1.11 Reactive oxygen species and antioxidant

Reactive Oxygen Species (ROS) is a phrase used to describe a number of reactive chemical species and free radicals derived from molecular oxygen. *Free radicals* are atoms or molecules that have unpaired valence electrons, which explain their instability and high reactivity. Radicals can have positive, negative or neutral charge. They are formed as necessary intermediates in a variety of normal biochemical reactions. There are many types of radicals, but those of most concern in biological systems are derived from oxygen. Ground state oxygen may be converted to ROS either by *energy transfer* or by *electron transfer* reactions. The former leads to the formation of *singlet oxygen*, whereas the latter results in the sequential reduction to *superoxide radical*, *peroxide radical* and *hydroxyl radical*.

Oxygen (O_2) Superoxide radical ($O_2^{\bullet -}$) Peroxide radical ($O_2^{\bullet -2}$) Hydroxyl radical (•OH)

When the level of ROS exceeds the normal level, a cell is said to be in a state of 'oxidative stress.' The enhanced level of ROS causes peroxidation of lipids (oxidations of unsaturated fatty acids), oxidation of proteins, damage to nucleic acids, enzyme inhibition and activation of programmed cell death pathway.

An *antioxidant* is a molecule that inhibits the oxidation of other molecules. Antioxidants remove free radical intermediates and inhibit oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols. Cells contain multiple types of antioxidants. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. Non-enzymatic antioxidants are represented by vitamin C, vitamin E, glutathione, carotenoids, flavonoids and other antioxidants.

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

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

$$H_2O_2 + 2\,GSH \xrightarrow{GPx} 2H_2O + GSSG$$ (GSH = Reduced glutathione and GSSG = Oxidized glutathione)

1.12 Enzymes

General features

An enzyme is a biocatalyst that increases the rate of chemical reaction without itself being changed in the overall process. It is a remarkable molecular device that determines the pattern of chemical transformations. Virtually all cellular reactions or processes are mediated by enzymes. Enzymes have several properties that make them unique.

- Most enzymes are proteins. With the exception of a small group of catalytic RNA molecules, all enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost.
- Enzymes are highly specific. They are specialized proteins and have a high degree of specificity for their substrates.
- Enzymes exhibit enormous catalytic power. It increases the rate of a reaction by lowering the activation energy. It changes only the rate at which equilibrium is achieved; it has no effect on the position of the equilibrium.

Enzymes can be divided into two general classes: **simple enzymes**, which consist entirely of amino acids and **conjugated enzymes**, contains a non-protein group called a **cofactor**, which is required for biological activity. Removal of cofactor from a conjugated enzyme leaves only protein component, called an *apoenzyme*, which generally is biologically inactive. The complete, biologically active conjugated enzyme (simple enzyme plus cofactor) is called a *holoenzyme*. A cofactor can be linked to the protein portion of the enzyme either covalently or non-covalently. Some cofactors are simple metal ions and other cofactors are complex organic groups, which are also called **coenzymes**. Cofactors which are tightly associated with the protein covalently or non-covalently are called *prosthetic group*.

Table 1.21 Vitamin B-complex and their coenzyme forms

Vitamin	*Coenzyme form*	*Reaction or process promoted*
Thiamine (B_1)	Thiamine pyrophosphate	Decarboxylation, aldehyde group transfer
Riboflavin (B_2)	FAD and FMN	Redox reaction
Pyridoxine (B_6)	Pyridoxal phosphate	Amino group transfer
Nicotinic acid (niacin)	NAD^+ and $NADP^+$	Redox reaction
Pantothenic acid (B_5)	Coenzyme A	Acyl group transfer
Biotin	Biocytin	Carboxylation
Folic acid	Tetrahydrofolic acid	One-carbon group transfer
Vitamin B_{12}	Deoxyadenosylcobalamin	Intramolecular rearrangements

The vitamins in the human diet that are coenzyme precursors are all water soluble vitamins.

Table 1.22 Example of some enzymes and their cofactors

Fe^{2+} or Fe^{3+}	Cytochrome oxidase, Catalase, Peroxidase, Xanthine oxidase
Cu^{2+}	Cytochrome oxidase, Lysyl oxidase, Superoxide dismutase
Zn^{2+}	Carbonic anhydrase, Alcohol dehydrogenase, Carboxypeptidase
Mg^{2+}	Hexokinase, Enolase, Glucose-6-phosphatase
Mn^{2+}	Arginase, Enolase, Pyruvate carboxylase
K^+	Pyruvate kinase
Ni^{2+}	Urease
Mo	Dinitrogenase, Xanthine oxidase
Se	Glutathione peroxidase

1.12.1 Naming and classification of enzyme

Many enzymes have common names. For example, trypsin, a proteolytic enzyme, is secreted by the pancreas. Common names provide little information about the reactions that enzymes catalyze. Many enzymes are named for their substrates and for the reactions that they catalyze, with the suffix-*ase* added. As for example, ATPase is an enzyme that helps in breaking down ATP, whereas ATP synthase is an enzyme that helps in synthesis of ATP. Because of the confusion that arose from these common names, an International Commission on enzymes was established to create a systematic basis for enzyme nomenclature.

The enzyme commission has developed a rule for naming enzymes. According to this rule, each enzyme is classified and named according to the type of chemical reaction it catalyzes. The *Enzyme Commission* (EC) has given each enzyme a number with four parts, like EC 2.7.1.2 (Hexokinase). The first three numbers define major *class*, *subclass*, and *sub-subclass*, respectively. The last number is a serial number in the sub-subclass, indicating the order in which each enzyme is added to the list.

Common name and EC numbers of some enzyme

Alcohol dehydrogenase	EC 1.1.1.1
Phosphofructokinase	EC 2.7.1.11
Glutamine synthetase	EC 6.3.1.2
Acetylcholinesterase	EC 3.1.1.7

Systematic classification

The first integer in the EC number designates the class of enzymes. There are six classes to which different enzymes belong. These classes are:

EC 1 Oxidoreductase

Oxidoreductase catalyzes oxidation-reduction reactions.

$A_{red} + B_{ox} \longrightarrow A_{ox} + B_{red}$

Example

Oxidases	Use oxygen as an electron acceptor but do not incorporate it into the substrate.
Dehydrogenases	Use molecules other than oxygen (e.g. NAD^+) as an electron acceptor.
Oxygenases	Directly incorporate oxygen into the substrate.
Peroxidases	Use H_2O_2 as an electron acceptor.

EC 2 Transferases

Transferases catalyze reactions that involve the transfer of groups from one molecule to another. Examples of such groups include amino, carboxyl, carbonyl, methyl, phosphoryl and acyl (RC=O). Common trivial names for the transferases often include the prefix *trans*.

A–B + C ⟶ A + B–C

Examples

Transcarboxylases	Transfers a carboxylate group to a substrate.
Transaminases	Transfer amino group from amino acids to keto acids.
Kinases	Transfer phosphate from ATP to a substrate.
Phosphorylases	Transfer inorganic phosphate to a substrate.

EC 3 Hydrolases

Hydrolases catalyze reactions in which the cleavage of bonds is accomplished by adding water.

$A–B + H_2O \longrightarrow A–H + B–OH$

Example

Phosphodiesterases	Cleave phosphodiester bonds.
Phosphatases	Remove phosphate from a substrate.
Peptidases	Cleave amide bonds such as those in proteins.

EC 4 Lyases

Lyases are enzymes that catalyzes the breaking of C—C, C—O, C—N, C—S and other bonds by means other than hydrolysis or oxidation. These bonds are cleaved by the process of elimination and the result in the formation of a double bond or a new ring, or conversely adding groups to double bonds.

A = B + HX ⟶ A–X + B–H

Example

Aldolases	Removal of aldehydes *via* elimination reactions.
Synthases	Link two molecules without involvement of ATP.
Dehydratases	Removal of H_2O *via* elimination reactions.
Decarboxylases	Removal of CO_2 *via* elimination reactions.

EC 5 Isomerases

Isomerases catalyze several types of intramolecular rearrangements and yield isomeric forms.

A–B ⟶ B–A

Example

Mutases	Catalyze the intramolecular transfer of functional groups.
Cis-trans isomerase	Catalyzes the isomerization of geometric isomers.
Epimerases	Catalyze the inversion of asymmetric carbon atoms.
Racemases	Interconvert L and D stereoisomers.

EC 6 Ligases

Ligases catalyze the formation of C—C, C—S, C—O, and C—N bonds with simultaneous hydrolysis of ATP. Other common names for ligases include *synthetases*, because they are used to synthesize new molecules.

$$A + B + ATP \longrightarrow A - B + ADP$$

Example

Carboxylases	Use CO_2 as a substrate.

Biochemical nomenclature distinguishes synthetases from synthases. *Synthases* catalyze condensation reactions in which no nucleoside triphosphate (ATP and GTP) is required as an energy source. *Synthetases* catalyze condensations that *do* use nucleoside triphosphate as a source of energy for the synthetic reaction. A synthase is a lyase and does not require any energy, whereas a synthetase is a ligase and thus requires energy.

1.12.2 What enzyme does?

A chemical reaction between two substances occurs only when an atom, ion, or molecule of one collides with an atom, ion, or molecule of the other. Only a fraction of the total collisions result in a reaction, because usually only a small percentage of the molecules interacting have the minimum amount of kinetic energy that a molecule must possess for it to react. When the reactants collide, they may form an intermediate product whose chemical energy is higher than the combined chemical energy of the reactants. In order for this transition state in the reaction to be achieved, some energy must enter into the reaction other than the chemical energy of the reactants. The transition state is the one with the highest free energy. The difference in free energy between the transition state and the reactants is called the *Gibbs free energy of activation* or simply the *activation energy*.

An enzyme lowers the activation energy of a reaction, thereby increasing the fraction of molecules that have enough energy to attain the transition state and making the reaction go faster in both directions. However, the catalyst does not change the relative energies of the initial and final states. The free energy of reaction, $\Delta G°$, remains unchanged in the presence of a catalyst, so the relative amounts of reactants and products at equilibrium are unchanged. In other words, a catalyst does not influence the position of equilibrium. It only increases the rate of a reaction by lowering the activation energy.

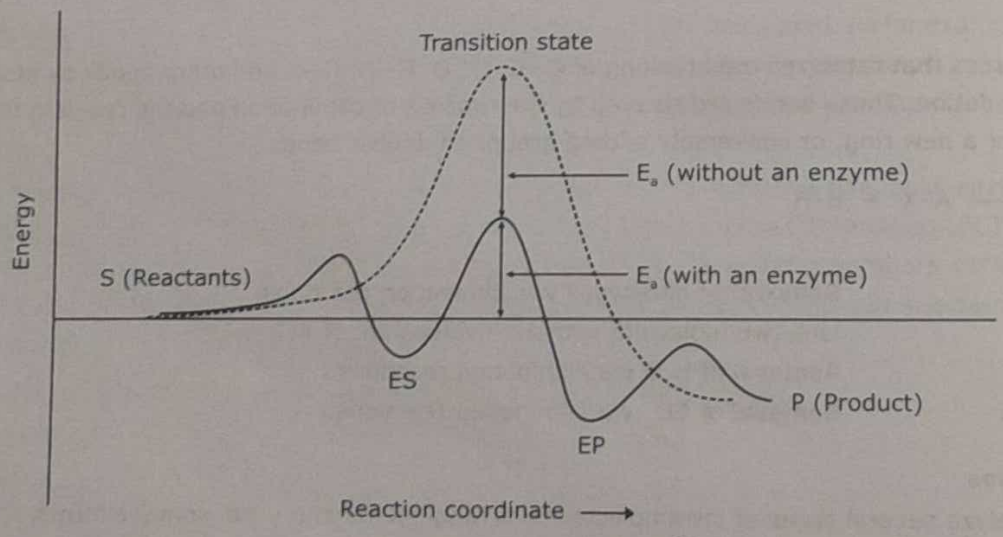


Figure 1.88 Energy profile of a simple enzyme-catalyzed reaction. The non-enzyme catalyzed reaction proceeds via a higher energy transition state and hence the reaction has a higher activation energy than the enzyme catalyzed reaction.

1.12.3 How enzymes operate?

In the first step of catalysis, the enzyme binds to the substrate, the compound to be catalyzed. The substrate binds to the active site of an enzyme by multiple weak non-covalent interactions like hydrophobic, H-bond, ionic interaction or reversible covalent bonds. The free energy released in the formation of a large number of weak interactions between the enzyme and the substrate is termed *binding energy*. *This binding energy is used to lower the activation energy*. Only the correct substrate can participate in most or all of the interactions with the enzyme and thus maximize binding energy. The full complement of such interactions is formed only when the substrate is converted into the transition state.

The binding of the substrate to the enzyme may be represented by:

$$E + S \rightleftharpoons E - S \rightleftharpoons E - P \rightleftharpoons E + P$$

Where E is the enzyme, S is the substrate, the product E – S is the enzyme-substrate complex and E – P is a complex of the enzyme and the product. The binding of enzyme and substrate is highly specific. A given enzyme usually binds to only one kind of substrate. The specificity of enzyme-substrate interactions arises mainly from hydrogen bonding, which is directional, and the shape of the active site, which rejects molecules that do not have a sufficiently complementary shape. The recognition of substrates by enzymes is accompanied by conformational changes at active sites, and such changes facilitate the formation of the transition state. However, the transition state is too unstable to exist for long. It collapses to either substrate or product, but which of the two accumulates is determined only by the free energy difference between the substrate and the product.

Enzyme-substrate complex

Two important models have been proposed to describe the binding process. The first, the **lock-and-key model**, assumes a high degree of similarity between the shape of the substrate and the geometry of the binding site on the enzyme.

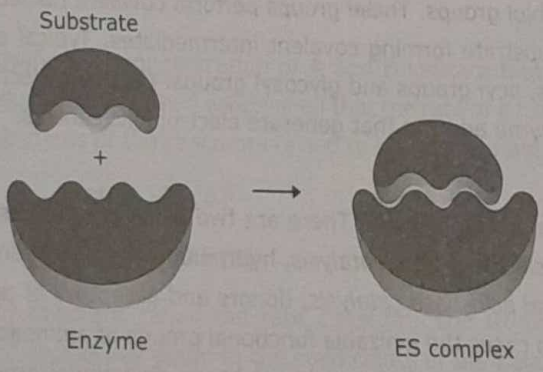


Figure 1.89 Lock-and-key model. In this model, the active site of the unbound enzyme is complementary in shape to the substrate.

The second model takes into account the fact that proteins have some three-dimensional flexibility. According to this **induced-fit model**, the binding of the substrate induces a conformational change in the enzyme that results in a complementary fit once the substrate is bound. The binding site has a different three-dimensional shape before the substrate is bound. When the substrate is bound and the transition state is subsequently formed, the bonds are rearranged. In the transition state, the substrate is bound close to atoms with which it has to react. As bonds are broken and new bonds are formed, the substrate is transformed into a product. The product is released from the enzyme.

Enzyme activity

Amounts of enzymes can either be expressed as molar amounts, or measured in terms of activity. Enzymes are usually present in very small quantities. So, a convenient method of enzyme quantification is a measurement of catalytic activity. Activity is then related to concentration. When enzymes are quantified relative to their activity rather than a direct measurement of concentration, the unit used to report enzyme levels are *activity units*. There are two standard units to express enzyme activity:

a. *Enzyme unit* (U) : The amount of enzyme causing transformation of 1 µmole of substrate per minute at 25°C under optimal conditions of measurement. 1 enzyme unit = 1 µmol min^{-1}.

b. *Katal* (kat) : The katal is the accepted SI unit of enzyme activity. One katal is that amount of enzyme that catalyzes the transformation of 1 mole of substrate per second. 1 katal = 1 mol sec^{-1}.

1U = 16.67 nanokat

1.12.4 Enzyme kinetics

Enzyme kinetics is the quantitative study of enzyme catalysis. Kinetic studies measure reaction rates and the affinity of enzymes for substrates and inhibitors. Kinetics also provides insight into reaction mechanisms.

The general principles of chemical reaction apply to enzyme-catalyzed reactions as well. In a chemical reaction of the form A + B → P, where A and B are the reactants and P is the product, the rate of reaction can be expressed in terms of either the rate of disappearance of one of the reactants or the rate of appearance of a product. The rate of disappearance of A is $-\Delta[A]/\Delta t$, where Δ symbolizes change, [A] is the concentration of A in moles/litre, and t is time. Likewise, the rate of disappearance of B is $-\Delta[B]/\Delta t$, and the rate of appearance of P is $\Delta[P]/\Delta t$. The rate of the reaction can be expressed in terms of any of these changes, because the rates of appearance of the product and disappearance of reactant are related by the stoichiometric equation for the reaction.

$$\text{Rate} = -\frac{\Delta[A]}{\Delta t} = -\frac{\Delta[B]}{\Delta t} = \frac{\Delta[P]}{\Delta t}$$

The negative signs for the changes in concentration of A and B indicate that A and B are being used up in the reaction, while P is being produced. It has been established that the rate of a reaction at a given time is proportional to the product of the concentrations of the reactants raised to the appropriate powers.

$$\text{Rate} \propto [A]^f [B]^g$$

or, as an equation,

$$\text{Rate} = k\,[A]^f [B]^g$$

where k is a proportionality constant called the *rate constant*. The exponents *f* and *g must be determined experimentally*. They are *not necessarily* equal to the coefficients of the balanced equation. The exponents in the rate equation are usually small whole numbers such as 1 or 2. (There are also some cases in which the exponent 0 occurs). The values of the exponents are related to the number of molecules involved in the detailed steps that constitute the mechanism. The *overall order* of a reaction is the sum of all the exponents. If, for example, the rate of a reaction A → P is given by the rate equation.

$$\text{Rate} = k[A]^1$$

where k is the rate constant and the exponent for the concentration of A is 1, then the reaction is *first order* with respect to A, and first order overall.

If the rate of a reaction is $A + B \rightarrow C + D$ then

$$\text{Rate} = k[A]^1\,[B]^1$$

(where k is the rate constant, the exponent for the concentration of A is 1, and the exponent for the concentration of B is 1), then the reaction is said to be first order with respect to A, first order with respect to B, and *second order* overall.

Many common reactions are first or second order. The possibility exists that exponents in a rate equation may be equal to zero, with the rate for a reaction A → B given by the equation.

$$\text{Rate} = k[A]^0 = k$$

Such a reaction is called zero order, and its rate, which is constant, does not depend on the concentrations of reactants, but depends on other factors such as the presence of catalysts.

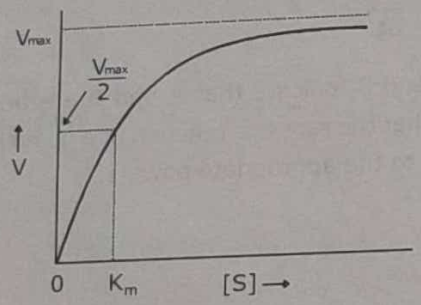
Kinetics of enzyme catalyzed reactions

A particularly useful model for the kinetics of enzyme-catalyzed reaction was devised in 1913 by *Leonor Michaelis* and *Maud Menten* when they studied the reaction in which the enzyme *invertase* hydrolyzes sucrose, a disaccharide, into monosaccharides, glucose and fructose. Michaelis-Menten described the relationship between reaction velocity and substrate concentration. To explain their results in the conversion of sucrose to glucose and fructose by the enzyme invertase, Michaelis and Menten proposed the following scheme of reactions:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1.1)$$

In this equation k_1 is the rate constant for the formation of the enzyme-substrate complex, ES, from the enzyme, E, and the substrate, S; k_{-1} is the rate constant for the reverse reaction, dissociation of the ES complex to free enzyme and substrate; and k_2 is the rate constant for the conversion of the ES complex to product P and the subsequent release of product from the enzyme.

According to Michaelis-Menten approach, when the rate (also called the velocity) of an enzyme catalyzed reaction is measured at varying substrate concentrations, the rate depends on the substrate concentrations [S]. At a relatively low concentration of substrate, initial velocity (V) increases almost linearly with an increase in a substrate concentration. The reaction reaches a maximum velocity (V_{max}) with an increase in substrate concentration and it doesn't increase any further by increasing the concentration of substrate.



The hyperbolic relationship between initial velocity (V) and substrate concentration [S] of an enzyme catalyzed reaction.

Concentration of substrate at which reaction velocity reaches half its maximum velocity is called **Michaelis constant** (K_m). It is the ratio of constants $(k_{-1} + k_2)/k_1$. The K_m is expressed as mole of substrate per litre. Lower value of K_m describes the greater affinity of the enzymes for the substrate.

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

Michaelis and Menten put forward a mathematical equation to establish the mathematical relationship among the quantities [E], [S], V_{max} and K_m.

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

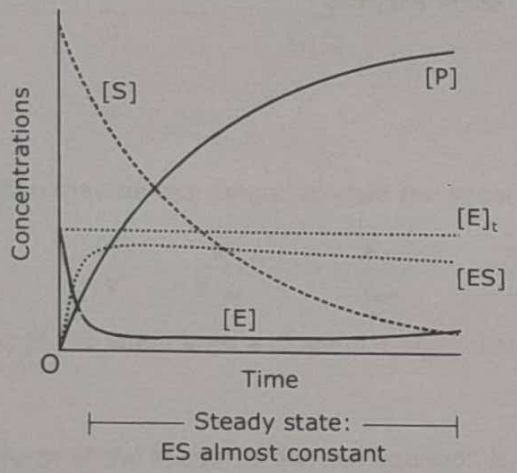
1. When $[S] << K_m$, then $V \propto [S]$
2. When $[S] >> K_m$, then $V = V_{max}$
3. When $[S] = K_m$, then $V = \frac{V_{max}}{2}$

According to the Michaelis Menten equation, the reaction velocity versus substrate concentration shows the *hyperbolic plot*. Michaelis and Menten noted two significant features about the kinetics of this reaction. When the substrate concentration was kept constant and the enzyme concentration was increased, the rate of reaction increased. But when the enzyme concentration was kept constant and the substrate concentration increased, the reaction rate increased to a maximum value and then leveled off. It is caused by the saturation of all the available enzyme binding sites by substrate molecules. Since at low concentration of the substrate, velocity is proportional to substrate concentration [S], the enzyme catalyzed reaction is a *first order reaction*. At high [S], velocity becomes virtually independent of [S] and approaches a maximal limit. Since rate is no longer dependent on [S] at high concentration, the enzyme catalyzed reaction obey *zero order kinetics*. Michaelis-Menten model is based on the assumptions. The following assumptions are made in driving the Michaelis-Menten rate equation:

1. The concentration of substrate [S] is much greater than the concentrations of enzyme [E].
2. The rate of formation of ES is equal to that of the breakdown of ES (*the steady state assumption*).
3. Very little accumulation of P, so the formation of enzyme-substrate complex from E + P is negligible.

Steady state assumption

The interpretation of the Michaelis-Menten model were refined by Briggs and Haldane by an assumption termed the steady state assumption. The steady state hypothesis states that the concentration of enzyme-substrate complex remains constant through much of the reaction. When the enzyme and substrate are first mixed, the concentration [ES] will rise rapidly from zero to a so called steady state level as illustrated in the figure below.



Change in concentrations over time for enzyme E, substrate S, complex ES and product P

For a simple enzyme-catalyzed reaction ($E + S \rightleftharpoons ES \rightarrow E + P$), the graph shows how the concentrations of substrate [S], free enzyme [E], enzyme-substrate complex [ES], and product [P] vary with time. After a very brief initial period, [ES] reaches a steady state in which ES is consumed approximately as rapidly as it is formed. So,

$$\frac{d[ES]}{dt} = 0$$

The amounts of E and ES are greatly exaggerated in the graph for clarity. Note that $[E]+[ES]=[E_t]$, or total enzyme concentration, and that [ES] actually falls very slowly as substrate is consumed, while [E] accordingly rises.

Significance of K_m and V_{max}

The Michaelis constant, K_m, varies considerably from one enzyme to another, and also with different substrates for the same enzyme. By definition, it is equal to the substrate concentration at half the maximum rate. In another way, K_m represents the substrate concentration at which half the enzyme active sites are filled by substrate molecules. The value of K_m is sometimes equated with the *dissociation constant* (K_S) of the enzyme-substrate complex, ES. However, this is true only when $k_2 << k_{-1}$ so that

$$K_m = \frac{k_{-1} + k_2}{k_1} = \frac{k_{-1}}{k_1} + \frac{k_2}{k_1} = K_S + \frac{k_2}{k_1}$$

Since K_s is the dissociation constant of the enzyme-substrate complex, as K_s decreases, the enzyme's affinity for substrate increases. K_m is therefore also a measure of the affinity of the enzyme for its substrate providing k_2/k_1 is small compared with K_s.

K_m depends on temperature, the nature of the substrate, pH, ionic strength, and other reaction conditions; therefore, its value serves to characterize a particular enzyme-substrate system under specific conditions. The maximum rate, V_{max}, has a well-defined meaning, both theoretically and empirically. It represents the maximum rate attainable; that is, it is the rate at which the total enzyme concentration is present as the enzyme-substrate complex. Mathematically it can be represented as

$$V_{max} = K_2 [E_t] \qquad E_t = \text{Total enzyme concentration}$$

Turnover number (K_{cat})

Turnover number of an enzyme is the number of substrate molecules converted into the product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. The term K_{cat} represents the kinetic efficiency of the enzyme. Its unit is sec^{-1}.

At saturating [S], $\qquad V = V_{max} = K_2 [E_t]$

$$\text{Thus, } K_2 = \frac{V_{max}}{E_t} = K_{cat}$$

Table 1.23 Values of K_{cat} (Turnover number) for some enzymes

Enzyme	K_{cat} (sec^{-1})
Catalase	40,000,000
Carbonic anhydrase	1,000,000
Acetylcholinesterase	14,000
Penicillinase	2,000
Lactate dehydrogenase	1,000
Chymotrypsin	100
DNA polymerase I	15
Lysozyme	0.5

Significance of K_{cat} and K_{cat} / K_m

An enzyme with a high K_m requires a higher substrate concentration to achieve a given reaction velocity than an enzyme with a low K_m for the same K_{cat}. The K_{cat} is the turnover number for the enzyme. At very low substrate concentrations, where $[S] << K_m$, most of the enzyme is free. Thus we can think of $[E] = [E_t]$, where E_t is the total concentration of enzyme. So that the Michaelis-Menten equation becomes

$$V = \frac{K_{cat}}{K_m} [E][S]$$

Thus, the ratio K_{cat} / K_m is equivalent to the rate constant for the reaction between the free enzyme and the free substrate. K_{cat} / K_m ratio called **specificity constant** is often thought of as a measure of enzyme efficiency. Note that either a large value of K_{cat} (which means rapid turnover) or a small value of K_m (which means high affinity for the substrate) will make K_{cat} / K_m large. A comparison of K_{cat} / K_m for the same enzyme with different substrates, or for two enzymes with their different substrates, is widely used as a measure of enzyme catalytic efficiency.

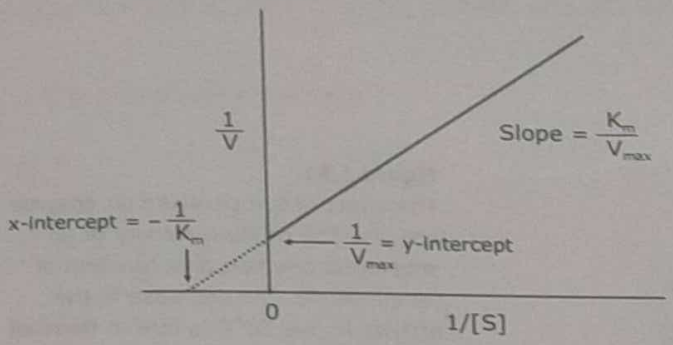
Table 1.24 Turnover number and specificity constants for some enzymes

Enzyme	K_{cat}	K_{cat} / K_m
Catalase	4×10^7	4×10^8
Carbonic anhydrase	4×10^5	1.5×10^7
Urease	1.0×10^4	4×10^5

Lineweaver-Burk plot (or Double reciprocal plot)

It is very difficult to determine the V_{max} directly from a plot of V against [S] and therefore K_m cannot readily be determined in this way either. To overcome these difficulties Michaelis-Menten equation can be rearranged in a number of ways to give convenient graphical representations. If an enzyme obeys Michaelis-Menten kinetics, a plot of the reciprocal of the reaction velocity 1/V as a function of the reciprocal of the substrate concentration 1/[S] transforms Michaelis-Menten equation into a straight line equation. Plot between the reciprocal of V and [S] is known as *Lineweaver-Burk* or *double reciprocal plot*.

Lineweaver-Burk equation : $$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$



Lineweaver-Burk graphical procedure for determining the two steady-state kinetic parameters in the Michaelis-Menten equation.

Hanes-Woolf plot

The Lineweaver-Burk equation may be rearranged to yield the linear equation for the *Hanes-Woolf plot*.

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

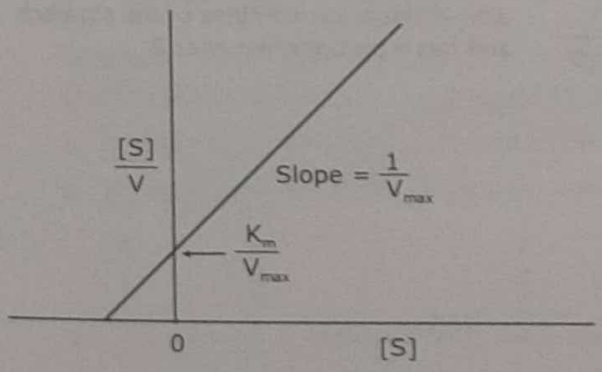
Thus, a plot of [S]/V versus [S] is linear with a slope of $1/V_{max}$. The intercept on the [S]/V axis gives K_m / V_{max}.

Eadie-Hofstee plot

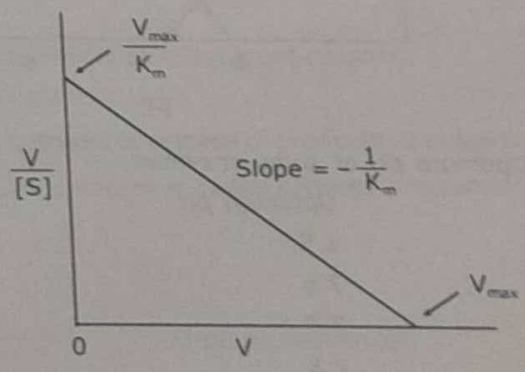
Another widely used linear form of the Michaelis-Menten equation is the *Eadie-Hofstee plot*, which is described by

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

The plot of V/[S] versus V is linear with slope of $-1/K_m$. The intercept on the V axis gives V_{max}.



Hanes plot



Eadie-Hofstee plot

Effect of temperature and pH

Effect of temperature

Since most of enzymes are proteins so they unfold and denature at high temperature. This denaturation causes a decrease or loss of catalytic activity, which in turn decreases the rate of reaction. Similarly low temperature also inactivates the enzyme. Within limits, the rate of an enzyme catalyzed reaction increases with increase in temperature because the greater kinetic energy of both enzyme and substrate molecules ensures more frequent collisions. Thus, an enzyme-catalyzed reaction achieves its maximum reaction at an intermediate temperature called *optimum temperature*. The optimum temperature varies greatly from enzyme to enzyme and especially from organism to organism. The reaction rate of most enzymes of homeotherms is maximum at about 40°C. Some enzymes retain activity at unusually high temperatures. For example, enzyme like Taq DNA polymerase present in thermophilic bacteria *Thermus aquaticus* has optimal temperature 72°C.

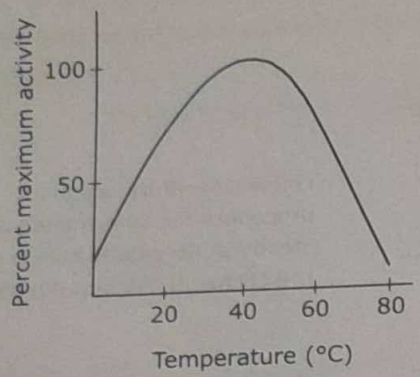


Figure 1.91
The effect of temperature on enzyme activity. The relative activity of an enzymatic reaction as a function of temperature. The decrease in the activity above 50°C is due to thermal denaturation.

Effect of pH

Enzymes are sensitive to pH. This pH dependence is due to the presence of charged amino acids at the active site. Variation in pH cause changes in overall charge of amino acids. Thus, each enzyme has its optimal pH at which rapid activity occurs. For example, optimal pH for pepsin (protein digesting enzyme) is 2 and for trypsin it is 8.5. When the activity is plotted against pH, a *bell-shaped curve* is usually obtained.

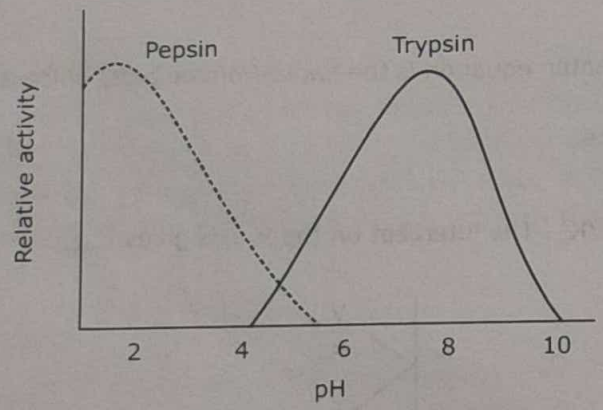


Figure 1.92
The pH activity profiles of two different enzymes. Trypsin, an intestinal protease, has a slightly alkaline pH optimum, whereas pepsin, a gastric protease, acts in the acidic confines of the stomach and has a pH optimum near 2.

Table 1.25 Optimum pH of some enzymes

Enzyme	*Optimum pH*
Pepsin	1.5
Catalase	7.6
Trypsin	7.7
Fumarase	7.8
Ribonuclease	7.8
Arginase	9.7

Problem

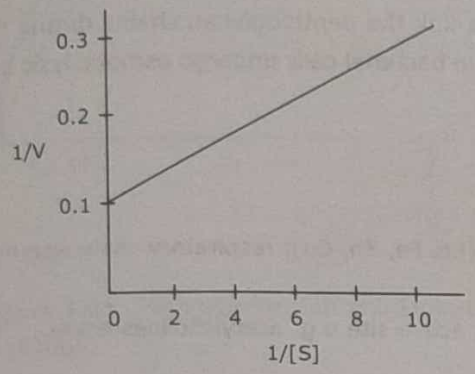
The following data are obtained for an enzyme-catalyzed reaction.

[S](mM)	V(mmol ml^{-1} min^{-1})
0.1	3.33
0.2	5.00
0.5	7.14
0.8	8.00
1.0	8.33
2.0	9.09

1. From a double-reciprocal plot of the data, determine V_{max}.
2. If the concentration of enzyme is 10^{-6} M, then calculate its turnover number.

Solution

1. $V_{max} = 1/0.1 = 10$ mmol ml^{-1} min^{-1}.



2. Turnover number = $V_{max}/[E_t] = 10/10^{-6} = 10^7$ min^{-1}.

Kinetics of bi or multireactant system

So far, we have considered enzyme catalyzed reactions with single substrate. This situation is not common. Usually, enzymes catalyze reactions are those in which two (or more) substrates take part. Consider the case of an enzyme catalyzing a reaction involving two substrates A and B, and yielding the products P and Q:

$$A + B \rightleftharpoons P + Q$$

Such a reaction is termed as *bisubstrate reaction*. In general, bisubstrate reactions proceed by one of the two possible routes:

1. Both A and B are bound to the enzyme and then reaction occurs to give P + Q:

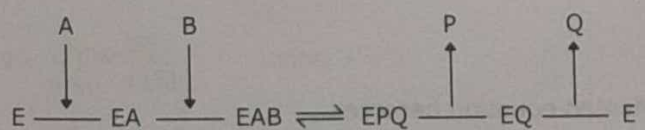
   $$E + A + B \rightleftharpoons E + P + Q$$

   Reactions of this type are defined as *sequential* or *single-displacement reactions*.

   Sequential reactions can be either of two distinct classes:

   a. If there is no obligatory order of addition of substrates or release of products, it is called *random sequential*.

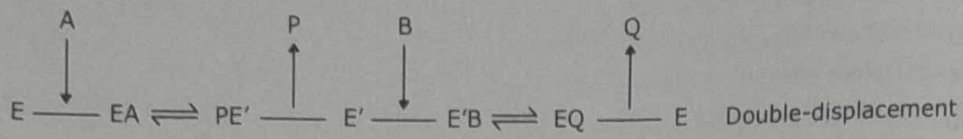
   b. If substrates add in an obligatory order, the mechanism is called *ordered sequential*.



Ordered sequential

In ordered sequential reactions, one substrate is obligated to bind to the enzyme before a second substrate. In random sequential mechanisms, there is no preference. In practice, there is usually some degree of order in binding.

2. The other general possibility is that one substrate, A, binds to the enzyme and reacts with it to yield a chemically modified form of the enzyme (E′) plus the product, P. The second substrate, B, then reacts with E′, regenerating E and forming the other product, Q. Reactions that fit this model are called *ping-pong* or *double-displacement reactions*.



1.12.5 Enzyme inhibition

Inhibition of enzyme activity may be *irreversible* or *reversible*. Irreversible inhibitors usually bound covalently to the enzyme and destroy the functional group in the active site. Most of irreversible inhibitors are toxic substances. The antibiotic penicillin acts as an irreversible inhibitor of the enzyme *glycopeptide transpeptidase* (also known as glycoprotein peptidase). Penicillin exerts its effects by covalently reacting with an essential serine residue in the active site of glycopeptide transpeptidase, an enzyme that acts to cross-link the peptidoglycan chains during the synthesis of bacterial cell walls. Once the cell wall synthesis is blocked, the bacterial cells undergo osmotic lysis and bacterial growth is halted.

Table 1.26 Examples of irreversible enzyme inhibitors

Name	*Mode of action*
Cyanide	Reacts with enzyme metal ions (i.e. Fe, Zn, Cu); respiratory chain enzymes are primary targets.
Diisopropyl phosphofluoridate (DIPF)	Inhibits enzymes with serine at active site e.g. acetylcholinesterase.
Sarin (nerve gas)	Like DIPF
Physostigmine	Like DIPF
Parathion (insecticide)	Like DIPF, but especially inhibitory to insect acetylcholinesterase.

In *reversible inhibition*, the inhibitor can dissociate from the enzyme. Reversible inhibitors involve the non-covalent binding with enzymes. Three common types of reversible inhibition are competitive, uncompetitive and noncompetitive inhibition.

Competitive inhibition

The structure of a competitive inhibitor closely resembles that of the enzyme's normal substrate. Because of its structure, a competitive inhibitor binds reversibly to the enzyme's active site. The inhibitor forms an enzyme-inhibitor complex (EI) that is equivalent to the ES complex. The effect of a competitive inhibitor on activity can be reversed by increasing the concentration of substrate. At high [S], all the active sites are filled with substrate, and reaction velocity reaches the value observed without an inhibitor.

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

$$+$$

$$I$$

$$\Updownarrow K_I \qquad K_I = \frac{[E][I]}{[EI]}$$

$$EI$$

In the presence of a competitive inhibitor, the Michaelis-Menten equation becomes

$$V = \frac{V_{max}[S]}{\alpha K_m + [S]} \qquad \text{Where, } \alpha = 1 + \frac{[I]}{K_I}$$

In a double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

In competitive inhibition, V_{max} stays same and K_m increases, but the inhibitor does not affect the turnover number of the enzyme. Clinical treatment of methanol poisoning is a classical example of the exploitation of competitive inhibitory mechanism. In the case of methanol poisoning, methanol in the body is converted to harmful formaldehyde by alcohol dehydrogenase. A high dose of ethanol is used to alleviate the effect of methanol because ethanol competitively binds with the active site of alcohol dehydrogenase.

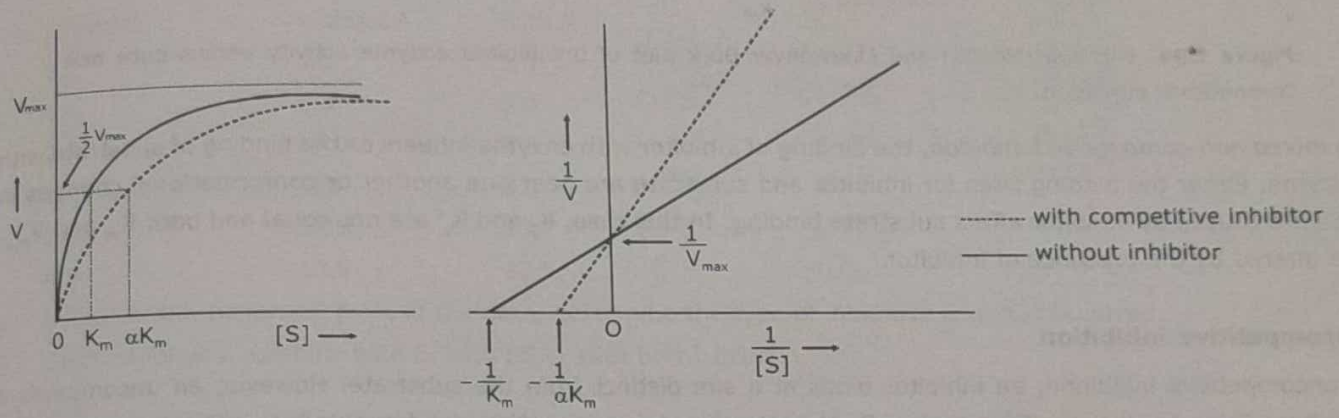


Figure 1.93 Michaelis-Menten and Lineweaver-Burk plot of uninhibited enzyme activity versus competitive inhibition.

Non-competitive inhibition

In non-competitive inhibition, the inhibitor binds to the enzyme at a site other than the active site. Inhibitor binding alters the enzyme's three-dimensional configuration and blocks the reaction. There are two types of non-competitive inhibition— pure and mixed. In *pure non-competitive inhibition*, substrate and inhibitor bind at different sites on enzyme and binding of inhibitor does not affect binding of substrate. The inhibitor binds to *either* free enzyme or the ES complex. Hence, inhibition is not reversed by increasing the concentration of substrate.

$$\begin{array}{ccccc} E + S & \rightleftharpoons & ES & \longrightarrow & E + P \\ + & & + & & \\ I & & I & & \\ \Big\downarrow\Big\uparrow K_I & & \Big\downarrow\Big\uparrow K_I' & & \\ EI & & ESI & & \end{array}$$

$$K_I = \frac{[E]\,[I]}{[EI]} \quad \text{and} \quad K_I' = \frac{[ES]\,[I]}{[ES\,I]}$$

Pure non-competitive inhibition occurs if $K_I = K_I'$. In this type of inhibition, *V_{max} decreases and K_m stays constant.* In the presence of pure non-competitive inhibitor, the Michaelis-Menten equation becomes

$$V = \frac{V_{max}[S]}{\alpha K_m + \alpha'[S]} \qquad \text{Where, } \alpha' = 1 + \frac{[I]}{K_I'} \text{ and } \alpha = 1 + \frac{[I]}{K_I}$$

$$V = \frac{V_{max}[S]}{\alpha K_m + \alpha[S]} \qquad \text{Since, } \alpha' = \alpha$$

In a double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha}{V_{max}}$$

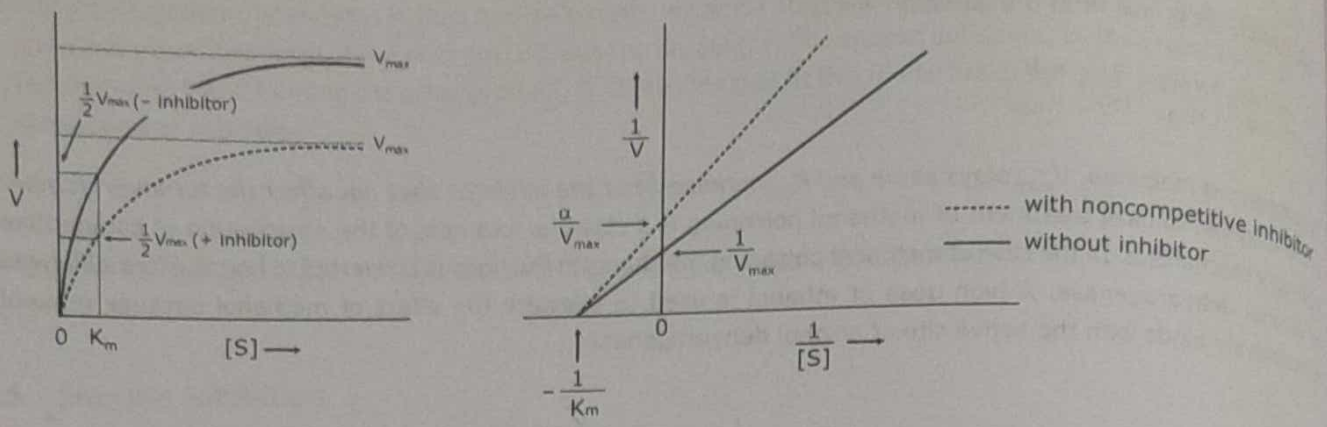


Figure 1.94 Michaelis-Menten and Lineweaver-Burk plot of uninhibited enzyme activity versus pure non-competitive inhibition.

In *mixed non-competitive inhibition*, the binding of inhibitor with enzyme influences the binding of substrate with enzyme. Either the binding sites for inhibitor and substrate are near one another or conformational changes in enzyme caused by inhibitor affect substrate binding. In this case, K_I and K_I' are not equal and both K_m and V_{max} are altered by the presence of inhibitor.

Uncompetitive inhibition

In uncompetitive inhibition, an inhibitor binds at a site distinct from the substrate. However, an uncompetitive inhibitor will bind *only* to the ES complex. On the other hand noncompetitive inhibitor binds to *either* free enzyme or the ES complex. In uncompetitive inhibition, apparent V_{max} and K_m both decrease.

$$E + S \rightleftharpoons ES \longrightarrow E + P$$
$$+$$
$$I$$
$$\Updownarrow K_i'$$
$$ESI$$

$$K_I' = \frac{[ES]\,[I]}{[ESI]}$$

In the presence of uncompetitive inhibitor, the Michaelis-Menten equation becomes

$$V = \frac{V_{max}\,[S]}{K_m + \alpha'\,[S]} \qquad \text{where, } \alpha' = 1 + \frac{[I]}{K_I'}$$

In a double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

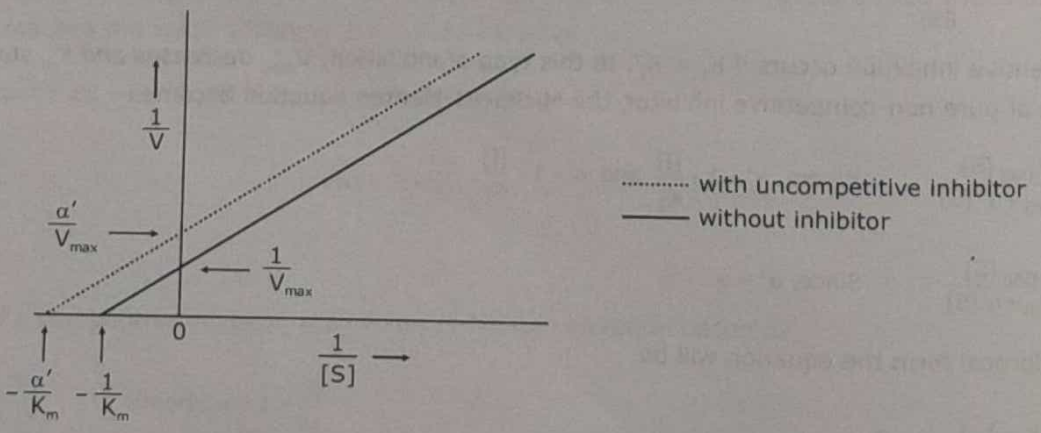


Figure 1.95 Lineweaver-Burk plot of uninhibited enzyme activity versus uncompetitive inhibition.

Table 1.27 Effects of inhibitors on the parameters of the Michaelis-Menten equation

Type of inhibition	V_{max}		K_m	
None	V_{max}		K_m	
Competitive	V_{max}	(No change)	αK_m	(Increased)
Pure noncompetitive	V_{max}/α	(Decreased)	K_m	(No change)
Uncompetitive	V_{max}/α'	(Decreased)	K_m/α'	(Decreased)

Problem

The following data are obtained for an enzyme-catalyzed reaction in the presence and absence of inhibitor A.

	V(mmol ml^{-1} min^{-1})				
[S](mM)	Without A	With A	$\frac{1}{[S]}$	$\frac{1}{V}$(without A)	$\frac{1}{V}$(A)
0.2	5.0	3.0	0.25	0.07	0.08
0.4	7.5	5.0	0.5	0.08	0.09
0.8	10.0	7.5	1.00	0.09	0.12
1.0	10.7	8.3	1.25	0.1	0.13
2.0	12.5	10.7	2.5	0.133	0.2
4.0	13.6	12.5	5	0.2	0.33

1. Using double-reciprocal plots of the data, determine the type of inhibition that has occurred.
2. Does inhibitor A combine with E, with ES or with both? Explain.

Solution

1. The double-reciprocal plots intersect on the y-axis, so the inhibition is competitive.
2. The inhibitor combined only with E, the free enzyme. A competitive inhibitor cannot combine with ES because the inhibitor and the substrate compete for the same binding site on the enzyme.

1.12.6 Regulatory enzymes

Regulatory enzymes exhibit increased or decreased catalytic activity in response to specific signals. Activities of regulatory enzymes can be modulated through reversible non-covalent binding by regulatory compounds or by reversible covalent modification.

Regulation by non-covalent modification : Allosteric enzymes

Allosteric enzymes (Greek words: *allo* meaning other and *steric* meaning place or site) are a class of regulatory enzymes which increases or decreases catalytic activities in response to certain signals. Allosteric enzymes function through *reversible*, noncovalent binding of regulatory compounds called *modulators* or *effectors*. The modulators for allosteric enzymes may be inhibitory or stimulatory. Often the modulator is the substrate itself; regulatory enzymes for which substrate and the modulator are identical are called **homotropic**. When the modulator is a molecule other than the substrate, the enzyme is said to be **heterotropic**.

Most allosteric enzymes are oligomeric (consisting of multiple subunits). Allosteric enzymes may have two functionally different binding sites. One of the site, the active site, binds the substrate and catalyzes the reaction. The other type of site, known as *allosteric* or *regulatory site* binds with molecule which is called a modulator. Modulator molecules are of two types: positive modulator (called *activators*) that enhance enzyme activity and negative modulator (or *inhibitors*) which inhibit enzyme activity. Binding of modulator causes conformational changes in the allosteric enzymes which influence their catalytic activity. Enzymes with several modulators generally have different specific binding sites for each.

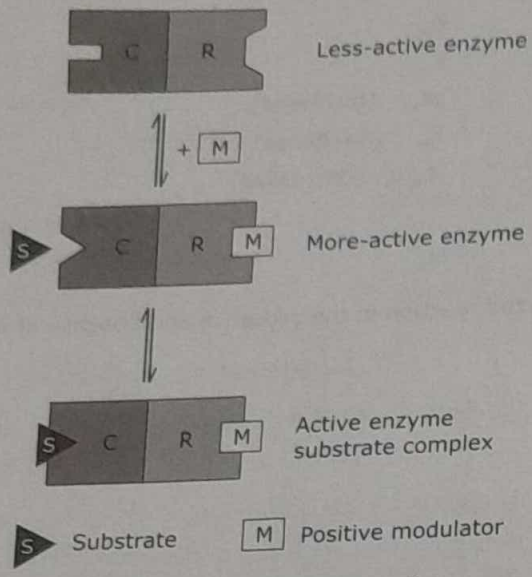
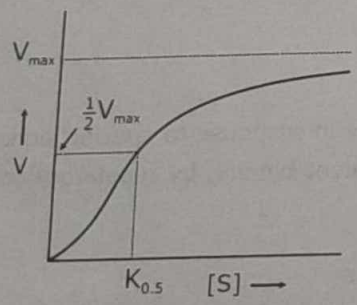
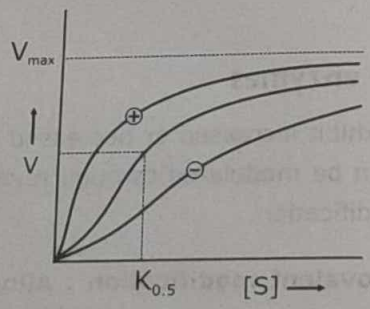


Figure 1.96
Allosteric enzymes are a class of regulatory enzymes which increases or decreases catalytic activities in response to modulators.

Allosteric enzymes don't obey Michaelis-Menten kinetics. Allosteric enzymes show relationships between V_0 and [S] that differ from Michaelis-Menten kinetics. They do exhibit saturation with the substrate when (S) is sufficiently high, produce a sigmoid saturation rather than the hyperbolic curve typical of non-regulatory enzymes. Sigmoid kinetic behavior generally reflects cooperative interactions between protein subunits. The principles are particularly well illustrated by a nonenzyme; O_2 binding to hemoglobin. Sigmoid kinetic behavior is explained by the *concerted* and *sequential models* for subunit interaction.



The sigmoid curve of a homotropic allosteric enzyme



The effects of a positive modulator (+) and a negative modulator (−) on heterotropic allosteric enzyme. The central curve shows the substrate-activity relationship without a modulator

Regulation by reversible covalent modification

Activity of some regulatory enzymes is modulated by *reversible covalent modification*. Covalent modification includes phosphorylation, adenylylation, methylation, uridylylation and ADP-ribosylation. Phosphorylation is the most common type of covalent modification. An important example of regulation by phosphorylation occurs in *glycogen phosphorylase* of muscle and liver, which catalyzes the glycogenolysis. Glycogen phosphorylase occurs in two forms: Glycogen phosphorylase *a* (active form) and glycogen phosphorylase *b* (less active form). In glycogen phosphorylase *a*, specific *Ser* residues, one on each subunit, are phosphorylated. Glycogen phosphorylase *a* is converted to the less active glycogen phosphorylase *b* by the enzymatic loss of these phosphoryl group. Glycogen phosphorylase *b* can be reconverted to glycogen phosphorylase *a* by the action of phosphorylase kinase.

Table 1.28 Some enzymes whose activity can be controlled by reversible covalent modification

Enzyme	*Modification*
Glycogen phosphorylase	Phosphorylation
Glycogen synthase	Phosphorylation
Acetyl-CoA carboxylase	Phosphorylation
Glutamine synthetase (*E. coli*)	Adenylylation
Nitrogenase	ADP-ribosylation

Feedback inhibition and feedback repression

In *feedback inhibition* (or end product inhibition), the end product of a biosynthetic pathway inhibits the *activity* of the first enzyme that is unique to the pathway, thus controlling production of the end product. The first enzyme in the pathway is an allosteric enzyme. Its allosteric site will bind to the end product of the pathway which alters its active site so that it cannot mediate the enzymatic reaction. It is a negative regulation. It prevents enzyme from acting. Enzymes can also be subject to positive regulation, in which the enzyme's activity is stimulated by regulatory molecule rather than being suppressed.

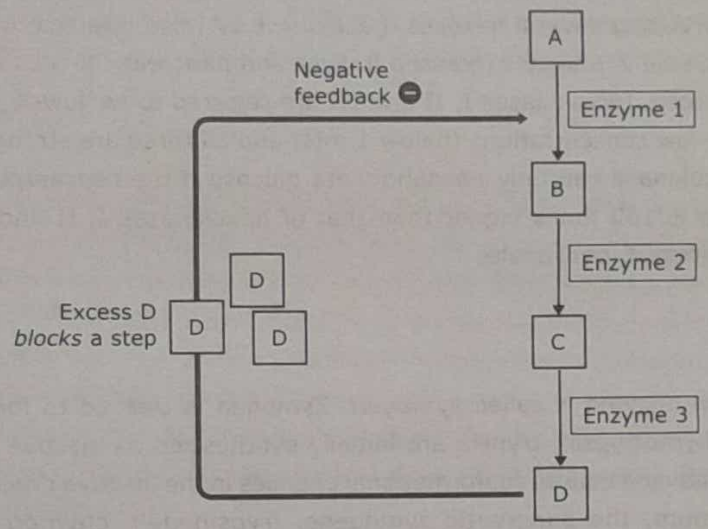


Figure 1.97 The pathway is regulated by the process of feedback inhibition. Compound D, the end product of the pathway, is the effector molecule that binds to the allosteric site of enzyme 1, the first enzyme in the pathway. When compound D is bound to the enzyme the catalytic (active) site of enzyme 1 is altered so that it is unable to react with its substrates and the synthesis of B is inhibited.

If a metabolic pathway branches, leading to the synthesis of two products, each end product can control its own synthesis without affecting the other.

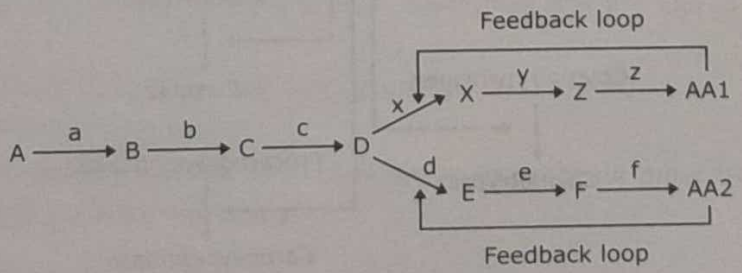


Figure 1.98 Generalized scheme for regulation of a branched metabolic pathway by the process of feedback inhibition.

The feedback inhibition is different from feedback repression. An inhibitory feedback system in which the end product produced in a metabolic pathway acts as a co-repressor and represses the *synthesis* of an enzyme that is required at an earlier stage of the pathway is called *feedback repression*.

1.12.7 Isozymes

Multiple forms of an enzyme that catalyzes the same reaction but differ from each other in their amino acid sequence composition, substrate affinity, V_{max}, and /or regulatory properties are described as *isozymes* (isoenzymes). A common example is lactate dehydrogenase (LDH) which catalyzes the reversible conversion of pyruvate to lactate. There are five LDH isoenzymes. Each is a homo- or heterotetramer of one or two different types of subunits called H and M. Two different subunits combine randomly with each other forming five isozymes.

HHHH (H_4)	LDH1	In the heart and RBCs
HHHM (H_3M)	LDH2	In the reticuloendothelial system
HHMM (H_2M_2)	LDH3	In the lungs
HMMM (HM_3)	LDH4	In the kidneys, placenta
MMMM (M_4)	LDH5	In the liver and striated muscle

Similarly, there are four important mammalian **hexokinase** isozymes that vary in subcellular locations and kinetics with respect to different substrates and conditions. They are designated hexokinases I, II, III and IV or hexokinases A, B, C and D. Hexokinases catalyze the phosphorylation of hexose (such as glucose). While hexokinases I, II and III are capable of phosphorylating several hexoses, hexokinase IV (also referred to as *glucokinase*) acts only on glucose as hexose. Glucokinase is mainly expressed in liver and pancreatic β-cells and is characterized by a low affinity to its substrate glucose. Hexokinases I, II and III are referred to as 'low-K_m' isozymes because of a high affinity for glucose even at low concentrations (below 1 mM) and all three are strongly inhibited by their product, glucose-6-phosphate. Glucokinase can only phosphorylate glucose if the concentration of this substrate is high enough (its K_m for glucose is 100 times higher than that of hexokinases I, II and III) and is not allosterically inhibited by its product, glucose-6-phosphate.

1.12.8 Zymogen

An inactive precursor of an enzyme is called *zymogen*. Zymogen is cleaved to form the active enzyme. Many proteolytic enzymes like chymotrypsin, trypsin are initially synthesized as inactive precursor chymotrypsinogen and trypsinogen. Specific cleavage causes conformational changes in the inactive precursor that expose the enzyme active site. In the duodenum, the pancreatic zymogens, *trypsinogen, chymotrypsinogen, proelastase* and *procarboxypeptidase* are converted into active enzymes by enteropeptidase and trypsin, as shown in figure 1.99.

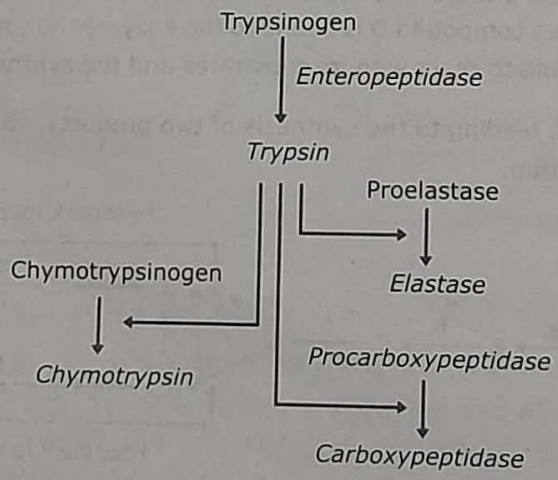


Figure 1.99 Activation of pancreatic zymogens.

Chymotrypsinogen, a single polypeptide chain of 245 amino acid residues, is converted to α-chymotrypsin, which has three polypeptide chains linked by two of the five disulfide bonds present in the primary structure of chymotrypsinogen.

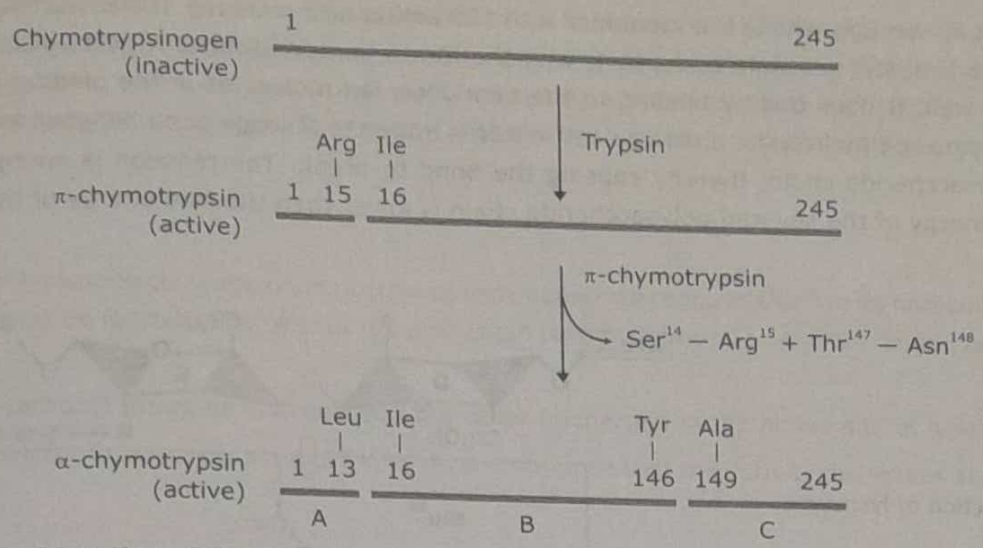


Figure 1.100 Formation of chymotrypsin (active) from chymotrypsinogen (inactive). The numbering of amino acid residues represents their positions in the primary sequence of the zymogen, chymotrypsinogen. The three polypeptide chains (A, B and C) of chymotrypsin are linked by disulfide bonds.