1.1.4 Factors Influencing Enzyme Activity

Various factors influence the activity of enzymes. These factors include substrate concentration, reaction temperature, pH of the buffer or reaction medium, oxidation, and radiation exposure. The effect of these factors on the rate of enzyme catalyzed reaction is discussed below.

Effect of substrate concentration

The effect of substrate concentration on enzyme activity can be described by plotting velocity (V) against substrate concentration [s], as shown in Fig. 1.1. The plot is a rectangular hyperbola with two distinct regions: (a) linear region, and (b) plateau region.

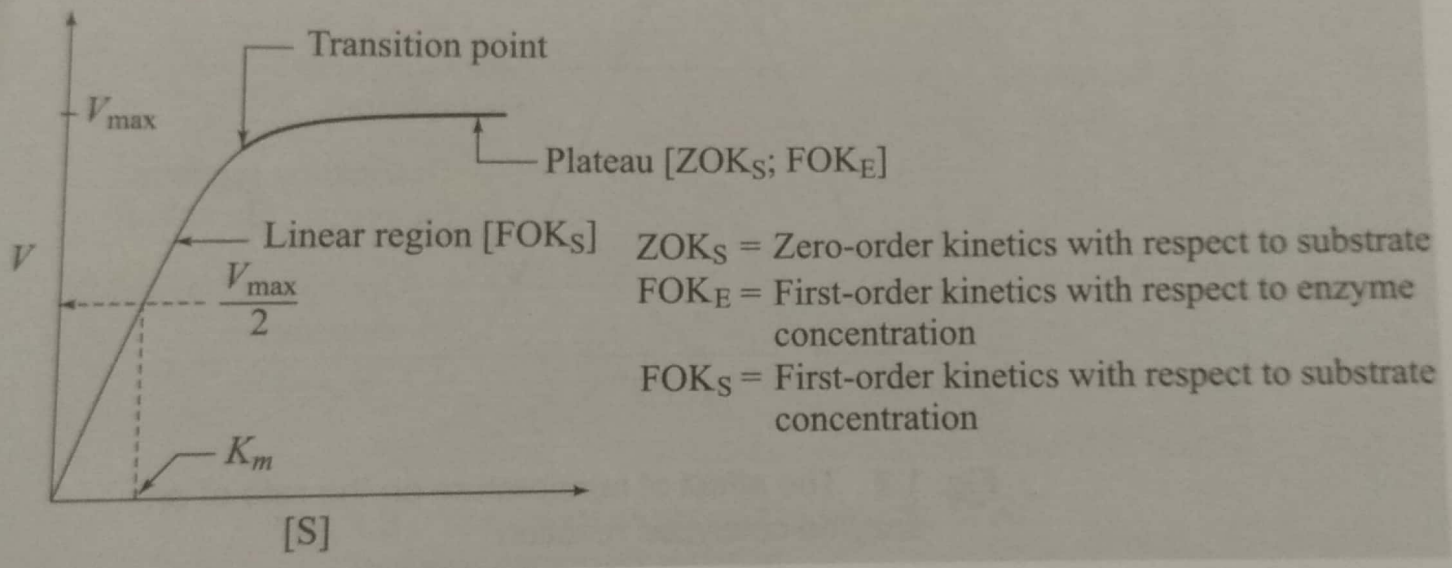


Fig. 1.1 Effect of substrate concentration on enzyme activity

Linear region At the initial stage of the reaction the velocity of the enzyme catalyzed reaction increases *linearly* with an increase in substrate concentration (shown as the linear region). This is also termed initial velocity of the reaction. At this stage, the substrate concentration is low and the reaction follows *first order* kinetics with respect to substrate concentration. Generally, it is assumed that the velocity of the enzyme-catalyzed reaction is directly proportional to the enzyme concentration in a reaction mixture.

Plateau region As the substrate concentration is raised gradually, the velocity ... a maximum (V_{max}). After V_{max} the velocity is *constant* even with an increase in ... concentration. This leads to a plateau region. At this region, the reaction rate ... *zero-order* kinetics with respect to substrate, but *first order* kinetics with respect ... zyme concentration.

The point in the graph, where the linear plot is *transformed* into a plateau is ... *the saturation point*. At this point the active site of the enzyme is completely ... with the substrate. Substrate concentration at which the rate is $V_{max}/2$, i.e. the rate ... of the maximal velocity is called k_m.

Effect of temperature

The effect of temperature on the rate of the enzyme catalyzed reaction is represent... plot of velocity versus temperature. This is shown in Fig. 1.2.
The plot is *bell shaped* with three regions:

- ascending segment,
- peak, and
- descending segment.

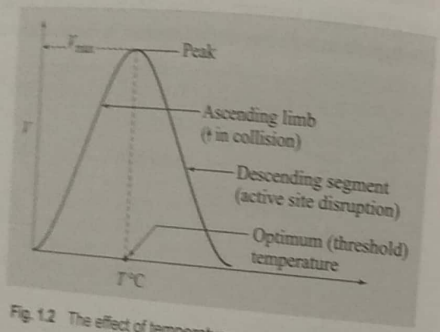


Fig. 1.2 The effect of temperature on the rate of an enzyme-catalyzed reaction

The rate of enzyme-catalyzed reaction increases with the initial rise in temperature in accordance with the *Arrhenius equation*:

$$K = A_e^{-\Delta G^*/RT}$$

where, K is the kinetic rate constant for the reaction,
A is the Arrhenius constant, also known as the frequency factor,
ΔG^* is the standard free energy of activation (kJ M^{-1}), which depends on entropic and enthalpic factors,
R is the gas law constant, and
T is the absolute temperature.

The initial rise in temperature increases the probability of effective *collision* between the reactive groups due to increase in kinetic energy, and hence the velocity increases *linearly* (shown as an ascending segment). The velocity "V" is enhanced for every 10°C is called *temperature coefficient*, denoted as Q_{10}. But after a certain limit of temperature, called the *optimum temperature*, the velocity reaches a *maximum* (peak), then decreases gradually (the descending segment). The descending segment represents that beyond the optimum temperature there is a fall in velocity. It may be due to covalent changes such as the deamination of asparagine residues, or noncovalent changes, such as the rearrangement of the protein chain, or inactivation by heat denaturation.

Effect of pH

The effect of pH on the velocity of an enzyme-catalyzed reaction is represented in Fig. 1.3.

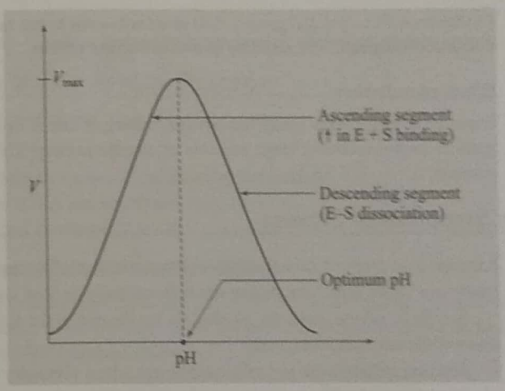


Fig. 1.3 The effect of pH on the velocity of an enzyme-catalyzed reaction

The *bell-shaped* curve of pH versus V is similar to that of T versus V. In the initial stage of the reaction the velocity increases with the increase in pH due to increase in enzyme-substrate *binding*. At a particular pH, called the *optimum pH* (usually 6–8) the velocity reaches a maximum. Beyond the optimum pH the 3-D structure of the enzyme is altered, leading to a *dissociation* of E–S complex and a fall in velocity.

As the optimum pH is between 6–8 most of the enzymes display a bell-shaped curve, but pepsin is an exception. The optimum pH of pepsin is 2 (which exists in acidic gastric juice for digesting proteins), and the curve, is shown in Fig. 1.4.

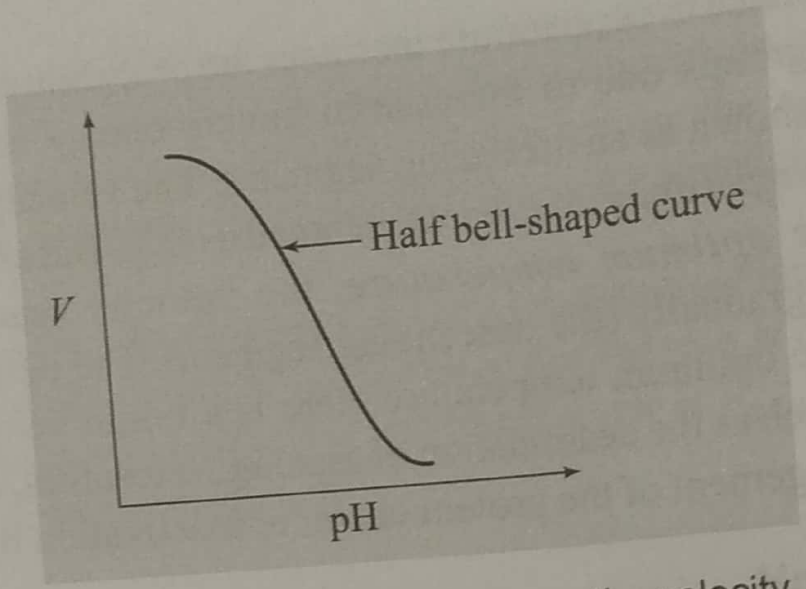


Fig. 1.4 The effect of pH on the velocity of pepsin

Effect of oxidation

Oxidation of the sulphydryl group (–SH) in the active site by the oxidizing agent leads to disulphide bridging (S–S), resulting in loss of enzyme activity.

Effect of radiation

Exposure to high energy (short wavelength) radiations like X-rays, β-rays and γ-rays, leads to *conformational change* and loss of enzyme activity. UV rays also *inactivate* enzymes.

Structure of Enzymes

2

MECHANISM OF ENZYME ACTION

Daniel Koshland Jr. proposed the Induced Fit Model of enzyme action.

LEARNING OBJECTIVES

After reading this chapter, the reader would be able to understand and appreciate the following:

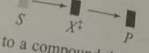
- Mechanistic enzymology and intermediary enzymology
- Transition state and activation energy
- Reaction coordinate diagram
- Strategies of enzyme action
- Multienzyme complex
- Mechanism of action and structure of some important enzymes

2.1 MECHANISTIC AND INTERMEDIARY ENZYMOLOGY

The study of interaction between the functional groups of enzymes with that of the substrate during catalysis is called *mechanistic enzymology*. The study of the enzyme-bound intermediate formed during catalysis is called *intermediary enzymology*.

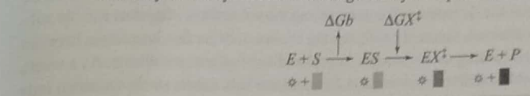
2.1.1 TRANSITION STATE AND ACTIVATION ENERGY

In biochemical reactions, the substrate (S) is converted to product (P) by passing through a high energy *transition state* ($X^{\ddagger}$).



Transition state refers to a compound that is intermediate in structure between the substrate and the product. The energy required by the substrate to form $X^{\ddagger}$ is called *activation energy* (also called *energy of activation* or *binding energy*). It is denoted as $\Delta G^{\ddagger}$. The energy of $X^{\ddagger}$ is greater than that of the substrate and the product. Due to translational and rotational motion, free substrates possess high entropy (Fig. 2.1).

An enzyme-catalyzed reaction can generally be represented as follows:



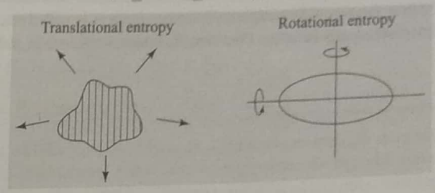


Fig. 2.1 Entropy of free substrate

2.1.2 ENZYMES ACT AS CATALYST BY LOWERING THE ACTIVATION ENERGY

Enzymes and substrates recognize each other through their structural complementarities (molecular recognition). The enzyme lures its specific substrate into its active site by weak interactions (hydrogen bonding, salt bridges, hydrophobic interaction, and Vander Waal's interaction) to form an enzyme substrate complex. This luring phenomenon is called the *Circe effect*. The free energy released during the weak interactions between enzyme and substrate is called *intrinsic binding energy* and it is denoted as ΔGb (Fig. 2.2).

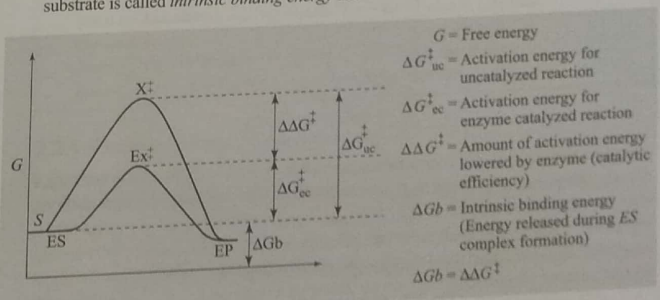


Fig. 2.2 A transition state diagram to show that enzymes catalyze the reaction by decreasing the activation energy

When the ES complex is formed, the entropy of substrate is lost. The entropy loss is denoted as $-T\Delta S$. Entropy loss generates mechanical strain. The active site of the

enzyme is properly oriented to bind tightly to the transition state than to the substrate itself. Therefore, the fit between the enzyme and the substrate is imperfect and the substrate is again strained. Strained conformation is necessary for fast interaction between reactive groups, leading to an increase in probability of effective collision. As a result, the strained conformation of substrate is distorted towards a more stable transition state $X^{\ddagger}$, thus forming $EX^{\ddagger}$ complex. This phenomenon is called *rack mechanism*. $S \rightarrow X^{\ddagger}$ distortion usually involves alterations in bonds, bond length, and bond angle.

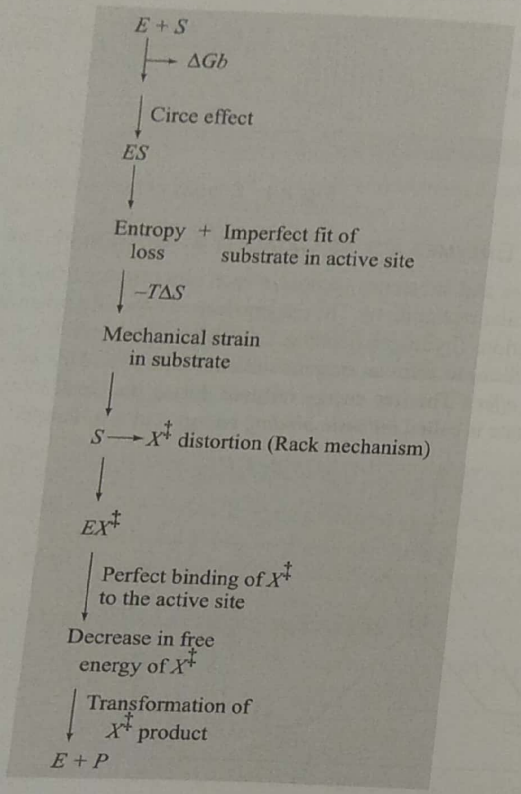


Fig. 2.3 Summary of the mechanism of enzyme action

The energy required by the substrate to form $X^{\ddagger}$ is called *activation energy* ($\Delta G^{\ddagger}$). also called *energy of activation* or *energy barrier* The dissociation constant for $EX^{\ddagger}$ is less than that of ES, i.e., $kX^{\ddagger} < kS$. The enzyme in $EX^{\ddagger}$ complex decreases the activation

energy and increases the rate by forming the product. *Enzymes act as catalysts by decreasing the activation energy.* The amount by which the activation energy is lowered by the enzyme is called *catalytic efficiency* and it is denoted as $\Delta\Delta G^{\ddagger}$. ΔGb acts as a major source of free energy for the enzyme to lower the activation energy. ΔGb is equal to the amount by which $\Delta G^{\ddagger}$ is lowered, i.e., $\Delta Gb = \Delta G^{\ddagger}$. This catalytic concept could be described by a *transition state diagram* (or reaction coordinate diagram), which is a plot of free energy (G) against the progress of the reaction (reaction coordinate) (Fig. 2.2). All the concepts discussed here are shown in Fig. 2.3.

2.2 Strategies of Enzyme Action

The catalytic efficiency of an enzyme is attributable to multiple mechanisms called *strategies*. Usually enzymes follow one or more of the six catalytic strategies, which include proximity effect (entropy reduction), preferential transition state binding, metal ion catalysis (electrophilic catalysis), covalent catalysis (nucleophilic catalysis), general acid basis catalysis, and concerted acid base catalysis. Table 2.1 provides an example for each strategy.

Table 2.1 Strategies of enzyme action with example

Mechanism/Strategy	Example
Proximity effect (entropy reduction)	Acetic anhydride synthase
Preferential transition state binding	Proline racemase
Metal ion catalysis	Carbonic anhydrase
Covalent catalysis	Chymotrypsin
General acid basis catalysis	Glucose mutase
Concerted acid base catalysis	RNAse

2.2.1 Proximity Effect

Formation of the transition state requires entropy reduction (ΔS). In bisubstrate reaction, ΔS tends to be low. In such cases, the catalysis is brought about by the *proximity effect*. It is also called as *propinquity effect* or *entropy reduction*. In this mechanism, the enzymes *abstract* the substrate from the dilute solution and bring them in proximity orientation (approximate orientation) in the active site. This allows bond polarization and increases the reaction rate. This phenomenon by which the enzyme increases the reaction rate by proper orientation or steering of reactive groups is called *orbital steering* (Fig. 2.4). Acetic anhydride synthase, which forms acetic anhydride from acetic acid, functions by the proximity effect.

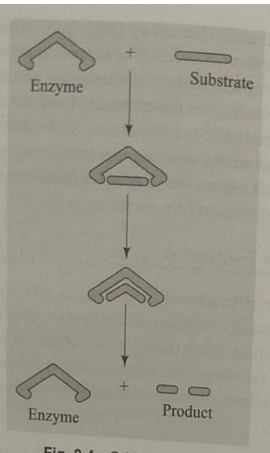


Fig. 2.4 Orbital steering

2.2.2 Preferential Transition State Binding

Preferential transition state binding (also called transition state stabilization or strain distortion) is probably the most important rate enhancement strategy of most of the enzymes. This is based on *Pauling's postulate* according to which "an enzyme recognizes and binds more tightly with the transition state than the substrate itself". The specific amino acid residues in the active site are oriented to fit tightly with the transition state to form a stable $EX^{\ddagger}$ complex. This leads to a decrease in the activation energy and the subsequent formation of the product at a higher rate. For example, proline racemase act by preferential transition state binding.

2.2.3 Metal Ion Catalysis (Electrophilic Catalysis)

Metal ions like Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} bind with enzymes, forming tight complexes called *metalloenzymes*. Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} bind with enzymes, forming loose complexes called *metal activated enzymes*. Some examples of metal ion catalysts are shown in Table 2.2.

Cytochrome oxidase, the terminal electron acceptor of electron transport chain, possesses copper at its active site. Carbonic anhydrase catalyzes the hydration of carbon dioxide using zinc at its active site. Magnesium is needed for the phosphorylation of hexose by hexokinase. Manganese is the metal catalyst for arginase reaction in the urea cycle. Urease-induced cleavage of urea is catalyzed by nickel. Selenium forms the active site component of glutathione peroxidase, thereby enhancing the rate of detoxification

Table 2.2 Examples of metal ion catalysts

Enzymes	Metal catalysts
Cytochrome Oxidase	Copper
Carbonic anhydrase	Zinc
Hexokinase	Magnesium
Arginase	Manganese
Urease	Nickel
Glutathione peroxidase	Selenium
Catalase	Ferrous
Nitrate reductase	Vanadium
Nitrogen-fixing enzyme	Cobalt

reaction. Detoxification of hydrogen peroxide into water and oxygen by catalase is enhanced by ferrous ion. Vanadium and cobalt forms the active site of nitrate reductase and nitrogen-fixing enzyme.

During metal ion catalysis, either metalloenzymes or metal-activated enzymes are formed first. Subsequently, the substrate binds to metalloenzymes or metal-activated enzymes forming a *ternary complex*. Four different types of ternary complexes are possible. They are as follows:

- Substrate bridge complex
- Enzyme bridge complex
- Simple metal bridge complex
- Cyclic metal bridge complex

A substrate bridge complex is formed only by metal-activated enzymes, whereas the other three types of complexes are formed by both metalloen-zymes and metal-activated enzymes. Figure 2.5 shows the ternary complexes and Table 2.3 describes the complexes with examples.

Metal ions act as catalysts by one or more of the following effects:

- Electrophilic effect
- Nucleophilic effect
- Binding energy enhancement
- Approximation and steric effect
- Strain effect
- Charge-masking effect

Table 2.3 Ternary complexes formed during metal ion catalysis

Ternary complex	Type of the complex	Example
Substrate bridge complex	Metal-activated enzyme	Phosphotransferase
Enzyme bridge complex	Metal-activated enzyme	Glutamine synthase
Simple metal bridge complex	Metalloenzyme Metal-activated enzyme	Alkaline phosphatase
Cyclic metal bridge complex	Metalloenzyme Metal-activated enzyme Metalloenzyme	Carbonic anhydrase

Substrate bridge complex

E---S---M

Enzyme bridge complex

S---E---M

Simple metal bridge complex

S---M---E

Cyclic metal bridge complex

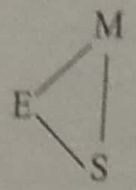


Fig. 2.5 Ternary complexes

Electrophilic effect As metal ions possess charges greater than +1 at neutral pH, they act as potent electrophiles and can form π/σ bonds with electron dense regions of the substrate. Thus, metal ions are called *super acids*. Alcohol dehydrogenase is an example.

Nucleophilic effect Metal ions (M) bind with water molecules and form M–H_2O complex. Metal-bound water is more acidic (ionizable) than free water. So, H^+ is lost forming MOH^- conjugate, which is a potent nucleophilic catalyst. Carbonic anhydrase is an example.

Binding energy enhancement Metal ion increases the binding energy by increasing the enzyme-substrate interaction and enhancing *the probability of collision*. For example, NMP kinase. Binding of metal ions to substrates also enhances catalysis. This concept can also be understood from the action of ribozyme (catalytic RNA; see Chapter 7). Conformational changes are required for the biological catalytic function of RNA molecules. In the Tetrahymena group I ribozyme reaction, a conformational change has been suggested to occur upon binding of the oligonucleotide substrate (S) or the guanosine nucleophile (G), leading to stronger binding of the second substrate.

The two substrates are bridged by a metal ion that coordinates both the non-bridging reactive phosphoryl oxygen of S and the 2-OH of G. These results suggest that the energy from the metal ion substrate interactions is used to drive the proposed conformational change. A central role of the bridging metal ion is responsible for the conformational change driving the action.

Approximation and steric effect The coordination sphere of the metal ion serves as three-dimensional *templates* for holding the reactive groups of the substrate and the enzyme in a specific steric orientation. This exerts a *stereochemical* control over the course of an enzyme-catalyzed reaction. Example, Pyruvate kinase and Adenylate kinase.

Strain effect Metal ions chelate with the substrate producing a strain. This causes conformational distortion of strained substrate into transition state for the subsequent formation of the product. For example, D-xylose isomerase.

Charge-masking effect Metal ions may mask or *shield* a nucleophile, thus preventing other likely side reactions. For example, histidine deaminase.

3

Enzyme Kinetics

Maud Leonora Menten formulated the Michaelis-Menten rate equation for enzyme kinetics.

Learning Objectives

After reading this chapter, the reader would be able to understand and appreciate the following:

- The definition of kinetics and the order of a reaction
- Michaelis–Menten kinetics
- Enzyme association, enzyme dissociation, and the pre-steady and steady state of a reaction
- The MM equation and the MM plot
- Transformation of the hyperbolic plot into a linear plot
- The kinetics of bisubstrate reaction
- Numerical problems related to enzyme kinetics

3.1 Definition

The study of the rate of an enzyme-catalyzed reaction and the influence of experimental parameters on the reaction rate is called *enzyme kinetics*. Enzyme kinetics helps in understanding the enzyme mechanism as facilitated by the three-dimensional structure and enzyme mutagenesis.

3.2 Order of a Reaction

The order of a reaction is the number of atoms or molecules whose concentration determines the rate of that reaction. During enzyme catalysis, we come across first-order kinetics and zero-order kinetics while measuring the rate of a reaction. Hence, a brief note on reaction rate and reaction order is essential for learning enzyme kinetics.

3.2.1 Zero-order Reaction

A zero-order reaction is one in which the rate of reaction is independent of the reactant concentration. This is represented in Fig. 3.1 that shows the plot of rate of a reaction versus concentration of a reactant or substrate [S].

3.2.2 First-order Reaction

A first-order reaction is one in which the rate of the reaction is dependent on the reactant concentration raised to the first power. If [S] is the substrate or reactant, then the rate or velocity of a first-order reaction is represented as

$$V \propto [S] \quad \text{or} \quad V = k[S]$$

where k is the rate constant.

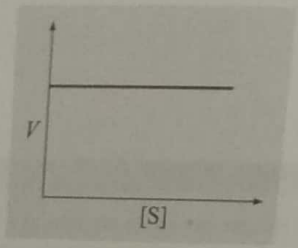


Fig. 3.1 Plot of substrate concentration versus rate for a zero-order reaction

3.2.3 Second-order Reaction

A second-order reaction is one in which the rate of a reaction is dependent on the substrate concentration raised to the second power. The rate of a second-order reaction is represented as

$$V \propto [S]^2$$

or $$V = k[S]^2$$

where k is the rate constant.

In other words, for a bisubstrate reaction, the rate is dependent on the product of the concentration of the two substrates, $[S_1]$ and $[S_2]$.

$$V \propto [S_1]\,[S_2]$$

or $$V = k\,[S_1]\,[S_2]$$

3.2.4 Third-order Reaction

A third-order reaction is one in which the rate of reaction is dependent on the cube of the substrate concentration

$$V \propto [S]^3$$

or $$V = k[S]^3$$

where k is the rate constant.

In other words, for a bisubstrate reaction, the rate is dependent on the product of the square of concentration of one substrate to the concentration of the other substrate raised to first power.

$$V \propto [S_1]\,[S_2]^2$$

or $$V = k\,[S_1]\,[S_2]^2$$

For a tri-substrate reaction, the rate is dependent on the product of the concentration of the three substrates, $[S_1]$, $[S_2]$, and $[S_3]$.

$$V \propto [S_1]\,[S_2]\,[S_3]$$

or $$V = k\,[S_1]\,[S_2]\,[S_3]$$

3.3 The Substrate Velocity Plot

An enzyme-catalyzed reaction is represented as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

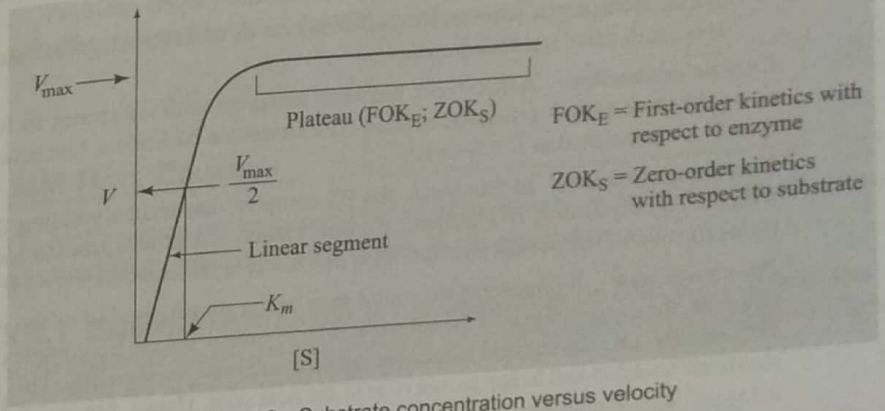


Fig. 3.2 Substrate concentration versus velocity

3.3.1 Segments of Hyperbola

A hyperbolic plot consists of two segments: a *linear segment* and a *plateau*. The analysis of a hyperbola reveals several kinetics, as listed below:

- First-order kinetics
- Zero-order kinetics
- Saturation kinetics
- Steady state kinetics

Linear segment A linear segment represents the initial stage of the reaction at a low substrate concentration. At this stage, V increases linearly with [S], i.e. $V \propto [S]$ or $V = k[S]$ where k is the rate constant. Here the reaction follows first-order kinetics with respect to the substrate (FOK_S).

Plateau It represents the later stage of the reaction at very high substrate concentration. At this stage, V is maximum, i.e. $V = V_{max}$. After V_{max} is attained, V remains constant and does not increase linearly with [S]. Here the reaction follows first-order kinetics with respect to the enzyme (FOK_E) and zero-order kinetics with respect to the substrate (ZOK_S). At V_{max}, the enzyme is completely saturated with substrate to form an enzyme-substrate complex (ES). Hence, the concentration of the free enzyme is zero. [E] = 0. This is called *saturation kinetics*. The substrate concentration at which the rate of the reaction is half of the maximal velocity is called K_m.

3.3.2 Michaelis-Menten Kinetic Postulates

The hyperbolic plot shown in Fig. 3.1 led Leonor Michaelis and Maud Menten in 19[illegible] to put forward two postulates:

1. During the catalytic process, two major events take place. They are *enzyme association* and *enzyme dissociation*.
2. During the catalytic process, two different periods or different states exist. They are *pre-steady state* and *steady state*.

Enzyme association In this event, enzymes associate with substrates to form an enzyme substrate complex denoted as [ES]. The formation of ES is a fast reversible process. It is represented as $E + S \rightarrow ES$.

Enzyme dissociation In this event, the ES complex dissociates yielding the product with the regeneration of free enzyme. ES dissociation is a slower process and therefore limits the rate of the overall reaction. This process is represented as $ES \rightarrow E + P$.

Pre-steady state It represents the initial state or the starting period of the reaction. In this state, the concentration of the substrate is large excess and the ES complex assembles very rapidly, i.e., the enzyme associates with the substrate very swiftly. This state is too short and unstable and hence passes over to the next state. Hence, pre-steady state kinetics is difficult to analyze.

Steady state It represents the second state or the post-initial period of the reaction that occurs immediately after the pre-steady state. In this state, the concentration of the enzyme-substrate complex as well the other reaction intermediates remain approximately constant (steady) over time. This state is relatively long and stable. Hence, steady state kinetics is easy to analyze and the velocity of an enzyme-catalyzed reaction generally reflect the steady state. Michaelis and Menten scoped their studies towards the steady state rate and hence the enzyme kinetics described by them is called the *steady state kinetics* or *Michaelis–Menten kinetics* or *MM kinetics*.

3.4 The Michaelis-Menten Reaction

According to Michaelis and Menten, the steady state analysis of an enzyme catalyzed reaction is represented as in Eqn. (3.1).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \tag{3.1}$$

This equation can also be called the MM reaction. According to this reaction, the enzyme (E) combines with the substrate (S) forming the ES with a rate constant k_1. ES can dissociate into either E + S with a rate constant k_{-1} or E+P with a rate constant k_2. ES dissociates into P at a velocity V and reaches a maximum velocity. For the MM reaction, k_2 is rate limiting; thus $k_2 <<< k_{-1}$ and hence it reduces to k_{-1}/k_1 which is defined as the *dissociation constant* for ES complex.

During the course of the reaction, the concentration of the free enzyme [E], the substrate-bound enzyme (saturated enzyme) [ES], and the total enzyme [E_T] can be correlated by the following equation:

$$[E_T] = [E] + [ES] \tag{3.2}$$

From the above postulate, the velocity of an enzyme-catalyzed reaction (V) is represented by an equation called the MM equation or the rate equation. It is given as follows:

$$V = \frac{V_{max}[S]}{K_m + [S]} \tag{3.3}$$

3.5 The MM Rate Equation

The MM equation can be derived via several steps using various parameters. These parameters and the derivation are dealt here in two separate titles.

3.5.1 Parameters Involved in the Derivation of MM Rate Equation

Some important parameters are considered while deriving the MM rate equation. These parameters are summarized in Table 3.1.

Table 3.1 Parameters used in the derivation of rate equation

Parameter	Representation
Concentration of the substrate	[S]
Concentration of the free enzyme	[E]
Concentration of the total enzyme	[E_T]
Concentration of the enzyme-substrate complex	[ES]
Rate constants for the forward reaction for ES formation	[k_1]
Rate constants for the forward reaction for ES dissociation	[k_2]
Rate constants for the reverse reaction for ES dissociation	[k_{-1}]
Maximal velocity	V_{max}

3.5.2 Derivation

Let us recall the enzyme-catalyzed reaction represented as in Eqn. (3.1):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

E combines with S forming ES with a rate constant k_1. ES can dissociate into eit E+S with a rate constant k_{-1} or E+P with a rate constant k_2.

The rate of ES formation is as follows:

$$\frac{d[ES]}{dt} = k_1 [E][S] \Rightarrow \frac{d[ES]}{dt} = k_1 \{[ET] - [ES]\}[S]$$

The rate of ES dissociation is as follows:

$$\frac{d[ES]}{dt} = k_{-1}[ES] + k_2[ES] \Rightarrow \frac{d[ES]}{dt} = (k_{-1} + k_2)[ES]$$

As per steady state kinetics, the rate of formation of ES (3.4) is equal to the rate o dissociation of ES (3.5). So equating (3.4) and (3.5), we get

$$k_1 \{[ET] - [ES]\}[S] = (k_1 + k_2)[ES]$$

Bringing the rate constants on one side, we get

$$\frac{(k_1 + k_2)}{k_1} = \frac{\{[ET] - [ES]\}[S]}{[ES]}$$

In the above equation, $(k_{-1} + k_2)/k_1$, the ratio of rate constants is denoted as K_m. Thus, Equation (3.7) becomes

$$K_m = \frac{\{[ET] - [ES]\}[S]}{[ES]}$$

Taking [S] as a common factor in the above equation, we get

$$K_m = \frac{[S][ET] - [S][ES]}{[ES]}$$

Cross multiplication of (3.9) gives

$$[S][ET] - [S][ES] = K_m[ES]$$

Rearranging (3.10), we get

$$[S][ET] = K_m[ES] + [S][ES]$$

Taking [ES] as a common factor in (3.11), we get

$$[S][ET] = [ES]\{K_m + [S]\}$$

Solving (3.12) for [S], we get

$$[S] = \frac{[ES]\{K_m + [S]\}}{[ET]}$$

(3.13)

In a reaction mixture, [ES] is difficult to measure. But V and V_{max} are easily measurable by a variety of methods. So it is essential to replace [ES] and [ET] in (3.13) by a measurable factor. This replacement is done based on the principle of saturation kinetics. Saturation kinetics states that 'at V_{max}, [E] is completely saturated with [S] to form [ES]'. Thus,

$$[E] = 0 \quad \text{(a)}$$

Since we get

$$[ET] = [E] + [ES], \quad [ET] = [ES] \quad \text{(b)}$$

Thus,

$$V = k_2[ES] \quad \text{(c)}$$

and

$$V_{max} = k_2[ET] \quad \text{(d)}$$

Solving (c) and (d) for k_2, we get

$$k_2 = \frac{V}{[ES]} \quad \text{(e)}$$

$$= \frac{V_{max}}{[ET]} \quad \text{(f)}$$

Combining (e) and (f), we get

$$\frac{V}{[ES]} = \frac{V_{max}}{[ET]} \quad \text{(g)}$$

Solving (g) for velocity terms, we get

$$\frac{V}{V_{max}} = \frac{[ES]}{[ET]} \quad \text{(h)}$$

Using (h), the immeasurable quantity [ES] is related to the measurable quantities V and V_{max}. Hence, (h) is used to solve (3.13).

Thus, substituting (h) in (3.13), we get

$$S = \frac{V}{V_{max}} \times K_m + [S] \quad (3.14)$$

Solving (3.14) for V, we get

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (3.15)$$

Equation (3.15) is called the MM equation or *rate equation* or *velocity equation*, where K_m is the MM constant.

3.6 Constants in Enzyme-Catalyzed Reaction

The velocity of an enzyme-catalyzed reaction is governed by two important constants.

1. k_{cat} – the catalytic constant, and
2. K_m – the MM binding constant.

3.6.1 The Catalytic Constant

Catalytic constant is the maximum number of substrate molecules converted to products per active site per second. It is denoted as k_{cat}. It is also called the *turnover number*. In a monosubstrate MM reaction, k_{cat} is the first-order rate constant for ES → EP conversion. A good substrate must have a large k_{cat}/K_m ratio. k_{cat} and k_{cat}/K_m values of some important enzymes are shown in Table 3.2. The k_{cat} value is highest for carbonic anhydrase, the fastest enzyme known.

Table 3.2 k_{cat} and k_{cat}/K_m values of some enzymes

Enzyme	Substrate	k_{cat} (S^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1}\,S^{-1}$)
Carbonic anhydrase	CO_2	1×10^6	1.2×10^{-2}	8.3×10^7
Fumarase	Malate	9×10^2	2.5×10^{-5}	3.6×10^7
Catalase	H_2O_2	4×10^7	1.1	4×10^7
Crotonase	Crotonyl CoA	5.7×10^3	2×10^{-5}	2.8×10^8
Triose phosphate isomerase	Glyceraldehyde-3-P	4.3×10^3	4.7×10^{-4}	2.4×10^8
β-lactamase	Benzyl Penicillin	2×10^3	2×10^{-5}	1×10^8

3.6.2 The Michaelis–Menten Binding Constant

In equation (3.1), the ratio of the rate constant of the forward reaction to that of the reverse reaction is called the Michaelis–Menten constant. It is denoted as K_m.

$$\text{i.e.,} \quad K_m = \frac{(k_{-1} + k_2)}{k_1}$$

K_m is defined as the substrate concentration when the reaction rate is half maximal, i.e.

$$K_m = [S] \quad \text{when} \quad V = V_{max}/2 \tag{3.16}$$

The above definition and Eqn. (3.16) can be proved as follows.

Proof for K_m

Consider the rate equation

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

Substituting $V = V_{max}/2$ in the above equation, we get

$$\frac{V_{max}}{2} = \frac{V_{max}\,[S]}{K_m + [S]} \tag{3.17}$$

Cross-multiplication of (3.17) gives

$$V_{max} \times K_m + [S] = 2 \times V_{max}[S] \tag{3.18}$$

As V_{max} is common for both sides, (3.18) can be rewritten as

$$K_m + [S] = 2.[S] \tag{3.19}$$

Solving (3.19) for k_m, we get

$$K_m = 2[S] - [S]$$

or

$$K_m = [S] \tag{3.20}$$

Thus, $K_m = [S]$ when V is $V_{max}/2$.

Other definitions for K_m

The MM constant can also be defined as follows:

- It is the substrate concentration [S] at which the enzyme is half-saturated
- It is the dissociation constant of ES if k_2 is much smaller than k_{-1} ($k_2 <<<< k_{-1}$).
- It is the [S] required for significant catalysis.
- It is the ratio of rate constant for the enzyme-catalyzed reaction:

Thus, $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$

For the above reaction, $K_m = \frac{k_{-1} + k_2}{k_2}$ (3.21)

Significance of K_m

1. K_m is constant for a particular enzyme and substrate. The K_m value of an enzyme is influenced by several factors like pH, temperature, and ionic strength. K_m values of some enzymes are shown in Table 3.3.
2. The fraction of enzyme site saturated with substrate is called the *fractional filling site*. K_m is used to calculate the fractional filling site at any substrate concentration by the following formula:

$$\text{Fractional filling site} = \frac{V}{V_{max}} = \frac{[S]}{[S] + K_m}$$

3. It is a measure of the strength of ES complex. K_m is inversely proportional to the strength of the ES complex.

4. It is a measure of E-S affinity. Lower the K_m higher the E-S affinity.
5. It is an indicator of the rate limiting step of a metabolic pathway. Highest K_m corresponds to slowest step of the pathway.

Table 3.3 K_m values of some enzymes

Enzyme	Substrate	K_m (μM)
β-galactosidase	Lactose	4000
Carbonic anydrase	CO_2	9000
Threonine dehydratase	L–threonine	5000
Catalase	Hydrogen peroxide	25000
Hexokinase	D–Fructose	1500

3.7 Transformation of MM Plot Into Linear Plot

It is very difficult to determine the limiting value of v (i.e. V_{max}) directly from the hyperbolic MM plot and therefore K_m cannot readily be determined. To overcome these difficulties, the MM equation can be rearranged in three different ways to give three different graphical representations – Lineweaver Burk (LB) plot, Eadie-Hofstee plot, and Hanes plot. In all these three representation, the equation of a straight line is

$$y = mx + c$$

where m equals the slope and c is the intercept on the Y-axis. The intercept on the X-axis equals $-c/m$. The plot versions of these three linear transforms are shown in Table 3.4. The LB plot draws the relationship between $1/V$ and $1/[S]$. The Eadie-Hofstee plot is obtained by plotting V against $V/[S]$ and Hanes plot is derived by plotting $[S]/V$ against $[S]$.

Table 3.4 Linear transforms of MM plot

Plot	X-axis	Y-axis
Lineweaver-Burk	$1/[S]$	$1/V$
Eadie-Hofstee	$V/[S]$	V
Hanes	$[S]$	$[S]/V$

3.7.1 The Lineweaver-Burk Transformation

The hyperbolic MM plot is algebraically manipulated and transformed into a linear Plot, in order to determine V_{max} accurately. Such a linear plot is called the Lineweaver-Burk plot (LB plot).

The LB plot

It is derived by taking the reciprocal of MM axis parameters, V and $[S]$. Hence it is also known as the, *double reciprocal plot*. The LB plot is shown in Fig. 3.3. The affinity of enzyme substrate is constant i.e. the affinity of enzyme does not increase with the increase in substrate concentration. This phenomenon is called *non-cooperativity*. Because of non-cooperativity, the LB plot is linear and the slope is constant. Table 3.5 compares the LB plot with the MM plot.

Thus, for the MM plot, the X-axis is [S] and the X-intercept is K_m; Y-axis is V and Y-intercept is V_{max}. For the LB plot, the X-axis is $1/[S]$ and the X-intercept is $-1/K_m$; Y-axis is $1/V$ and Y-intercept is $1/V_{max}$.

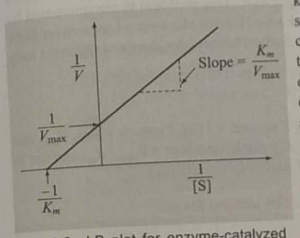


Fig. 3.3 LB plot for enzyme-catalyzed reaction

Table 3.5 Comparison of MM plot and LB plot

Parameters	MM plot	LB plot
X-axis	$[S]$	$1/[S]$
X-intercept	K_m	$-1/K_m$
Y-axis	V	$1/V$
Y-intercept	V_{max}	$1/V_{max}$
Rate equation	$V = \frac{V_{max}[S]}{K_m + [S]}$	$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$

The LB equation

The MM rate equation (3.15) is transformed into LB rate equation as follows:

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

Taking reciprocal of the above equation, we get

$$\frac{1}{V} = \frac{K_m[S]}{V_{max}[S]} \tag{3.22}$$

Taking $V_{max}[S]$ as the common denominator, we get

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{[S]}{V_{max}[S]} \tag{3.23}$$

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{3.24}$$

Equation (3.24) is called the LB equation.

3.7.2 The Eadie-Hofstee Transformation

In LB plot, the extrapolation across the $1/V$ axis to determine the value of $-1/K_m$ reaches the edge of the graph before reaching the 1/[S] axis. This problem is circumvented by Eadie-Hofstee (EH) plot. An advantage of an Eadie-Hofstee plot over a Lineweaver Burk plot (which plots $1/V$ versus 1/[S]) is that the Eadie-Hofstee plot does not require a long extrapolation to calculate K_m.

The Eadie-Hofstee plot (EH plot)

The EH plot (Fig. 3.4) is obtained by plotting V against $V/[S]$. From this plot, V_{max} and K_m can be determined. The slope of EH plot = K_m; the X-intercept = V_{max}/K_m; and the Y-intercept = V_{max}. Table 3.6 compares the MM plot with the EH plot.

Table 3.6 Comparison of MM plot and EH plot

Parameters	MM plot	EH plot
X-axis	[S]	V/[S]
X-intercept	K_m	V_{max}/K_m
Y-axis	V	V
Y-intercept	V_{max}	V_{max}
Rate equation	$V = \frac{V_{max}[S]}{K_m + [S]}$	$V = -K_m \frac{V_{max}}{[S]} + V_{max}$

The Eadie-Hofstee equation

The Eadie-Hofstee equation is derived from the LB equation (3.24) as follows:

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

Multiplying LB equation by a factor V. V_{max}, we get

$$\frac{1}{V} V V_{max} = \frac{K_m}{V_{max}} \frac{1}{[S]} V V_{max} + \frac{1}{V_{max}} V V_{max} \quad (3.25)$$

On simplifying (3.25), we get

$$V_{max} = K_m \frac{V}{[S]} + V \quad (3.26)$$

Solving (3.26) for V, we get

$$V = -K_m \frac{V}{[S]} + V_{max} \quad (3.27)$$

Equation (3.27) is called the EH equation.

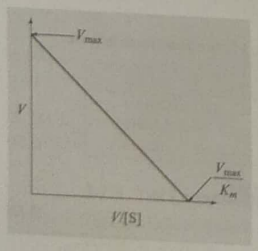


Fig. 3.4 Eadie-Hofstee plot

3.7.3 The Hanes Transformation

The LB plot is further modified to give a straight line that intercepts Y-axis only. Such a plot without an X-intercept is called the Hanes plot.

The Hanes plot

The Hanes plot is derived by plotting $[S]/V$ against [S] (Fig. 3.5). The slope of Hanes plot = $1/V_{max}$ and its Y-intercept = K_m/V_{max}. Table 3.7 compares the Hanes plot with the MM plot.

Table 3.7 Comparison of MM plot and Hanes plot

Parameters	MM plot	Hanes plot
X- axis	[S]	[S]
X-intercept	K_m	$-K_m$
Y-axis	V	$[S]/V$
Y-intercept	V_{max}	K_m/V_{max}
Rate equation	$V = \frac{V_{max}[S]}{K_m + [S]}$	$\frac{[S]}{V} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}}$

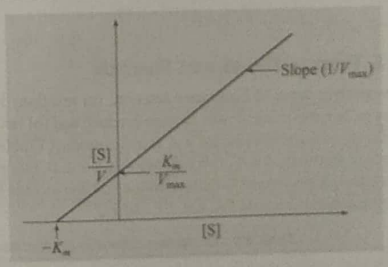


Fig. 3.5 Hanes plot

The Hanes equation

It is derived from the LB equation (3.24) as follows:

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

Multiplying the LB equation by [S] on both sides, we get

$$\frac{1}{V}[S] = \frac{K_m}{V_{max}} \times \frac{1}{[S]} \times [S] + \frac{1}{V_{max}} \times [S] \quad (3.28)$$

Solving (3.28) for [S]/V, we get

$$\frac{[S]}{V} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}} \tag{3.29}$$

Equation (3.29) is called the Hanes equation.

In recent years, microcomputers are used for data analysis to obtain the 'best fit' values of V_{max} and K_m. Using computer programs, the data is fitted directly to the MM rate equation (15). A number of such programs are commercially available, e.g. Origin & Originpro, Olpacube, Sigma plot, Wolfram alfa, etc.

4

Enzyme Inhibition

Alexandar Fleming discovered Penicillin, a suicide inhibitor.

Learning Objectives

After reading this chapter, the reader would be able to understand and appreciate the following:

- Types of enzyme inhibition and enzyme inhibitors
- MM plot and LB plot for different types of reversible inhibition
- Applications and examples for reversible inhibitors
- Types of irreversible inhibition
- Examples of irreversible inhibitors
- Protocol and examples of active site mapping

4.1 Enzyme Inhibitors

Cellular enzymes are inhibited by low molecular weight compounds like drugs, antibiotics, toxins, and certain metabolites. Such compounds are called *inhibitors*. Sulphonamide and heavy metal ions are a few examples of enzyme inhibitors. Enzyme inhibition is the process of decreasing the rate of an enzyme activity. It serves as a major control mechanism in the biological system.

4.2 Types of Enzyme Inhibition

Enzyme inhibition is of two types: *reversible inhibition* and *irreversible inhibition*. Reversible inhibition is a temporary process and can be relieved, but irreversible, inhibition cannot be relieved and it is a permanent process. Reversible inhibition is of three types – *competitive inhibition*, *non-competitive inhibition*, and *uncompetitive inhibition*. Irreversible inhibition is of two types – *group-specific inhibition* and *suicide inhibition*. Different types of enzyme inhibition are shown in Table 4.1. Examples for different types of inhibitors are shown in Table 4.2.

Table 4.1 Types of enzyme inhibition

Reversible inhibition	Irreversible inhibition
Competitive inhibition	Group-specific inhibition
Non-competitive inhibition	Suicide inhibition • Mechanism-based inhibition • Latent inhibition • Affinity label inhibition • Suicide substrate inhibition • Trojan horse substrate inhibition
Uncompetitive inhibition	

Table 4.2 Different types of inhibitors

Types of inhibitors	Examples
Competitive inhibitor	Sulphonamide
Non-competitive inhibitor	Heavy metal ions
Uncompetitive inhibitor	L-phenylalanine
Group-specific inhibitor	Diisopropyl phosphofluoridate (DIPF)
Suicide inhibitor	Bromoacetol

4.2.1 Reversible Inhibition

The three types of inhibition (competitive, uncompetitive, and non-competitive) and the examples for each type are discussed under this section.

Competitive inhibition

In competitive inhibition, the substrate and the inhibitor exhibit structural similarity due to which they compete for the same active site. Competitive inhibitors are also called *structural analogs*. Competitive inhibition is represented in Fig. 4.1.

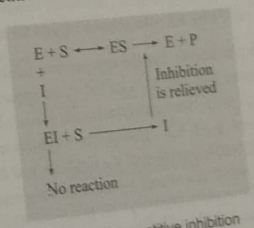


Fig. 4.1 Competitive inhibition

Competitive inhibitors function by decreasing the proportion of enzyme avail... for substrate binding. Competitive inhibition can be relieved by increasing the substr... concentration because at high substrate concentration, competitive inhibitor will be d... placed from the active site by *Le Chatelier principle* and the V_{max} can be attained. ... knowing the velocity of an enzyme-catalyzed reaction in the presence or the absence o... an inhibitor, the degree of an inhibition in % can be calculated from the following for... mula:

$$\text{Degree of inhibition} = I = 1 - \frac{V_i}{V_0} \times 100$$

MM plot and LB plot Figure 4.2 shows the MM plot and LB plot for competitively inhibited reactions.

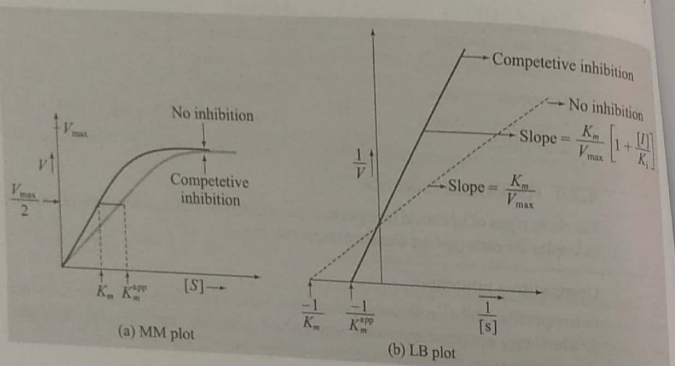


Fig. 4.2 MM plot and LB plot for competitive inhibition

The MM plot shows that the competitive inhibitor increases the K_m value keeping the V_{max} constant. The new K_m is called as the apparent K_m and is denoted as K_m^{app}. Thus, in the LB plot, $1/v$ (Y-intercept) is constant, while $-1/K_m$ (X-intercept) is decreased. The new X-intercept is $-1/K_m^{app}$. Competitive inhibitor increases K_m by a factor of $1 + [I]/K_i$, where $[I]$ = concentration of the inhibitor and K_i = dissociation constant for the EI complex. K_m^{app} and K_i are related by the following equation:

$$K_m^{app} = K_m\left(1+\frac{[I]}{K_i}\right)$$

The new slope of LB plot is,

$$\frac{K_m}{V_{max}} = 1 + \frac{[I]}{K_i}$$

The rate equation of a reaction inhibited by competitive inhibitor is given as

$$V_i = \frac{[S]V_{max}}{K_m\left(1+\frac{[I]}{K_i}\right)+[S]}$$

Examples for competitive inhibitors Some examples of competitive inhibitors include:

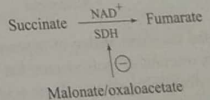
- Malonate
- Oxaloacetate
- Sulphonamide
- Methotrexate
- Allopurinol

Table 4.3 lists a few enzymes and their competitive inhibitors.

Table 4.3 Enzymes and their competitive inhibitors

Enzymes	Competitive inhibitors
Succinate dehydrogenase	Malonate
Folate synthesizing enzyme	Sulphonamide
Dihydrofolate reductase	Methotrexate
Xanthine oxidase	Allopurinol

Malonate/oxaloacetate inhibits succinate dehydrogenase Succinate dehydrogenase (SDH) is a classic example for competitively inhibited enzymes. During the TCA cycle, SDH converts succinate to fumarate.



Malonate and oxaloacetate being the structural analogs of succinate (Fig. 4.3) compete with succinate for binding with the active site of SDH.

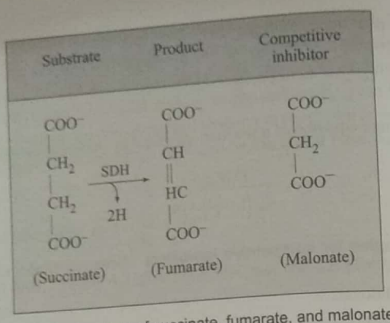
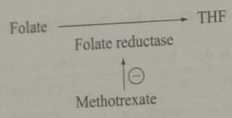
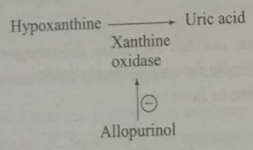


Fig. 4.3 Structure of succinate, fumarate, and malonate

Methotrexate inhibits dihydrofolate (DHF) reductase Tetrahydrofolate (THF) is a coenzyme derived from folate by the enzyme reductase. THF is used as a coenzyme in 'one carbon' transfer. Methotrexate being a structural analog of folate inhibits reductase and hence, folate synthesis.

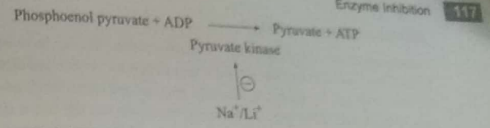


Allopurinol inhibits xanthine oxidase During purine metabolism, hypoxanthine is converted into uric acid by a metalloenzyme – xanthine oxidase.



Allopurinol, the structural analog of hypoxanthine, binds to xanthine oxidase and prevents the redox reaction of molybdenum ion in the active site and inhibits the enzyme, thereby impairing the synthesis of uric acid.

Metal ions also act as competitive inhibitors During glycolysis, pyruvate kinase converts phosphoenol pyruvate to pyruvate. Sodium and lithium ions inhibit pyruvate kinase.



Sulphonamide inhibits folate biosynthesis Sulphonamide inhibits the enzyme that catalyzes the biosynthesis of folate.

Applications of competitive inhibitors Various applications of competitive inhibitors are summarized in Table 4.4.

- Competitive inhibitors are used to treat *intoxication* caused by the product of a particular enzyme. For example, methanol poisoning and ethyl glycol poisoning can be treated by inhibiting alcohol dehydrogenase using ethanol as an inhibitor.
- Sulphonamide is used to inhibit *bacterial growth* by preventing folate synthesis, thereby preventing folate-catalyzed one carbon transfer reactions.
- Allopurinol is used to treat *gout*. Gout is a disease characterized by high serum levels of uric acid, the final *product* of purine degradation. It is formed by xanthine oxidase activity. Allopurinol is used to treat gout by impairing the xanthine-oxidase catalyzed synthesis of uric acid.
- Methyl pyrazole is used to treat *metabolic acidosis*, depression, and renal stone formation by inhibiting the formation of glycolic acid and oxalic acid.

Table 4.4 Applications of competitive inhibitors

Substrate	Enzyme	Product	Toxic effect of the product	Competitive inhibitor that prevents the toxic effect of the product
Methanol (Industrial solvent)	Alcohol dehydrogenase (ADH)	Formaldehyde Formic acid	Retinal damage Acidosis Depression CNS	Ethanol
Ethylene glycol (Automobile antifreeze)	ADH	Glycolic acid Oxalic acid	Metabolic acidosis Depression Renal stone	Methyl pyrazole
Isopropanol	ADH	Acetone	CNS Depression, Gastritis Vomiting, Hemorrhage	Other alcohol
Hypoxanthine	Xanthine oxidase	Uric acid	Gout	Allopurinol

Non-competitive inhibition

In non-competitive inhibition, the substrate and the inhibitor have no structural similarity and hence do not compete for the same active site. Non-competitive inhibitors bind to the *allosteric site* (*regulatory site*) of the enzyme and alters the three-dimensional structure of the active site so that the substrate cannot fit into the active site. A non-competitive inhibitor binds either to the free enzyme or to the enzyme substrate complex. Non-competitive inhibition is represented in Fig. 4.4.

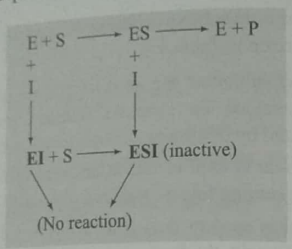


Fig. 4.4 Non-competitive inhibition

Non-competitive inhibitors function by decreasing the turnover number of the enzyme. Unlike competitive inhibition, non-competitive inhibition cannot be relieved at a higher substrate concentration, so the V_{max} cannot be attained. When the non-competitive inhibitor binds to the enzyme at the regulatory site, the shape of the active site changes so that it can no longer bind its substrate or catalyze the production of product. The enzyme will remain inhibited until the non-competitive inhibitor leaves the regulatory site. Figure 4.5 shows the MM plot and the LB plot for non-competitive inhibition.

MM plot and LB plot The MM plot shows that the non-competitive inhibitor decreases the V_{max} keeping the K_m constant. The new V_{max} is called apparent V_{max}, denoted as V_{max}^{app}. Thus, in the LB plot, $1/V$ (Y-intercept) is increased, while $-1/K_m$ (X-intercept) is constant. The new Y-intercept is $1/V_{max}^{app}$. Non-competitive inhibitor decreases V_{max} by a factor of $1 + [I]/K_i$. The new slope is

$$\frac{K_m}{V_{max}} = 1 + \frac{[I]}{K_i}$$

In the presence of a non-competitive inhibitor, the rate equation is given as:

$$V_i = \frac{[S]V_{max}}{([S] + K_m)\ 1 + \dfrac{[I]}{K_i}}$$

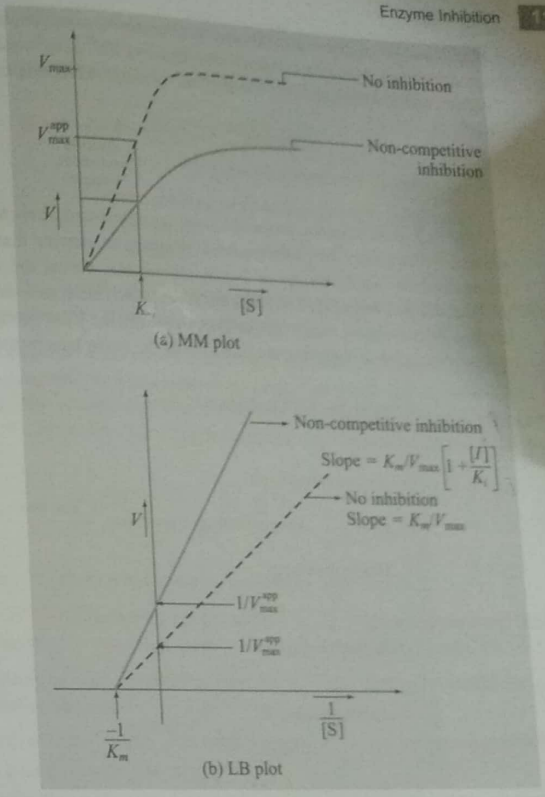


Fig. 4.5 MM plot and LB plot for non-competitive inhibition

Examples for non-competitive inhibitors Some examples of competitive inhib include:

- Heavy metal ions
- EDTA
- Fluoride
- Hydrogen sulphide
- Cyanide
- Pepstatin

Heavy metals Enzymes containing active sulphur, oxygen, and nitrogen are non-competitively inhibited by heavy metal ions like Pb^{2+}, Hg^{2+}, and Ag^{2+}. Heavy metals interact with the thiol group of the enzyme forming mercaptide complex which is inactive.

$$E-SH + Hg^{2+} \longrightarrow \underset{\substack{\text{(Inactive}\\ \text{mercaptide}\\ \text{Complex)}}}{E-S-H} + H^+$$

Pb^{2+} is known to inhibit *porphobilinogen synthase* and *ferrochelatase* by binding to the active thiol group. Porphobilinogen synthase is an enzyme that synthesizes porphobilinogen from two molecules of δ-aminolevulinate, during the second step of heme biosynthetic pathway (Fig. 4.6). This enzyme is also called as δ-aminolevulinate dehydratase. Ferrochelatase catalyzes the synthesis of heme from protoporphyrin IX by inserting ferrous ion to the substrate (last step of the heme biosynthetic pathway).

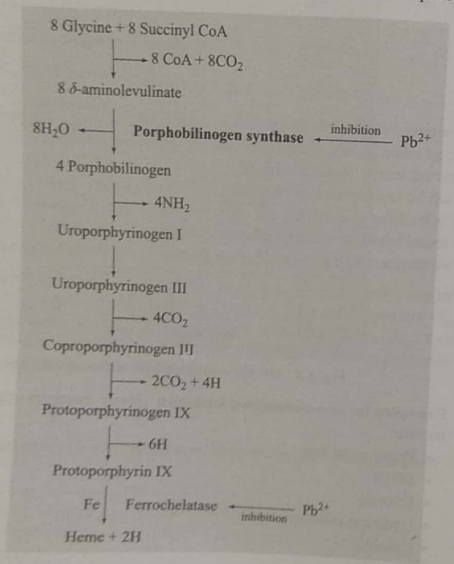
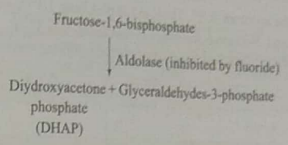


Fig. 4.6 Biosynthetic pathway of heme (Inhibition of porphobilinogen synthase and ferrochel[illegible]ead is highlighted)

Fluoride Aldolase is an enzyme that converts fructose-1,6-bisphosphate to diydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) during the fourth step of glycolytic pathway.



Fluoride ion inhibits aldolase by binding to active Mg^{2+} or Mn^{2+}. Thus, fluoride is used as a *preservative* for blood glucose analysis.

Hydrogen sulphide and cyanide Hydrogen sulphide (H_2S) and cyanide (CN^-) inhibit iron-containing enzymes like superoxide dismutase (SOD) and catalase (CAT). Superoxide dismutase catalyzes the dismutation of superoxide anion radical forming hydrogen peroxide. Catalase catalyzes the detoxification of hydrogen peroxide to water and oxygen.

$$O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Pepstatin Pepstatin non-competitively inhibits *renin*.

EDTA EDTA (chelating agent) functions as a non-competitive inhibitor of *metalloenzymes* by removing the metal ion cofactors like Mg^{2+} and Ca^{2+}.

Applications of non-competitive inhibitors Non-competitive inhibitors are used for the following purpose:

- To relieve heavy metal poisoning, e.g. lead poisoning and mercury poisoning
- To relieve cyanide poisoning
- Fluoride is used as blood preservative for subsequent analysis of glucose.

Uncompetitive inhibition

Uncompetitive inhibitors have no affinity for substrates. It binds to the allosteric site of the enzyme-substrate complex only, but not to the free enzyme. Uncompetitive inhibition is represented in Fig. 4.7.

$$E + S \longrightarrow ES + I \longrightarrow \underset{\text{(inactive)}}{ESI} \longrightarrow \text{No reaction}$$

Fig. 4.7 Uncompetitive inhibition

Figure 4.8 shows the MM plot and the LB plot for uncompetitive inhibition. MM plot shows that in the presence of uncompetitive inhibitor, V_{max} is increased and K_m is decreased. The new V_{max} is called the apparent V_{max}, denoted as V_{max}^{app} and the new K_m is called the apparent K_m^{app}. Thus, in LB plot, both $1/V$ (Y-intercept) and $-1/K_m$ (X-intercept) are altered. However, the slope is unaltered, it is K_m/V_{max}.

In the presence of an uncompetitive inhibitor, the rate equation is given as:

$$V_i = \frac{[S]V_{max}}{K_m + [S]\ 1 + \frac{[I]}{K_i}}$$

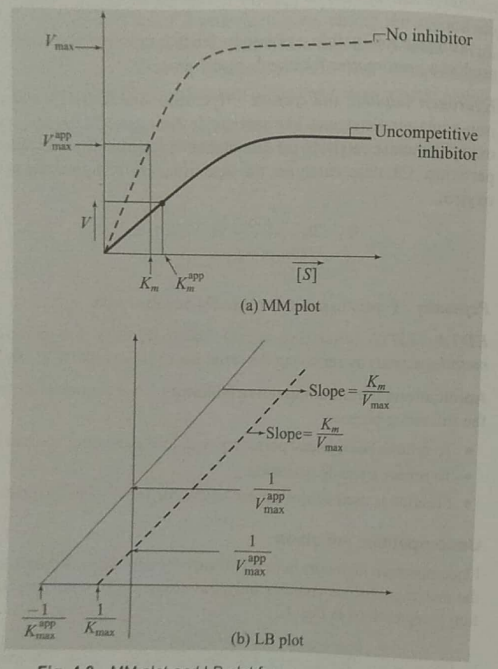


Fig. 4.8 MM plot and LB plot for uncompetitive inhibition

Examples for uncompetitive inhibition Uncompetitive inhibition is rare in monosubstrate reactions, but it is common in bisubstrate reactions showing double displacement mechanism.

L-Phenylalanine L-phenylalanine is an uncompetitive inhibitor for intestinal alkaline phosphatase. Three types of reversible inhibition discussed so far are summarized in Table 4.5.

Table 4.5 Summary of Reversible inhibition

Types of reversible inhibition	Alteration in MM plot		Alteration in LB plot			Target for the inhibitor	For inhibitor
	K_m	V_{max}	$-1/K_m$	$1/V_{max}$	Slope		
Competitive	↑	=	↓	=	$\frac{K_m}{V_{max}} = 1 + \frac{[I]}{K_i}$	Active site of enzyme	Malonate Oxaloacetate Methotrexate Allopurinol
Non-competitive	=	↓	=	↑	$\frac{K_m}{V_{max}} = 1 + \frac{[I]}{K_i}$	Allosteric site of enzyme	CN^-, H_2S, F^-, Pb^{2+}, Ag^{2+}, Hg^{2+}
Uncompetitive	↑	↓	↓	↑	$\frac{K_m}{V_{max}}$	Allosteric site of enzyme substrate complex	L-phenylalanine

↑ refers to increase; ↓ refers to decrease; = refers to constant (no change)

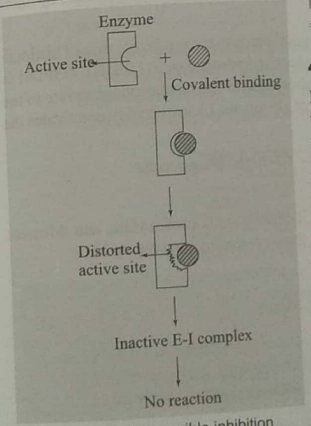


Fig. 4.9 Irreversible inhibition

4.2.2 Irreversible Inhibition

Irreversible inhibitors bind covalently with the functional groups of the active site, thereby permanently disturbing the catalytic activity leading to total inactivation of the enzyme. Irreversible inhibition is represented in Fig. 4.9.

Irreversible inhibition is of two types:

- Group-specific inhibition
- Suicide inhibition

Group-specific inhibition

Group-specific reagents are chemical compounds that react with specific functional groups of the active site amino acids. Common examples of these reagents include Diisopropyl-phosphofluoridate (DIPF), Iodoacetamide, and Iodoacetate (Table 4.6).

Table 4.6 Common group-specific agents that function as irreversible inhibitors

Name of the compound	Enzymes inhibited
Diisopropyl-phosphofluoridate (DIPF)	Enzymes with active serine residue
Iodoacetamide	Enzymes with active cysteine residue
Iodoacetate	Enzymes with active histidine residue, thiol residue, carboxylate residue, and thioester group.

Examples for group-specific inhibition DIPF, iodoacetamide, and iodoacetate are common group-specific inhibitors. Actions of these inhibitors are given in this section.

DIPF DIPF is a prominent component of insecticides and nerve gas. It combines covalently with the serine hydroxyl group in the catalytic triad of chymotrypsin and acetylcholine esterase (AChE) – an enzyme involved in the transmission of nerve impulse (Fig. 4.10). DIPF binds with the active hydroxyl group of the enzyme and forms an inactive complex with the liberation of a hydrogen ion and a fluoride ion.

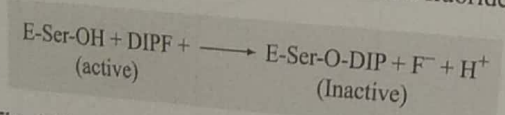
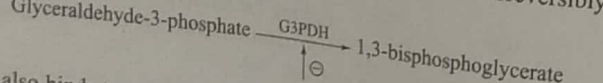


Fig. 4.10 Irreversible inhibition of active serine by DIPF

Iodoacetamide Iodoacetamide inhibits enzymes with active cysteine residues by binding to the thiol (–SH) group.

Iodoacetate (IA) Iodoacetate (alkylating agent) binds to the imidazole ring of histidine residue and inhibits glyceraldehyde-3-phosphate dehydrogenase (G3PDH). G3PDH is an enzyme that converts Glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the presence of NAD and Pi during the fifth step of glycolysis. IA irreversibly inactivates the enzyme, thereby inhibiting glycolysis.



IA also binds to other functional groups including thiol, carboxylate, and thioester groups. Figure 4.11 shows the inhibition of a thiol-containing enzyme by IA.

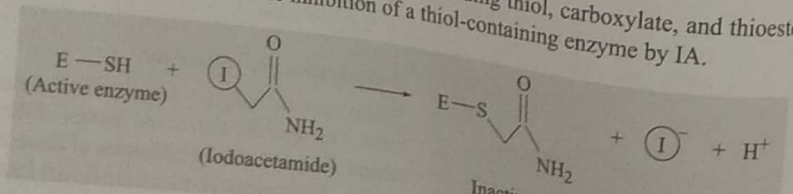


Fig. 4.11 Irreversible inhibition of active thiol by Iodoacetate

Suicide inhibition

Substrate analogs have a high affinity for active sites and hence label the functional groups of active sites by irreversible covalent bonds. These analogs are then converted into a toxic intermediate by normal catalysis. The toxic intermediate in turn inhibits the enzyme. This process is called *suicide inhibition*. Such substrate analogs are called by the following names:

- Suicide inhibitors
- Affinity labels
- Latent inhibitors
- Mechanism-based inhibitors
- Trojan horse substrates.

Triose phosphate isomerase, lysozyme, beta hydroxyl decanoyl dehydrase, and isoleucyl-tRNA synthetase are the known examples of enzymes inhibited by affinity labels. Majority of the affinity labels have been synthesized based on halomethyl ketones or epoxides.

Criteria for suicide inhibitors Any effective suicide inhibitors should have three criteria. The inhibitor should be chemically inactive in the absence of the target enzyme. It should be activated specifically by its target enzyme. The inhibitor should associate more rapidly with the target enzyme than it dissociates. Suicide inhibitors that fulfill these criteria include acetylenic compounds, β, γ-unsaturated compounds and β-halo compounds.

Enzymes with pyridoxal phosphate Enzymes that use pyridoxal phosphate as their coenzyme (Table 4.7) are the chief target for suicide inhibitors. Enzymes like β-aspartate decarboxylase, aspartate amino transferase, alanine racemase are inhibited by Chloroalanine. β-trifluoroalanine is the suicide inhibitor for serine dehydratase.

Table 4.7 Examples of enzymes with pyridoxal phosphate as coenzymes

Enzyme	Suicide inhibitor
β-aspartate decarboxylase	Chloroalanine
Aspartate amino transferase	Chloroalanine
Alanine racemase	Chloroalanine
Serine dehydratase	β-trifluoroalanine

Flavin-linked monoamine oxidase Monoamine oxidase (MAO) is responsible for the deamination of monoamines such as adrenaline, noradrenaline, dopamine, and serotonin which function as neurotransmitters. MAO covalently binds to flavin moiety by the thiol group of cysteine residue.

N,N-dimethyl propargyl amine (NNDPA) shown in Fig. 4.12 is an acetylenic suicide inhibitor that inactivates MOA by alkylating the flavin on the 5th nitrogen. The 5th nitrogen of flavin attaches to the acetylenic carbon. Imbalance in neurotransmitters results in Parkinson's disease and mental depression. For example, Parkinson's disease is associated with low levels of dopamine, and depression is associated with low levels of other monoamines. Hence, NNDPA is used for the treatment of Parkinson's disease and mental depression.

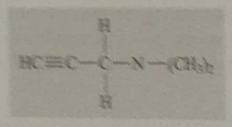


Fig. 4.12 Structure of NNDPA

Deprenyl, shown in Fig. 4.13, is another compound that functions as a suicide inhibitor for MOA. It is also used to treat Parkinson's disease and depression.

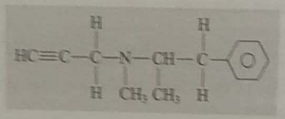


Fig. 4.13 Structure of Deprenyl

Examples for suicide inhibitors The examples of suicide inhibitors include: Penicillin, *D*-3-decanoyl-*N*-acetyl cysteine, *N, N*-dimethyl propargyl amine, Tosyl phenylalanine chlormethyl ketone (TPCK), and 3-bromoacetol (Table 4.8).

Table 4.8 Examples of some enzymes and their suicide inhibitors

Enzyme	Suicide inhibitor (substrate analogs)
Glycopeptide transpeptidase	Penicillin
D-3-hydroxy decanoyl ACP dehydratase	*D*-3-decanoyl-*N*-acetyl cysteamine
Monoamine oxidase (MAO)	*N,N*-dimethyl propargyl amine Deprenyl
Chymotrypsin	Tosyl phenylalanine chlormethyl ketone (TPCK)
Triose phosphate isomerase	3-bromoacetol
Alanine racemase	Chloroalanine
Serine dehydratase	β-trifluoroalanine

Penicillin Glycoprotein peptidase is an enzyme that cross-links peptidoglycan chain during bacterial cell wall synthesis. Penicillin consists of a thiazolidine ring fused to a β-lactam ring to which a variable group R is attached (Fig. 4.14). A reactive peptide bond in the β-lactam ring covalently attaches to a serine residue in the active site of the glycopeptide transpeptidase forming an inactive complex called penicilloyl-enzyme complex.

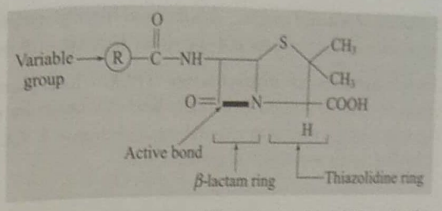


Fig. 4.14 Structure of penicillin, an irreversible inhibitor of active serine residue

The conformation of penicillin around its reactive peptide bond resembles the transition state of the normal glycoprotein peptidase substrate. Consequently, the bacterial cell wall synthesis is blocked leading to osmotic lysis and bacterial cell death. The bond between the enzyme and penicillin is indefinitely stable; that is, penicillin binding is irreversible. The inhibitory reaction is illustrated in Fig. 4.15.

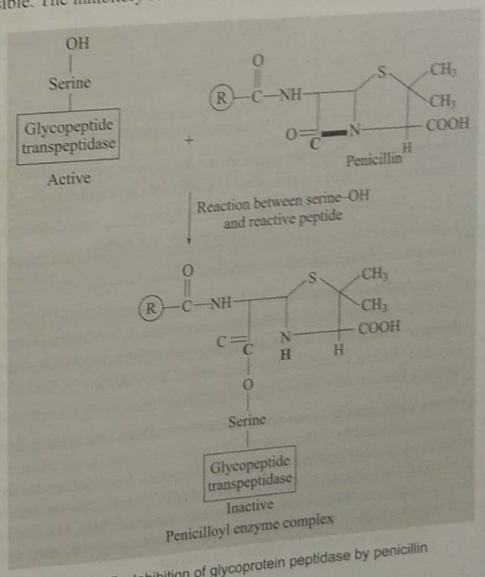


Fig. 4.15 Inhibition of glycoprotein peptidase by penicillin