by key metabolites provide the normal means of the metabolic fine control superimposed on the coarse control achieved by the regulation of the synthesis and the breakdown of the active enzyme.

(ii) External interference with metabolism by drugs, pesticides etc. often depends on the inhibition of the enzyme.

(iii) Inhibitors and inactivators provide a powerful tool for the studying the chemical mechanisms of the enzyme action.

1.11. (A) Irreversible Inhibitors:

Some agents once bound to the enzyme can not be removed from the enzyme molecules. The inhibitions caused by such agents are called irreversible inhibition and the agents are called irreversible inhibitors. For example, Iodo-acetate binds to glyceraldehyde-3-phosphate dehydrogenase and inhibits its activity irreversibly.

1.11. (B) Reversible Inhibitors:

Some agents bind to enzyme molecules under one condition and inhibit the activity of the enzyme. These agents can dissociate from the enzyme under different conditions and the enzyme gains its full activity. Such inhibitors are called reversible inhibitors. For example Malonate ion
binds to the succinate dehydrogenase and decreases its activity but it can be removed from succinate dehydrogenase by dialysis and the enzyme gains its full activity.

Generally there are three types of reversible inhibitors:

(i) Competitive Inhibitor:

These inhibitors bind to the free enzyme but not to the enzyme substrate complex. The competitive inhibition is schematically shown below.

\[
\begin{align*}
E + S & \xrightleftharpoons[k_{-1}]{k_1} E + S \\
I & \xrightleftharpoons[K_I]{K_I} E + P
\end{align*}
\]

(1.11.1)

The expression for the steady state velocity of the enzyme catalysed reaction in presence of competitive inhibitors is given by

\[
v = \frac{V_{\text{max}} [S]}{[S] + K_m (1 + \frac{[I]}{K_I})}
\]

(1.11.2)

where, \([I]\) is the concentration of the inhibitor and \(K_I\) is the dissociation constant for the enzyme inhibitor complex \([EI]\). The Lineweaver-Burk plots in presence of different concentrations of competitive inhibitors are shown in Fig. 1.11 (a).
The characteristic feature of a competitive inhibitor is that, it does not change the value of $V_{\text{max}}$, hence the intercept on Y-axis is not effected by the presence of inhibitor where as the slope of the double reciprocal plot is changed.

(ii) Uncompetitive Inhibitor:

The second type of a reversible inhibitor is uncompetitive inhibitor. The uncompetitive inhibitor binds to only the enzyme substrate complex $[ES]$. The binding of an uncompetitive inhibitor is schematically shown below.

\[ E + S \rightarrow E \cdot S \leftarrow E + P \quad (1.11.3) \]

\[ I \quad K_1 \]

\[ ESI \]

The expression for the steady state velocity of enzyme catalysed reaction in the presence of the uncompetitive inhibitor is given by

\[
v = \frac{V_{\text{max}} [S]}{[S] + K_m} \left[ 1 + \frac{[I]}{K_1} \right] \quad (1.11.4)
\]

where, $[I]$ is the concentration of the inhibitor and $K_1$ is the dissociation constant for the enzyme inhibitor complex. The Lineweaver-Burk plots in
the presence of different concentrations of uncompetitive inhibitors are shown in Fig- 1.11 (b).

The characteristic feature of an uncompetitive inhibitor is that, it affects $V_{\text{max}}$ and $K_m$ by the same factor giving a family of parallel lines for different values of the concentrations of [I].

(iii) Non-Competitive Inhibitor:

The third type of reversible inhibitor is the non-competitive inhibitor, which can bind to both the free enzyme [E] and the enzyme substrate complex [ES]. The schematic diagram of a simple non-competitive inhibition, in which the dissociation constant for enzyme inhibitor complex and substrate bound enzyme inhibitor complex are same, is shown below.

\[
\begin{align*}
E + S & \rightleftharpoons E S \\
& \rightleftharpoons E + P \\
I & \rightleftharpoons EI \\
& \rightleftharpoons ESI
\end{align*}
\]

The expression for the steady state velocity of the enzyme catalysed reaction in the presence of a non-competitive inhibitor is given below,

\[
v = \frac{V_{\text{max}} [S]}{1 + \frac{[I]}{K_I}} = \frac{[S]}{1 + \frac{[S]}{K_m}}
\]
where [I] is the concentration of the inhibitor and $K_i$ is the dissociation constant for the enzyme-inhibitor complex. The Lineweaver-Burk plots in presence of different concentrations of the non-competitive inhibitor are shown in Fig. 1.11(c).

The characteristic feature of a non-competitive inhibitor is that it affects both the slope and the intercept in the Lineweaver-Burk plot.

**1.11(C) Determination of Inhibition Constant ($K_i$):**

The standard procedure for determining the inhibition constant ($K_i$), from the steady state kinetic data is to plot $1/v$ vs $1/[S]$ at several inhibitor concentrations and to do a secondary plot of the slope or intercept against the concentrations of [I] and to evaluate the value of $K_i$ from the secondary plot. For example, in case of the competitive inhibition, a plot of the slope against [I] should give a straight line with a negative abscissa intercept equal to $K_i$. It is shown in the following equation:

$$Slope = (Uninhibited \ slope) - (1 + [I] / K_i)$$

The plot is shown in following Fig. 1.11 (d).
Fig.1.11 Lineweaver-Burk plots in presence of reversible inhibitors\textsuperscript{28}.

(a) Competitive inhibitor; (b) Uncompetitive inhibitor;
(c) Non-competitive inhibitor; (d) Determination of $K_I$ for Competitive inhibition.
1.12 Theories of Enzyme Catalysis:

For the detailed discussion on the theory of enzyme catalysis, one is referred to the recent work by Fersht and coworkers. However, a brief description of the theory is attempted here.

Ever since Haldane suggested that the binding energies of substrates may be used to distort the substrates to the structures of the products, theoreticians have explored the various ways in which the binding energy of the enzyme and the substrate may be used to lower the activation energy of the chemical steps.

The transition state theory is particularly useful in analysing the theories of enzyme catalysis. This approach is applied to a single substrate enzyme following Michaelis–Menten mechanism. It can be seen that binding energy automatically lowers the activation energy of $k_{cat}/K_m$ and some of the binding energy may be used to lower the activation energy of $k_{cat}$.

1.13 Role of Molecular Biology in the Development of Enzymology

The recent developments in the molecular biology have revolutionized the studies aimed at understanding the structural functional relationship of the enzyme molecules. The genes of the enzymes could be isolated, cloned in suitable vectors and sequenced, over expressed in suitable vectors and large amounts of enzymes could be produced. This has facilitated the structural and mechanistic studies on enzymes which was not possible in many cases due to the insufficient amounts of enzymes present in the natural sources.
The native enzyme could be crystallized in the form of single protein crystals and the three dimensional structure of the enzyme could be determined. The different steps involved in the enzyme catalysis by the enzyme could be identified using fast reaction kinetic techniques, their rate constants could be determined and a mechanism for the catalysis by the enzyme could be proposed. The detailed structural mechanistic relationship could be studied by the combined efforts of three groups of research workers—one generating mutant enzyme molecules using site directed mutagenesic techniques, the second group determining the structures of mutant molecules by X-rays crystallography and by other techniques and third group analyzing the kinetics and mechanism of the mutant enzyme molecules. Detailed structural functional relationship in cases of a number of enzymes has been obtained by such collaborative attempts.

The design of the new catalytic activities or binding site by systematic changes of single amino acid requires a lot of scientific efforts and is difficult to achieve. However, the development of directed evolutions of proteins has provided a method for improving the catalytic efficiency of the enzymes which is currently being used for improving the catalytic efficiencies of the enzymes.

1.14 Single Molecule Enzymology:
1.14.1 Movies of Molecular Motion and Chemical Reactions of Single Molecules:

In recent years, rapid advances in the patch clamp technique, atomic force microscopy, optical tweezers and fluorescence microscopy have permitted making single-molecule "movies" in situ at the millisecond to second time scale. Unlike molecular dynamics simulation, these techniques have low time resolutions, but their single-molecule sensitivities allow probing of slow conformational motion, which are otherwise masked in ensemble-average experiments. Moreover, chemical reactions can now be observed on a single-molecule basis. For example, enzymic turnovers of a few motor proteins, a nuclease and a flavoenzyme have been monitored optically in real time.

Our knowledge of enzyme kinetics has come primarily from experiment conducted on large ensembles of enzyme molecules, in which concentration changes over time are measured. In a single molecule experiment, the concentration of the molecule being studied becomes meaningless in discussing chemical kinetics. However, this does not negate the fundamental principles of chemical kinetics. The chemical kinetics can be cast in terms of single molecule probabilities. Thinking of the chemical kinetics in the terms of a single molecule is not only pertinent to the ever increasing single-molecule studies but is also insightful and very often more informative.

Such "single-molecule" thinking is also useful in understanding the chemistry in the living cells. In a living cell, the number of enzyme
molecules in a cellular component may not be large. Under this situation, the concentration in a small probe volume is no longer a constant but a fluctuating quantity, as molecule react or diffuse in and out of the probe volume. In fact, the reaction rate (and diffusion rate) can be extracted from the analyses of the concentration fluctuation. This approach is referred to as fluctuation correlation spectroscopy and has been recently conducted with single-molecule sensitivity. A typical fluctuation correlation spectroscopy trace, however, is averaged from a large number of molecules diffusing one or a few at a time in and out of a fixed probe volume. There are situations in which we need to focus on the behavior of a single molecule. For example, DNA exists as a “single molecule” inside a bacterial cell. The trajectory of a DNA-enzyme complex can be tracked. In another example, a single receptor protein at a particular spot in a membrane can be interrogated by optical or scanning probe microscopy. Studies in a similar line have been extensively carried out on ion channel proteins with the patch clamp technique.

1.14.2 Single-molecule Real-time Studies:

The single molecule experiments allow direct biochemical reactions. Single-molecule spectroscopy is capable of capturing reaction intermediates.

The single-molecule experiments allow determination of static and dynamic disorder. Static disorder is the stationary heterogeneity of a property within a large ensemble of molecules. Dynamic disorder is the time-dependent fluctuation of the property of an individual molecule.
Distributions of molecular properties of an ensemble are usually broad because of the both static and dynamic disorders. The distribution is difficult to determine by ensemble-average measurement. The ensemble-average measurements can not distinguish between static and dynamic disorders.

1.14.3. Single-molecule Enzymatic Assay:

A recent single-molecule enzymatic assay by Xue and Yeung\textsuperscript{70} has revealed static disorder in enzymatic turnover rates of genetically identical and electrophoretically pure enzyme molecules. In a capillary tube containing a solution of highly diluted enzyme molecules (lactate dehydrogenase) and concentrated substrate molecules (lactate and NAD\textsuperscript{+}), each enzyme molecule a discrete zone of thousands of NADH molecules after 1 h of incubation. The zones were then eluted by capillary electrophoresis and monitored by natural fluorescence of NADH. The enzyme molecule had a broad and asymmetrical distribution of activity, which was otherwise masked by ensemble-average measurements. The heterogeneity was found to be static at the time scale because the same enzyme molecule produces the same zone intensity after another incubation period. The microscopic origin of the static disorder observed in an interesting subject that deserves future research.

Such experiments are capable of determining static disorder but not dynamic disorder. Dynamic disorder in enzymatic turnover rates has been observed by real time single molecule experiments, as is discussed below.
1.14.4 Viewing Single-molecule Enzymatic Reactions by Fluorescence:

Consider the example of cholesterol oxidase, a 53kDa flavoprotein that catalyses the oxidation of cholesterol by oxygen. The active site of an enzyme(E) involves a FAD, which is naturally fluorescent in its oxidized form but not in its reduced form. The FAD is reduced by a cholesterol molecule to FADH₂ and is oxidized by molecular oxygen. As shown in Fig. (given below) 1.05, fluorescence turns on and off as the redox state of the FAD toggles between the oxidized and reduced states. Each on-off cycle corresponds to an enzymatic turnover.

![Diagram of cholesterol oxidase reaction]

*Fig. 1. Enzymatic cycle of cholesterol oxidase and real-time observation of enzymatic turnovers of a single cholesterol oxidase molecule. Each on-off cycle in the emission intensity trajectory corresponds to an enzymatic turnover. *ct*, count; *ch*, channel.*
The turnover trajectory contains detailed information about the chemical dynamics.

The single-molecule fluorescence measurements are carried out with an inverted fluorescence microscope, as described elsewhere. It is desirable to study immobilized molecules to avoid the complications of the diffusion process. The samples are thin film agarose gel of 99% water. The single-enzyme molecules are confined in the gel with no noticeable translational diffusion. In contrast, small substrate molecules still diffuse freely. Though confined in the polymer matrix, the enzyme molecules freely rotate within the gel, which was evidence by a polarization modulation experiment, as previously described. This means that the enzyme molecules do not bind to the polymer matrix. Control experiments were done to ensure the conventional enzymatic assays gave the same results in gel in solution. Polyacrylamide gel, which has small size, has also been used to confine proteins, such as green fluorescence proteins (GEP).

The turnover trajectories contain detailed dynamic information, which is extractable from statistical analyses. Good statistical analyses require long trajectories. The lengths of the trajectories are limited by photobleaching through photochemistry on the excited state. Better photostability for the FAD chromophore in protein than for dye molecules; has been observed, most likely because of the protection by the protein. Trajectories with more than 500 turnovers and $2 \times 10^6$ detected photons (detection efficiency, 10%) have been recorded. The analysis of the single molecule trajectories has revealed the validity of chemical kinetics.
particularly the Michelis-Menten mechanism for the averaged behaviours of many turn overs of a single molecule but it does not provide an accurate picture of the real time behaviour of a single molecule. On a single molecule basis, the rate for the activation step is fluctuating and this is not a small effect.

By studying individual molecules, it has been found that each enzyme molecule is different in its catalytic efficiency and catalytic efficiency changes slowly with time. The catalytic constants for different molecules differ as much as factor 4. There are many possible explanations but the most likely is that enzyme can exist in different conformations that interchange slowly. Whatever may be the explanation, single molecule real time enzymology will allow investigation of molecular and cellular dynamics at a level of great detail.

1.15 Forces Involved in Enzyme-Substrate Interaction:

Four fundamental non-covalent interactions are involved in the substrate enzyme interaction. These are electrostatic interactions, hydrogen bond, van der Waals forces and hydrophobic interactions\(^1\).

(a) Electrostatic Interaction:

A charged group on a substrate can attract an effectively charged group on enzyme. The force of such an electrostatic attraction is given by Coulomb’s law,

\[
F = \frac{q_1 q_2}{D r^2}
\]
in which $q_1$ and $q_2$ are the charges of the two groups, $r$ is the distance between them and $D$ is the dielectric constant of the medium. This kind of attraction is also called ionic bond, salt-linkage, and salt-bridge or ion-pair. The distance between positively charge groups in an optimal electrostatic attraction is 2.8 Å. The attraction is strongest in a vacuum where $D$ is 1 and weakest in the medium, such as water where $D$ is 80.

(b) **Hydrogen Bonding:**

Hydrogen bonding is another weak interaction, which plays important role in enzyme-substrate interaction. The bond energies range form 3 to 7 Kcal/mol. Hydrogen bonds are stronger than van der Waals bonds but much weaker than covalent bonds. The length of a hydrogen bond is intermediate between that of a covalent bond and van der Waals bond. An important feature of hydrogen bonds is that they are highly directional. The strongest hydrogen bonds are those in which the donor hydrogen and acceptor atoms are collinear. The $\alpha$-helix, a recurring motif in proteins, is stabilized by hydrogen bonds between amide (\(>\text{N–H}\)) and carbonyl (\(>\text{C=O}\)) groups.

![Figure 1-8](image_url)

*Figure 1-8*  
Schematic diagram of hydrogen bonding between an amide and a carbonyl group in an $\alpha$-helix of a protein.
(c) **van der Waals Interaction:**

This is non-specific attractive force and comes into play when only two atoms are 3 to 4 Å apart. The van der Waals bond energy of a pair of atoms is about 1 Kcal/mol. Though weaker and less specific than electrostatic and hydrogen bonds van der Waals interactions are equally important in substrate enzyme interaction.

(d) **Hydrophobic Interaction:**

Non-polar molecules or groups tend to cluster together in water. This puts some order Fig. 1.15 in the water molecules in immediate vicinity of non-polar molecules. If the size of the non-polar cluster increases, the orderliness of the water molecules in immediate vicinity of the non-polar molecules decreases. This is the origin of hydrophobic interactions and it plays very important role in protein structures and functions.

**References:**


