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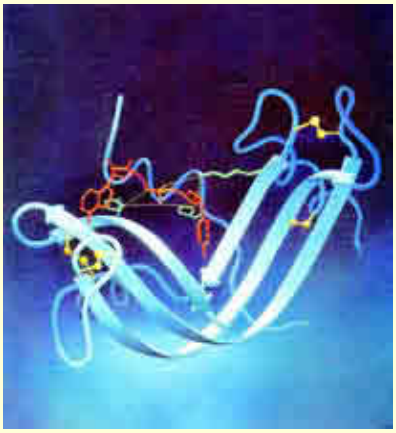


Bovine pancreatic ribonuclease S in complex with a non-hydrolyzable substrate analogue, the dinucleotide of the phosphonate UpcA.

CHAPTER

18

Enzymes-III
Mechanism of Enzyme Action

ENERGY MECHANICS OF ENZYMATIC REACTIONS

A simple enzymatic reaction might be written :

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

where, E, S and P represent enzyme,substrate and product, respectively. ES and EP are complexes of the enzyme with substrate and with the product, respectively.

For a better understanding of the kinetics of a chemical reaction, the two terms, reaction equilibria and reaction rates must be differentiated. The function of a catalyst is to increase the *rate* of a reaction. Catalysts do not affect reaction *equilibria*. Any reaction, such as $S \rightleftharpoons P$, can be described by a **reaction coordinate diagram** (Fig. 18–1). Energy in biological systems is described in terms of free energy, G. In the coordinate diagram, the free energy of the system is The free energy of the system is plotted against the progress of the reaction. Energy diagrams describe the energetic course of the reaction (vertical axis) and the progressive chemical changes (horizontal axis) as the substrate is converted to product.

S = free energy of the substrate

P = free energy of the product

$\neq$ = transition state

$\Delta G^{\neq}$ = activation energies for the two reactions, $S \rightarrow P$ and $P \rightarrow S$

$\Delta G^{\circ\prime}$ = overall standard free-energy change in moving from S to P

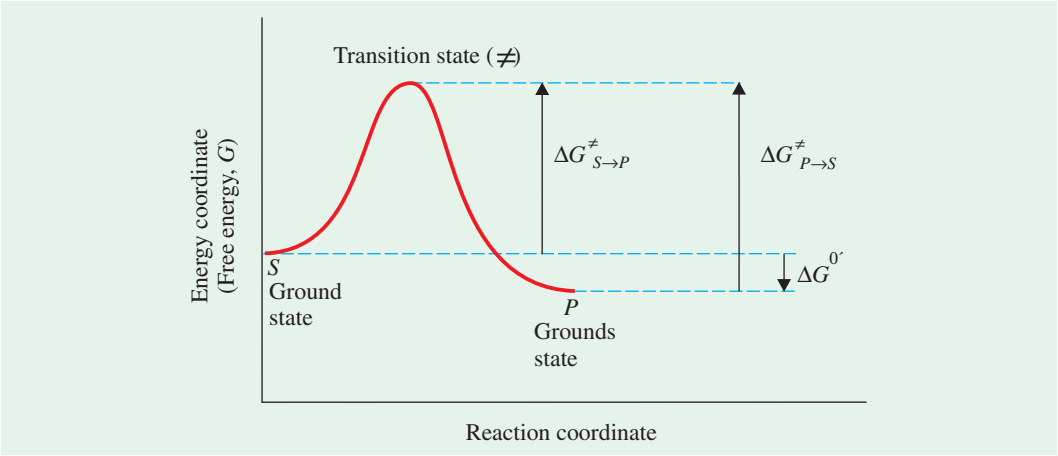


Fig. 18–1. Free energy diagram for a simple chemical reaction, S ⇌ P

described in terms of free enery, *G*. In the coordinate diagram, the free energy of the system is plotted against the progress of the reaction (or reaction coordinate). In its normal stable form or **ground state**, any molecule contains a specific amount of free energy. Chemists express the free energy change for this reacting system under standard set of conditions (temperature, 298 K; partial pressure of gases, each 1 atm or 101.3 kPa ; pH = 0; concentration of solutes, each 1M) and call this as **standard free-energy change, ΔG°**. Because biochemical systems commonly involve H+ concentrations far from 1M, biochemists define a constant $\Delta G^{\circ\prime}$, the standard free-energy change at pH 7.0.

The equilibrium between S and P reflects the difference in the free energy of their ground states. In the example shown in Fig., the free energy of the ground state of P is lower than that of S, hence $\Delta G^{\circ\prime}$ for the reaction is negative and the equilibrium favours P. This equilibrium is *not* affected by any catalyst. A favourable equilibrium, however, *does not* mean that S → P conversion is fast. The rate of a reaction, in fact, depends on an entirely different parameter. There exists an energetic barrier between S and P that represents the energy required for alignment of reacting groups, bond rearrangements and other changes needed for the reaction to occur in either direction. To undergo reaction, the molecules must overcome this barrier or "energetic hill" and therefore must be raised to a higher energy level. At the top of energy hill is a point at which decay to the S or P state is equally probable, which is downhill either way. This is called the **transition state** and should not be confused with a reaction intermediate. It is simply a moment of fleeting molecules in which certain events (bond breakage, bond formation, charge development etc) have proceeded to decide the future course of the reaction, *i.e.*, a collapse to either substrate or product. The difference between the energy levels of the ground state and the transition state is called the **Gibbs free energy of activation** or simply **activation energy** and is symbolized by $\Delta G^{\neq}$. The double dagger (≠) denotes a thermodynamic quantity of a transition state. The rate of reaction reflects this activation energy; *a higher activation energy corresponds to a slower reaction.* Reaction rates can be increased by raising the temperature, thereby increasing the number of molecules with higher energy to overcome this energy barrier. As an alternate, the activation energy can be lowered by adding a catalyst (Fig. 18–2). Thus, *catalysts enhance reaction rates by lowering activation energies.*

The ES and EP intermediates occupy minima in the energic process curve of the enzyme-catalyzed reaction. The terms $\Delta G_{NE}^{\neq}$ and $\Delta_{GE}^{\neq}$ correspond to the activation energies for the nonenzymatic and enzymatic reactions. The activation energy for the overall process is lower when the enzyme catalyzes the reaction. Note that for both reactions, the free energy for S and P, called the $\Delta G^{\circ\prime}$ of the reaction, is the same.

The catalysts affect the reaction rates, not the reaction equilibria. And so are the enzymes, the bidirectional arrows put in the equation on page make the point clear: any enzyme that catalyzes the reaction, S → P also catalyzes the reverse reaction, P → S. Its only role is to accelerate the

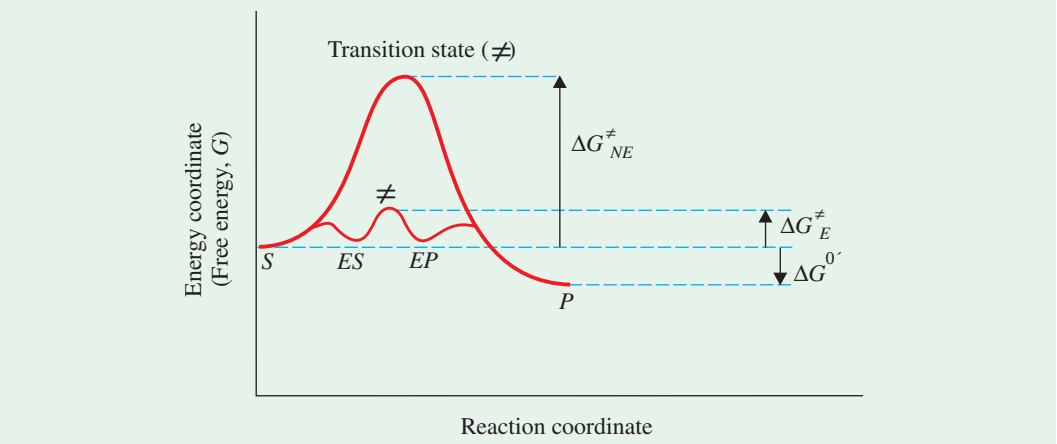


Fig. 18–2. Energy diagram, comparing the nonenzymatic and enzymatic reactions, S → P

interconversion of S and P. The enzyme is not consumed in the process, and the equilibrium point remains unaffected. However, the reaction reaches equilibrium much faster when the appropriate enzyme is present because the rate of the reaction is increased.

Practically, any reaction may have several steps involving the formation and decay of transient (unstable) chemical compounds called **reaction intermediates.** When the S → P reaction is catalyzed by an enzyme, the ES and EP complexes are intermediates and they occupy valleys in the reaction coordinate diagram (Fig. 18–2). When several steps occur in a reaction, the overall rate is determined by the step(s) with the maximum activation energy. This is called the **rate limiting step.** In practice, the rate limiting step can vary with reaction conditions. It may, however, be inferred from the above discussion that reaction *equilibria* are intimately linked with $\Delta G^{o\prime}$ and reaction *rates* are linked with $\Delta G^{\neq}$.

The energy of activation is a measure of the energy needed for the conversion of molecules to the reactive state. An inorganic catalyst lowers the activation energy and the organic catalyst (i.e., an enzyme) further decreases this. For example, decomposition of H_2O_2 requires 18,000 cal/mol ; this is lowered to 11,700 when colloidal platinum acts as catalyst and is further lowered to 2,000 when an enzyme catalase *catalyzes* the reaction. It is , thus, evident that enzymic catalysts are far more efficient in lowering the energy of activation than the nonenzymic catalysts for the same reaction. A perusal of Table 18–1 supports the above statement.

Table 18–1. Activation energy for enzymic and nonenzymic catalyses

S.No.	*Reaction*	*Catalyst*	*Activation Energy (expressed as cal/mol)*
1.	Decomposition of hydrogen peroxide	None	18,000
		Colloidal platinum	11,700
		Catalase	2,000
2.	Hydrolysis of ethyl butyrate	Hydrogen ion	16,800
		Hydroxyl ion	10,200
		Lipase	4,500
3.	Hydrolysis of sucrose	Hydrogen ion	25,600
		Invertase	10,000
4.	Hydrolysis of casein	Hydrogen ion	20,600
		Trypsin	12,000

(Adapted from White, Handler and Smith, 1964)

MICHAELIS-MENTEN HYPOTHESIS

The discovery by **Michaelis** that keratin, the major ingredient of hair, is soluble in thioglycolic acid made possible the development of the home permanent industry.

Leonor Michaelis and Maud L. Menten (1913), while studying the hydrolysis of sucrose catalyzed by the enzyme *invertase*, proposed this theory. Their theory is, however, based on the following assumptions :

1. Only a single substrate and a single product are involved.
2. The process proceeds essentially to completion.
3. The concentration of the substrate is much greater than that of the enzyme in the system.
4. An intermediate enzyme-substrate complex is formed.
5. The rate of decomposition of the substrate is proportional to the concentration of the enzyme-substrate complex.

The theory postulates that the enzyme (E) forms a weakly-bonded complex (ES) with the substrate (S). This enyzme-substrate complex, on hydrolysis, decomposes to yield the reaction product (P) and the free enzyme (E). These reactions may be symbolically represented as follows :

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

Although one may not feel any difficulty (at least theoretical) in describing the kinetics of these reactions, yet the difficulty is encountered when one starts to determine the concentration of ES or even S practically. The same difficulty was experienced by Michaelis and Menten, who devised an equation where these immeasurable quantities were replaced by those which could be easily measured experimentally.

Following symbols may be used for deriving Michaelis-Menten equation :

(E_t) = total concentration of enzyme

(S) = total concentration of substrate

(ES) = concentration of enzyme-substrate complex

$(E_t) - (ES)$ = concentration of free enzyme

The rate of appearance of products (*i.e.*, velocity, V) is proportional to the concentration of the enzyme-substrate complex.

$$V = k \neq (ES) \qquad ...(1)$$

The maximum reaction rate, V_m will occur at a point where the total enzyme E_t is bound to the substrate. Then the maximum concentration of ES will be equal to the total enzyme concentration, E*t*. Thus :

$$V_m = k \times (Et) \qquad ...(2)$$

Dividing equation (1) by (2,) we get :

$$\frac{V}{V_m} = \frac{(ES)}{(E_t)} \qquad ...(3)$$

With the help of this equation, one can easily measure the immeasurable quantities, (ES) and (E_t), in terms of the reaction rates experimentally.

Now coming back to the reversible reaction, $E + S \rightleftharpoons ES$, one can write the equilibrium constant for dissociation of ES as K_m which is equal to :

$$K_m = \frac{(E_t) - (ES) \times (S)}{(ES)} \qquad ...(4)$$

or $$(ES) \times K_m = (E_t) \times (S) - (ES) \times (S)$$

$$\text{or} \quad (ES) \times K_m + (ES) \times (S) = (E_t) \times (S)$$

$$\text{or} \quad (ES) \times [K_m + (S)] = (E_t) \times (S)$$

$$\text{or} \quad \frac{(ES)}{(E_t)} = \frac{(S)}{K_m + (S)} \qquad ...(5)$$

Substituting the value of $\frac{(ES)}{(E_t)}$ from equation (3) to equation (5), we get :

$$\frac{V}{V_m} = \frac{S}{K_m + (S)}$$

$$\text{or} \quad V = \frac{V_m \times (S)}{K_m + (S)} \qquad ...(6)$$

$$\text{or} \quad K_m = (S)\left[\frac{V_m}{V} - 1\right] \qquad ...(7)$$

Equation (6) is called as **Michaelis-Menten equation.** This can be used to calculate K_m after experimentally determining the reaction rates at various substrate concentrations. This equilibrium constant, K_m, is usually called **Michaelis constant.** *It is a measure of the affinity of an enzyme for its substrate.* Referring to the equation (4), the greater the concentration of ES complex, the lower is the concentration of free enzyme and consequently the lower is the value of K_m.

For experimental determination of K_m, the velocity of the reaction (relative activity of the enzyme) is measured as a function of substrate concentration. When $V = \frac{1}{2}V_m$, it will be seen from equation (6) that K_m is numerically equal to the substrate concentration or in other words K_m is equal to the concentration of the substrate which gives half the numerical maximal velocity, V_m. Thus, it is possible to determine K_m. The K_m, shown in Fig. 18–3, is indicated to be 0.017 M. It is noteworthy that *for any enzyme-substrate system, K_m has a characteristic value which is independent of the enzyme concentration.*

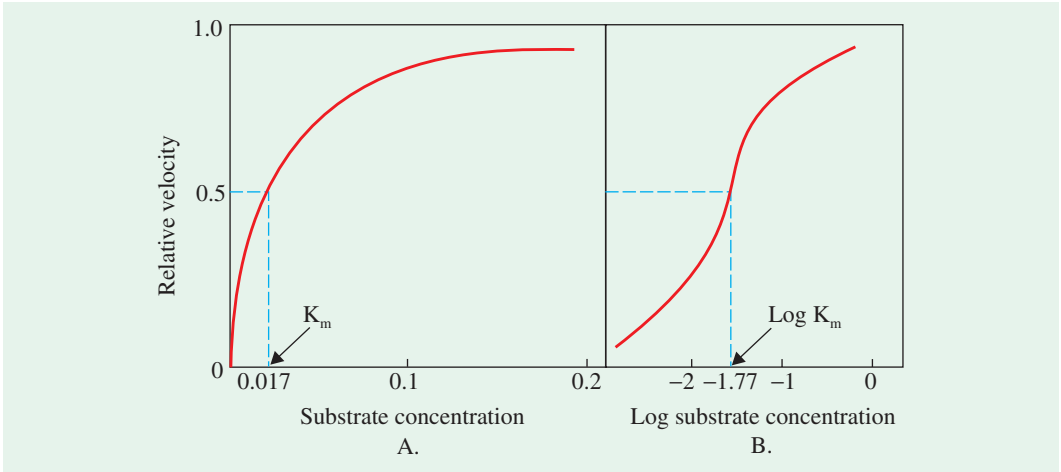


Fig. 18–3. Relative initial velocity as a function of substrate concentration (A) and as a function of the logarithm of the substrate concentration (B) for the action of yeast invertase on sucrose

(Adapted from White, Handler and Smith, 1964)

Lineweaver-Burk equation. The method described above for the detertmination of K_m is somewhat complex and, therefore, simpler methods have been devised. Two such methods (Fig. 18–4) are given below :

First method. A convenient means of evaluating K_m and V_m is to plot kinetic data as the reciprocals

of *V* and [S]. Such a double reciprocal plot was proposed by Hans Lineweaver and Dean Burk in 1934. If one takes the reciprocal of Michaelis-Menten equation, the following equation is obtained :

$$\frac{1}{V} = \frac{K_m + (S)}{V_m \times (S)}$$

or

$$\frac{1}{V} = \frac{K_m}{V_m} \times \frac{1}{(S)} + \frac{1}{V_m} \qquad ...(8)$$

This is known as **Lineweaver-Burk equation.** This equation is of the form, $y = mx + b$, if one considers the variables to be $\frac{1}{V}$ and $\frac{1}{(S)}$. When one plots the graph against these two variables, a straight line is obtained. The slope of this line corresponds to $\frac{K_m}{V_m}$ and the $\frac{1}{V}$ intercept corresponds to $\frac{1}{V_m}$. Since V_m can be determined from the intercept, the K_m may also be calculated.

Second Method. Another graphical method for the measurement of K_m from experimental data on V as a measure of (S) makes use of the above Lineweaver-Burk equation. Multiplication of both sides of this equation by (S) gives :

$$\frac{(S)}{V} = \frac{K_m}{V_m} + \frac{(S)}{V_m} \qquad ...(9)$$

A plot of $\frac{(S)}{V}$ versus (S) gives a straight line. The intercept of the line on $\frac{(S)}{V}$ axis is $\frac{K_m}{V_m}$ and the slope is $\frac{1}{V_m}$.

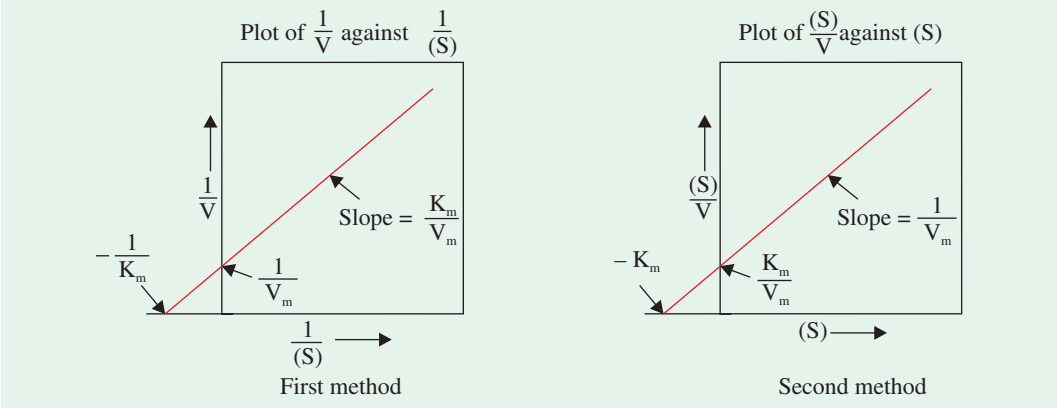


Fig. 18–4. Two methods of obtaining linear plots involving measurement of initial velocity, V and substrate concentration, (S)

V_m and K_m can be obtained from the intercept on the slope and the abscissa.

(Adapted from Fruton and Simmonds, 1960)

A Lineweaver-Burk plot provides a quick test for adherence to Michaelis-Menten kinetics and allows easy evaluation of the critical constants. As we shall see, it also allows discrimination between different kinds of enzyme inhibition and regulation. A disadvantage of Lineweaver-Burk plot is that a long extrapolation is often required to determine K_m, with corresponding uncertainty in the result. Consequently, other ways of plotting the data are sometimes used. One such alternative is to rearrange equation (6) into the form,

$$V = V_m - K_m \frac{V}{[S]} \qquad ...(10)$$

and graph V versus V/[S]. This yields what is called an **Eadie-Hofstee plot** (Fig. 18–5)

Significance of K_m and V_m Values

The **Michaelis constant (K_m)** values of the enzymes differ greatly. However, for most of the enzymes, the general range is between 10^{-1} and 10^{-6} M (refer Table 18–2). The K_m value depends on the particular substrate and on the environmental conditions such as temperature and ionic concentration.

The K_m value signifies two meanings :

(*a*) K_m is the concentration of substrate at which half the active sites are occupied. If K_m is known, the fraction of the sites filled, f_{ES}, at any substrate concentration, can be calculated from :

$$f_{ES} = \frac{V}{V_m} + \frac{(S)}{K_m + (S)} \quad ...(11)$$

(*b*) K_m is related to the rate constants of the individual steps in the catalytic scheme,

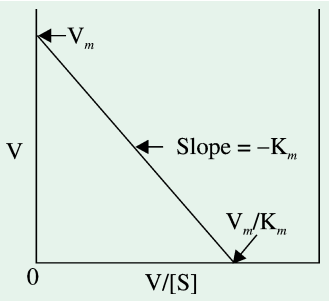


Fig. 18-5. An Eadie-Hofstee plot Graphing V versus V/[S], one obtains V_m at (V/[S]) = 0 and K_m from the slope of the line.

Table 18–2. Values of Michaelis constant (Km) of some enzymes

Enzyme	*Substrate*	K_m*
1. Lysozyme	Hexa-N-acetylglucosamine	6×10^{-6} M
2. Penicillinase	Benzylpenicillin	5×10^{-5} M
3. β-galactosidase	Lactose	4×10^{-3} M
4. Chymotrypsin	Acetyl-L-tryptophanamide	5×10^{-3} M
5. Carbonic anhydrase	Carbon dioxide	8×10^{-3} M
6. Pyruvate carboxylase	pyruvate	4×10^{-4} M
	ATP	6×10^{-5} M
7. Arginine-tRNA synthetase	tRNA	4×10^{-7} M
	Arginine	3×10^{-6} M
	ATP	3×10^{-4} M
8. Hexokinase	ATP	4×10^{-4} M
	D-glucose	5×10^{-5} M
	D-fructose	15×10^{-4} M
9. Sucrase	Sucrose	0.016 – 0.04 M
10. α-glucosidase	Methyl-α -D-glucoside	0.037 – 0.075 M
11. β-glucosidase	Methyl-β-D-glucoside	0.060 – 1.12 M
12. Pepsin	Ovalbumin	4.5%
13. Trypsin	Casein	2.0%
14. Urease	Urea	0.025 M
15. Catalase	Hydrogen peroxide	0.025 M
16. Lipase (Esterase)	Ethyl butyrate	> 0.03 M
17. Phosphatase	Glycerophosphate	< 0.003 M
18. Xanthine oxidase	Xanthine	4×10^{-7} M
19. Succinic dehydrogenase	Succinate	5×10^{-7} M
20. Amylase	Starch	0.8 – 0.25%
21. Dipeptidase	Glycylleucine	0.02 – 0.07 M

* K_m values are not absolute constants but depend on the temperature, substrate and source of enzyme.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

where k_1, k_2 and k_3 are the rate constants for the 3 reactions. If k_2 is much greater than k_3 ($K_2 >> k_3$), *i.e.*, the dissociation of ES complex is more rapid than formation of P and regeneration of E, then

$$K_m = \frac{k_2}{k_1} \qquad ...(12)$$

The dissociation constant of ES complex is given by,

$$K_{ES} = \frac{(E)\,(S)}{(ES)} = \frac{k_2}{k_1} \qquad ...(13)$$

In other words, K_m is equal to the dissociation constant of the ES complex if k_3 is much smaller than k_2 ($k_3 << k_2$). When this condition is reached, K_m is a measure of the strength of ES complex. The high K_m value indicates weak binding whereas the low K_m value signifies strong binding. It may, however, be emphasized that K_m indicates the affinity of the ES complex only when $k_2 >> k_3$.

The **maximal rate (V_m)** represents the turnover number of an enzyme, if the concentration of the active sites (E*t*) is known, since,

$$V_m = k_3\,(E_t) \qquad ...(14)$$

A 10^{-6} M solution of *carbonic anhydrase*, for instance, catalyzes the formation of 0.6 M carbonic acid per second when it is fully saturated with substrate. Hence, k_3 is 6×10^5 sec^{-1}. *The kinetic constant, k_3 is known as the turnover number.*

HERMANN EMIL FISCHER

(LT, 1852-1919)

Emil Fischer, a German, was the son of a wealthy merchant. He graduated from the Gymnasium of Bonn in 1869. After an abortive foray into the business world, he entered the University of Bonn in 1871 to study chemistry under Frederick August Kekule (LT, 1829-1867), master of structural chemistry. After receiving his doctoral degree from the University of Strasbourg in 1874, he taught at the Universities of Erlangen and Wurzburg, eventually becoming Professor of Chemistry at the University of Berlin in 1892 while succeeding Hofmann. He published his discovery of phenylhydrazine in 1875. Later in 1899, he turned to a detailed study of proteins. It was in the study of structural chemistry to the study of proteins that Fischer saw the possible future collaboration of biology and chemistry. He developed the *theory of the 'peptide bond'*, the chemical linkage by which all amino acids are joined together to form their respective proteins. He also chemically synthesized an *octadecapeptide*, composed of 3 leucine + 15 glycine residues. Frequently referred to as the **father of biochemistry**, Fischer received the second-ever **Nobel Prize in Chemistry (1902)** for his work on the synthesis of purines and sugars. Fischer's collection of 9,000 reference compounds is housed in the Department of Biochemistry, the University of California at Berkeley. The prized collection was a gift of H.O.L. Fischer, a biochemist and the only one of Fischer''s three sons to survive World War I. Following the deaths of his two sons in World War I, Emil Fischer committed suicide.

ACTIVE SITE

As the substrate molecules are comparatively much smaller than the enzyme molecules, there should be some specific regions or sites on the enzyme for binding with the substrate. Such sites of attachment are variously called as **'active sites'** or **'catalytic sites'** or **'substrate sites'**.

Although the enzymes differ widely in their properties, the active site present in their molecule possesses some common features (Fig. 18–6). These are litsed below :

1. The active site occupies a relatively small portion of the enzyme molecule.
2. The active site is neither a point nor a line or even a plane but is a 3-dimensional entity. It is made up of groups that come from different parts of the linear amino acid sequence. For example, as already stated, *lysozyme* (refer Fig. 17–16) has 6 subsites in the active site. The amino acid residues located at the active site are 35, 52, 59, 62, 63 and 107.
3. Usually the arrangement of atoms in the active site is well defined, resulting in a marked specificity of the enzymes. Although cases are known where the active site changes its configuration in order to bind a substance which is only slightly different in structure from its own substrate.

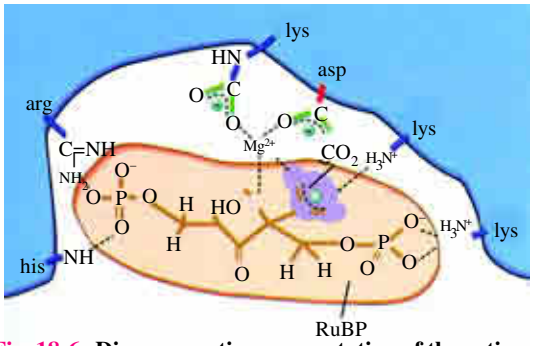


Fig. 18-6. Diagrammatic representation of the active site of the enzyme ribulose bisphosphate carboxylase (Rubisco) showing the various sites of interaction between the bound substrates (RuBP and CO_2) and certain amino acid side chains of the enzyme

In addition to determining the substrate-binding properties of the active site, these noncovalent interactions alter the properties of the substrate in ways that accelerate its conversion to products.

(*After Harris DA, 1995*)

4. The active site binds the substrate molecule by relatively weak forces.
5. The active sites in the enzyme molecules are grooves or crevices from which water is largely excluded. It contains amino acids such as aspartic acid, glutamic acid, lysine serine etc. The side chain groups like —COOH, —NH_2, —CH_2OH etc., serve as catalytic groups in the active site. Besides, the crevice creates a micro-environment in which certain polar residues acquire special properties which are essential for catalysis.

Fischer's Lock and Key Model

Previously, the interaction of substrate and enzyme was visualized in terms of a lock and key model (also known as *template model*), proposed by Emil Fischer in 1898. According to this model, the union between the substrate and the enzyme takes place at the active site more or less in a manner in which a key fits a lock and results in the formation of an enzyme substrate complex (Fig. 18–7).

To quote **Fischer (1868)** himself, "There is a relation between the unknown structure of an active enzyme and that of substrate, they are complementary and the one may be said to fit the other as a key fits a lock."

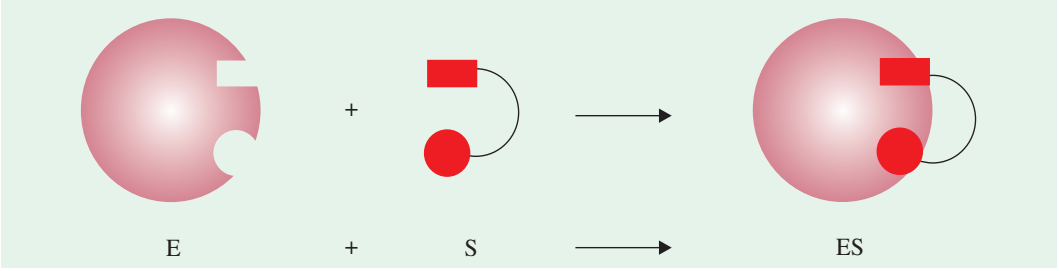


Fig. 18–7. Formation of an enzyme-substrate complex according to Fischer's lock and key model

In fact, the enzyme-substrate union depends on a *reciprocal fit* between the molecular structure of the enzyme and the substrate. And as the two molecules (that of the substrate and the enzyme) are involved, this hypothesis is also known as the **concept of intermolecular fit.** The enzyme-substrate complex (Fig. 18–8) is highly unstable and almost immediately this complex decomposes to produce

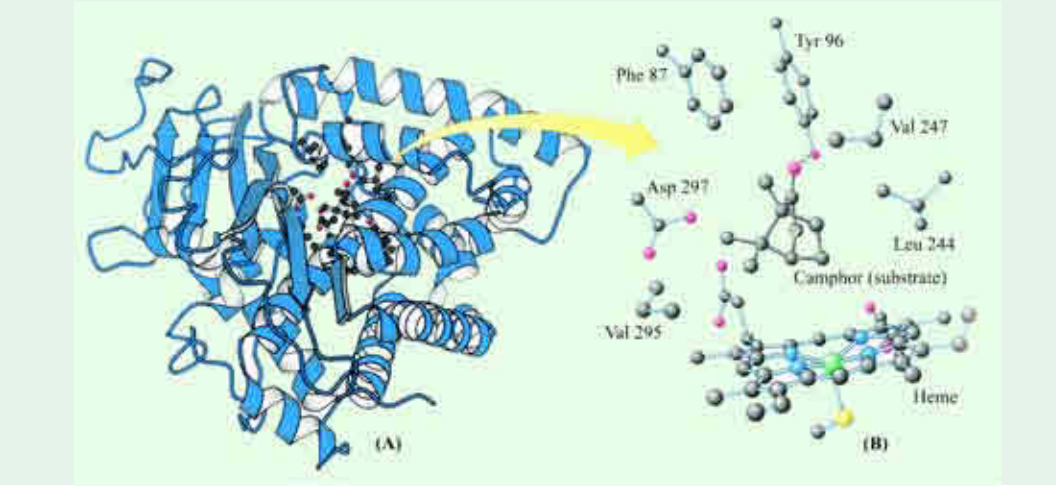


Fig. 18–8. Structure of an enzyme–substrate complex

(A) The enzyme cytochrome P-450 is illustrated bound to its substrate camphor.

(B) In the active site, the substrate is surrounded by residues from the enzyme. Note also the presence of a heme cofactor.

the end products of the reaction and to regenerate the free enzyme. The enzyme-substrate union results in the release of energy. It is this energy which, in fact, raises the energy level of the substrate molecule, thus inducing the *activated state*. In this activated state, certain bonds of the substrate molecule become more susceptible to cleavage.

Evidences Proving the Existence of an ES Complex:

The existence of an ES complex during enzymatically-catalyzed reaction has been shown in many ways :

1. The ES complexes have been directly observed by electron microscopy and x-ray crystallography.
2. The physical properties of enzymes (*esp*., solubility, heat sensitivity) change frequently upon formation of an ES complex.
3. The spectroscopic characteristics of many enzymes and substrates change upon formation of an ES complex. It is a case parallel to the one in which the absorption spectrum of deoxyhemoglobin changes markedly, when it binds oxygen or when it is oxidized to ferric state.
4. Stereospecificity of highest order is exhibited in the formation of ES complexes. For example, D-serine is not a substrate of tryptophan synthetase. As a matter of fact, the D-isomer does not even bind to the enzyme.
5. The ES complexes can be isolated in pure form. This may happen if in the reaction, $A + B \rightleftharpoons C$, the enzyme has a high affinity for the substrate A and also if the other reactant B is absent from the mixture.
6. A most general evidence for the existence of ES complexes is the fact that at a constant concentration of enzyme, the reaction rate increases with increase in the substrate concentration until a maximal velocity is reached.

Koshland's Induced Fit Model

An important but unfortunate feature of Fischer's model is the rigidity of the active site. The active site is presumed to be pre-shaped to fit the substrate. In order to explain the enzyme properties more efficiently, Koshland, in 1958, modified the Fischer's model. Koshland presumed that the enzyme molecule does not retain its original shape and structure. But the contact of the substrate *induces*

some configurational or geometrical changes in the active site of the enzyme molecule. Consequently, the enzyme molecule is made to *fit* completely the configuration and active centres of the substrate. At the same time, other amino acid residues may become buried in the interior of the molecule. Koshland's hypothesis has recently been confirmed by Lipscomb.

To explain the theory, a hypothetical illustration may be given (Fig. 18–9). The hydrophobic and charged groups both are involved in **substrate binding.** A phosphoserine (-P) and the -SH group of cysteine residue are involved in **catalysis.** Other amino acid residues not involved in either substrate binding or catalysis are lysine (Lys) and methionine (Met). In the absence of substrate, the substrate binding and catalytic groups are far apart from each other. But the proximity of the substrate induces a conformational change in the enzyme molecule aligning the groups for both substrate binding and catalysis. Simultaneously, the spatial orientation of other regions is also changed so that the lysine and methionine are now much closer.

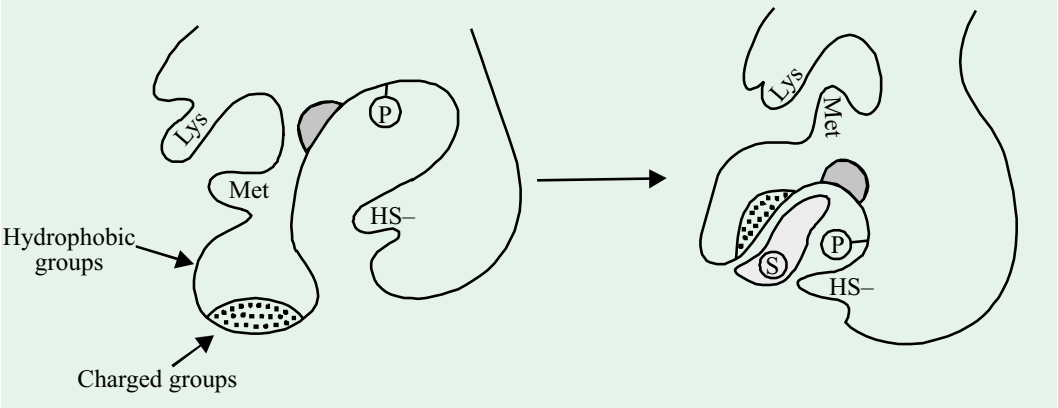


Fig. 18–9. Conformational changes brought about by induced fit in an enzyme molecule

(Modified from Daniel E. Koshland Jr., 1964)

An illustration of the competitive inhibitor or substrate analogue may also be given (Fig. 18–8). On contact with the true substrate, all groups are brought into correct spatial orientation. But attachment of a competitive inhibitor, which is either too "slim" or too "bulky", induces incorrect alignment.

As to the sequence of events during the conformational changes, 3 possibilities exist (refer Fig. 18–8). The enzyme may first undergo a conformational change (A), then bind substrate (B). An alternative pathway is that the substrate may first be bound (C) and then a conformational change (D) may occur. Thirdly, both the processes may occur simultaneously (E) with further isomerization (F) to the final conformation.

Originally little more than an attractive hypothesis, Koshland's model has now gained much experimental support. Conformational changes during substrate binding and catalysis have been demonstrated for various enzymes such as *phosphoglucomutase, creatine kinase, carboxypeptidase* etc.

ENZYME REACTION RATES

A plot of V (velocity, or reaction rate) versus substrate concentration in an enzymatically- catalyzed reaction produces a hyperbolic curve (Fig. 18–10) which is representative of the reaction kinetics of a noncooperative enzyme. The hyperbolic curve is analogous to the oxygen-dissociation curve of myoglobin. The plot shows that the velocity increases with substrate concentration until maximum V (V_{max}) is approached asymptotically, after which larger concentrations of substrate do not significantly enhance the reaction rate. In the lower region of the curve, the reaction approaches *first-order kinetics*, which means that v is a direct function of substrate concentration because

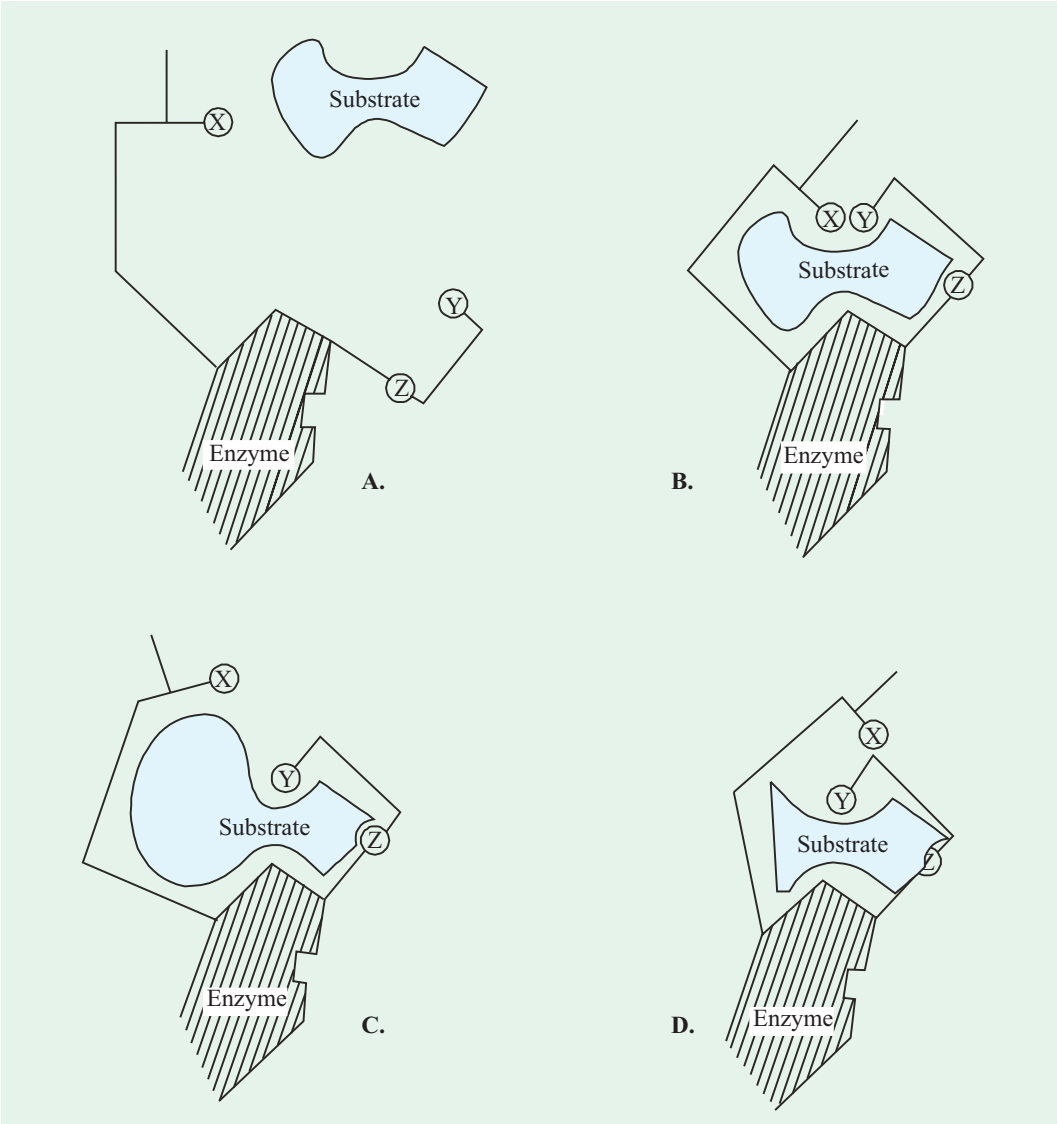


Fig. 18–10. Schematic model of flexible active site mechanism

Black lines indicate protein chains containing catalytic groups X and Y and binding groups Z.

A. Substrate and enzyme dissociated.

B. Substrate with induced change of protein chains to bring X and Y into proper alignment for reaction.

C. Bulky groups added to substrate prevent proper alignment of X and Y.

D. Deleted group eliminates buttressing action on chain containing X so that the complex has incorrect alignment of X and Y.

(From Daniel E. Koshland Jr., 1964)

the active sites of the enzyme molecules are not saturated. At the plateau at the upper portion of the plot, the reaction approaches *zero-order kinetics* because the active sites of all the enzyme molecules are saturated and the reaction rate is, therefore, independent of further increases in substrate concentration. For the intermediate portion of the curve, as the enzyme approaches substrate saturation,

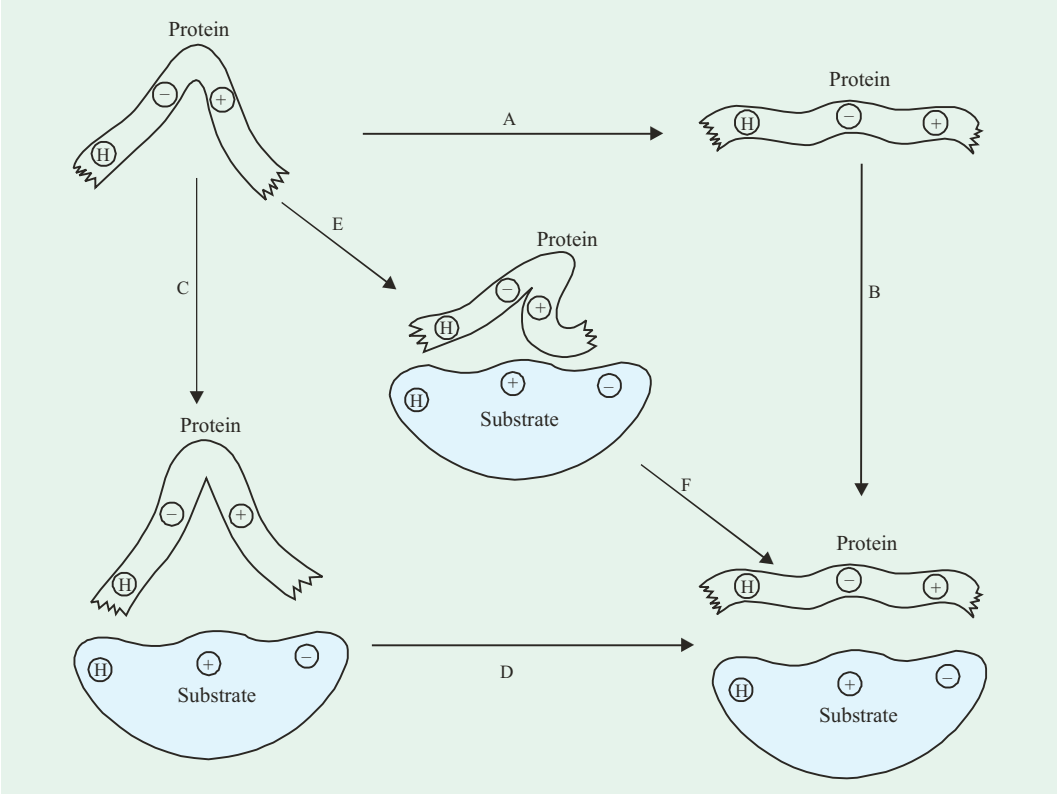


Fig. 18–11. Alternative pathways for a substrate-induced conformational change

(Adapted from Koshland and Neet, 1968)

kinetics are mixed zero and first order in substrate concentration. Routine enzyme assays are designed to follow zero-order kinetics to avoid the influence of substrate concentration on reaction velocity.

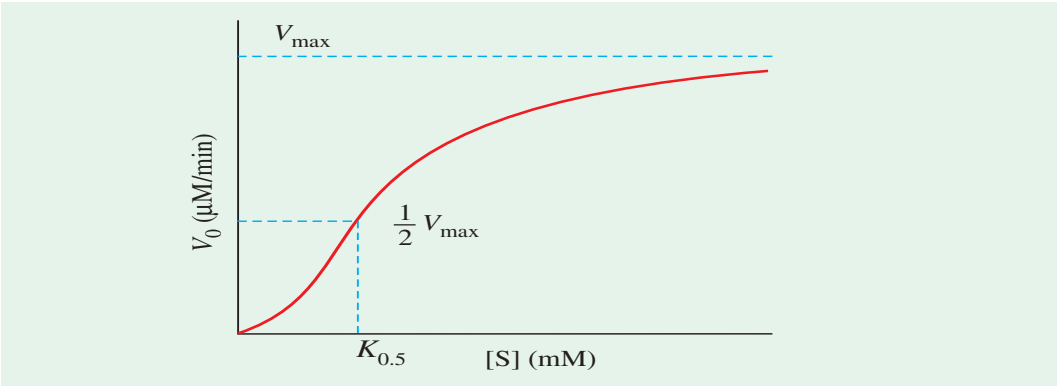


Fig. 18–12. Effect of substrate concentration on the velocity of an enzyme-catalyzed reaction

Under such conditions, measured rates are directly proportional to the concentration of the enzyme itself. In contrast, *uncatalyzed reactions do not show this saturation effect.*

MODIFIERS OF ENZYME ACTIVITY

The catalytic activity of certain enzymes is reversibly altered by certain inorganic and organic molecules called modifiers. Those molecules which increase the enzyme activity are called *positive modifiers* or *activators* and those which decrease the enzyme activity as *negative modifiers* or *inhibitors*.

Many metals act both as postitive and negative modifiers, whereas certain organic molecules retard enzyme activity, thus acting as negative modifiers.

Inorganic Modifiers (Enzyme Activators)

Certain enzymes, apart from a requirement of a coenzyme, also need a metal ion for full activity. Removal of the metal often results in partial or total loss of enzyme activity. The activity may, however, be restored by replacing the original or a similar metal ion. Some such metal ions (or cations) are K^+, Cu^+, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} etc. Mg participates in phosphate-transfer reactions and Fe, Cu and Mo are required in oxido-reduction reactions. Certain mechanisms as to how the metal ions bring about activation are given beolw :

Metals are the common inorganic modifiers. Besides accelerating the rate of enzymatically-catalyzed reactions, metals also inhibit the rate of such reactions.

1. **Direct participation in catalysis.** Certain metals may directly participate in the oxidoreduction reactions by undergoing a valence change and thus function in electron transport system. Fe, for exmaple, functions similarly in *cytochromes* or in *catalase*.
2. **Formation of a metallosubstrate.** Sometimes a metal combines with the substrate to form a metallosubstrate (MS) which, in fact, is the true substrate for the enzyme and forms an enzyme-metal-substrate (EMS) complex. This complex, later on, decomposes to produce the reaction product (P) and regenerate the enzyme and the metal.

$$S + M \rightleftharpoons MS \xrightarrow{+E} EMS \longrightarrow E + M + P$$

3. **Formation of a metalloenzyme.** A metal ion may first combine with an enzyme to form a metalloenzyme (ME) which then combines with the substrate forming an enzyme-metal-substrate (EMS) complex.

$$E + M \rightleftharpoons EM \xrightarrow{+S} EMS \longrightarrow E + M + P$$

4. **Alteration of equilibrium constant.** Metals may also change the nature of the reactants so that the apparent equilibrium constant of the reaction is also altered.
5. **Conformational change in the enzyme.** Metal ions may also bring about conformational change in the enzyme molecule, converting it into an active form. In such a case, the metal may be linked at a point far remote from the substrate and may serve to maintain an active tertiary or quaternary structure.

Regulation of Enzyme Activity

The activity of the enzymes is controlled by certain mechanisms which are enumerated below :

1. *Zymogen activation.* Some enzymes (esp., digestive and coagulating ones) are synthesized in inactive forms. These inactive forms of enzymes are called **proenzymes** or **zymogens**. Thus, zymogens are precursors of enzymes. They are not active on their own but can be active after conversion. Generally, prefix *pro-* or suffix *-ogen* is added to enzyme's name to denote its zymogen such as prothrombin, proelastase, trypsinogen, pepsinogen, fibrinogen etc. It is difficult to argue as to why cells should make precursors for some enzymes and not others, rather than merely forming the active enzymes in all cases. Perhaps, the precursors are evolutionary anachronisms or else they are required at times or places when the active enzymes would injure cells.

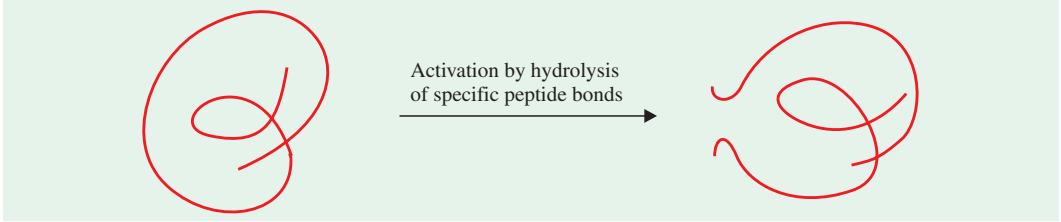


Fig. 18–13. Zymogen activation by hydrolysis of specific peptide bonds

The *precursors* should be converted to the active forms before they can catalyze a chemical reaction. *This type of activation usually involves cleavage of the peptide bond* (Fig. 18–13). Often, a part of the zymogen is also removed. Examples of some common precursor systems are given below :

(A) Chymotrypsin is synthesized by the exocrine cells of the pancreas in its precursor form, chymotrypsinogen. Hydrolysis by trypsin converts chymotrypsinogen into the active form, chymotrypsin.

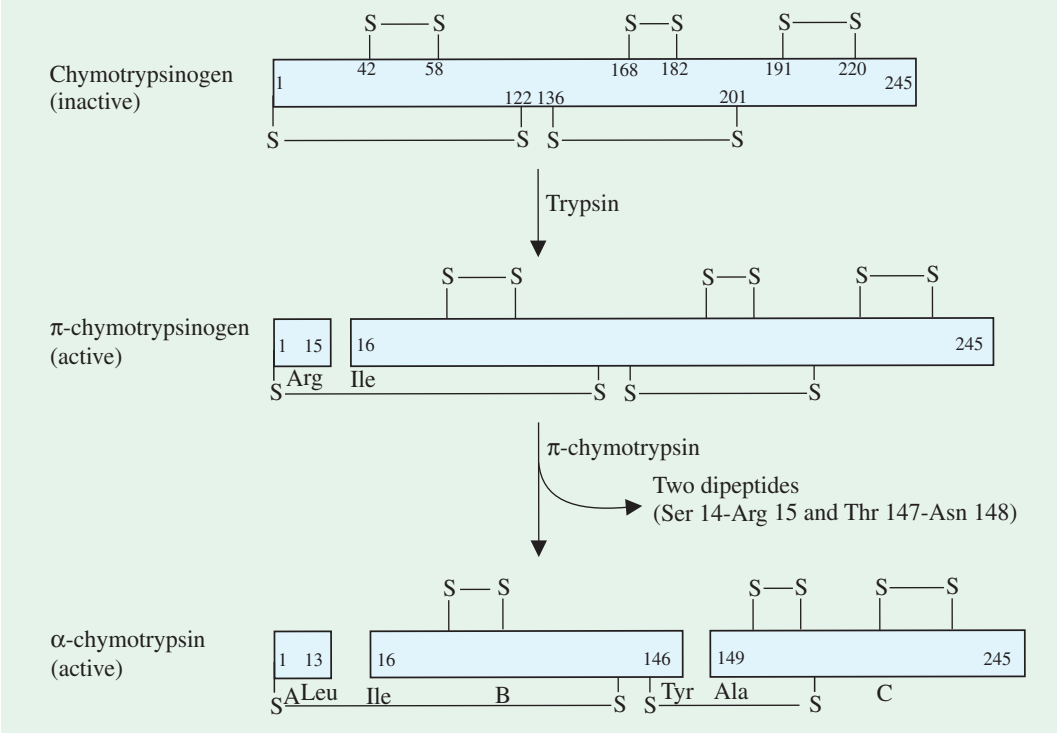


Fig. 18–14. Proteolytic activation of chymotrypsinogen

The 3 chains of α-chymotrypsin are linked by two interchain disulfide bonds (A to B and B to C).

$$\text{Chymotrypsinogen} \xrightarrow{\text{Trypsin}} \text{Chymotrypsin}$$

Chymotrypsinogen is composed of a single polypeptide chain with 245 amino acid residues (representing all the 20 standard amino acids) and 5 intrachain disulfide bridges (Fig. 18–14). It is virtually devoid of enzymatic activity and is converted into a fully active enzyme when the peptide bond linking Arg^{15} and Ile^{16} is cleaved by trypsin. The resulting enzyme, called π- **chymotrypsin**, then acts on other π-chymotrypsin molecules. Two dipeptides (between residues 14 and 15 and residues 147 and 148) are removed to yield α-**chymotrypsin**, the stable form of the active enzyme. The 3 resulting chains (designated A, B and C) in α-chymotrypsin remain covalently linked to each other by two interchain disulfide bonds, although 3 more (intrachain) disulfide bonds, one in chain B and two in chain C, are also present in the molecule. The additional cleavages made in converting π-chymotrypsin into α -chymotrypsin are superfluous, because π- chymotrypsin is already fully active. The striking feature of this activation process is that *cleavage of a single specific peptide bond transforms the protein from a catalytically inactive form into one that is fully active.*

(B) An intestinal enzyme termed enterokinase converts *trypsinogen*, a proenzyme also secreted

by pancreas, to trypsin. Trypsin acts upon peptide linkages involving the carboxyl group of arginine and lysine.

$$\text{Trypsinogen} \xrightarrow{H^+ \text{ or Pepsin}} \text{Trypsin}$$

(C) Another instance is the proteinase of the gastric juice called as pepsin. This is derived from its proenzyme *pepsinogen*, secreted by gastric mucosa. Pepsinogen is converted to the active form, pepsin, both by the acidity of the gastric juice and by pepsin itself. This process is, therefore, called as **autocatalysis.** During this process, a polypeptide is liberated from the proenzyme.

$$\text{Pepsinogen} \xrightarrow{\text{Enterokinase}} \text{Pepsin} + \text{a polypeptide}$$

Autocatalytic activity of an ezyme precursor may be graphically represented. If a graph of time versus enzyme activity is plotted, a sigmoid (S-shaped) curve is obtained (Fig. 18–15).

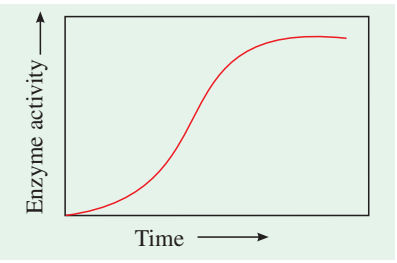
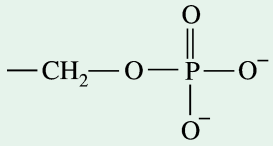


Fig. 18–15. Autocatalytic activation of an enzyme precursor

2. *Covalent modification*. The activity of serine-containing enzymes is regulated by the covalent insertion of a small group on an enzyme molecule. For example, the activity of the enzymes, that synthesize and cleave glycogen, is regulated by the attachment of a phosphoryl group to a specific serine residue on these enzymes. This modification can be reversed by hydrolysis.



Phosphorylated derivative of a serine residue

3. *Feedback inhibition.* Some enzymes catalyze the synthesis of small molecules (such as amino acids) in a number of steps. The enzyme catalyzing the first step in this biosynthesis is inhibited by the end product of the reaction. Such type of regulatory mechanism, which is called feedback inhibition (Fig. 18–16), is beautifully illustrated by the biosynthesis of isoleucine from threonine.

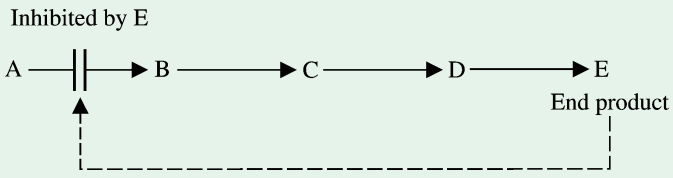


Fig. 18–16. Feedback inhibition of the first enzyme in a multistep reaction by reversible binding of the end product

The reaction completes in 5 steps. The first step reaction is catalyzed by the enzyme *threomine aminase.* The activity of this enzyme is inhibited upon accumulation of high quantities of isoleucine. Isoleucine binds to a different site from threonine. This is called *allosteric interaction.* However, when the level of isoleucine drops sufficiently, the enzyme reactivates and isoleucine is resynthesized.

Organic Modifiers (Enzyme Inhibitors)

Compounds which convert the enzymes into inactive substances and thus adversely affect the

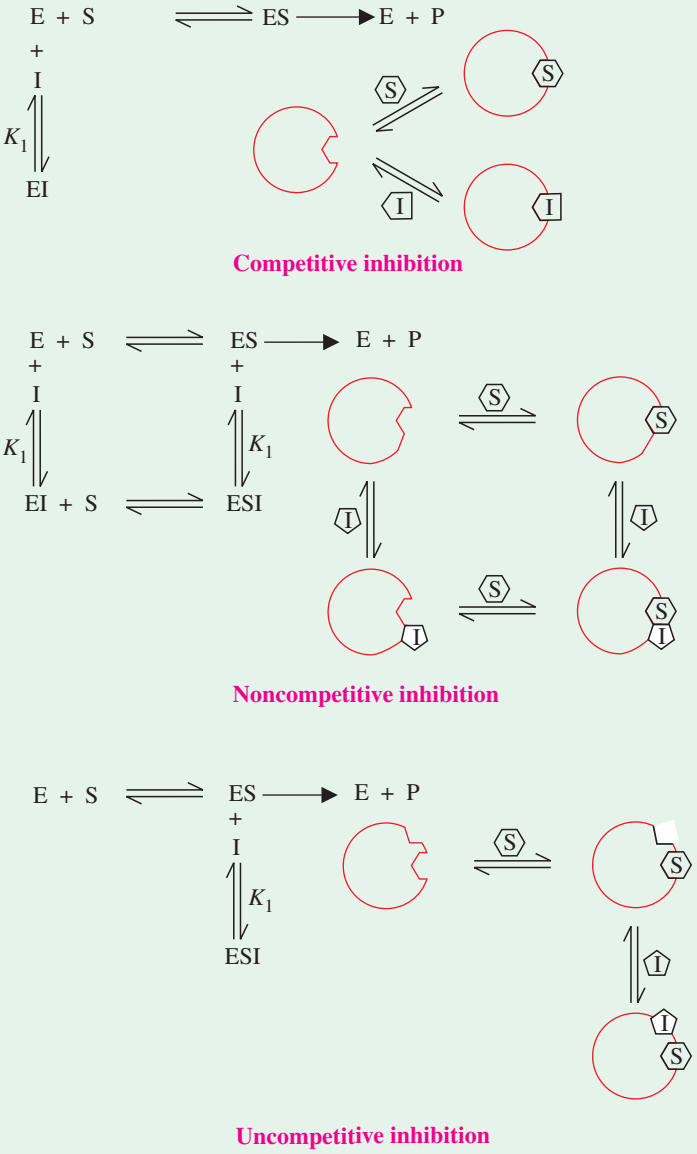


Fig. 18–17. Three types of reversible enzyme inhibition

Competitive inhibitors bind to the enzyme's active site. Noncompetitive inhibitors generally bind at a separate site termed allosteric site. Uncompetitive inhibitors also bind at an allosteric site but they bind only to the ES complex.

rate of enzymatically-catalyzed reaction are called as *enzyme inhibitors*. Such a process is known as *enzyme inhibition*. Two broad classes of enzyme inhibitors are generally recognized : reversible and nonreversible, depending on whether the enzyme-inhibitor (EI) complex dissociates rapidly or very slowly.

Reversible Enzyme Inhibition

A reversible inhibitor dissociates *very rapidly* from its target enzyme because it becomes very loosely bound with the enzyme. Three general types of reversible inhibition (Fig. 18–17) are

distinguished : competitive, noncompetitive and uncompetitive, depending on three factors:

a. whether the inhibition is or is not overcome by increasing the concentration of the substrate,

b. whether the inhibitor binds at the active site or at allosteric site, and "

c. whether the inhibitor binds either with the free enzyme only, or with the enzyme substrate complex only or with either of the two.

A. Competitive or Substrate analogue inhibition. This type of competition occurs at the active site (Fig. 18-18). Here the structure of the inhibitor (I) closely resembles with that of the substrate (S). It may, thus, combine with the enzyme (E), forming an enzyme-inhibitor (EI) complex rather than an ES complex. The inhibitor, thus, competes with the substrate to combine with the enzyme. *The degree of inhibition depends upon the relative concentrations of the substrate and the inhibitor.* Thus, by increasing the substrate concentration and keeping the inhibitor concentration constant, the amount of inhibition decreases and conversely a decrease in substrate concentration results in an increased inhibition. It may, however, be noted that in competitive inhibition, the enzyme can bind substrate (forming an ES complex) or inhibitor (EI), but not both (ESI). Thus, we see that a competitive inhibitor diminishes the rate of the reaction by reducing the proportion of the enzyme molecules bound to a substrate.

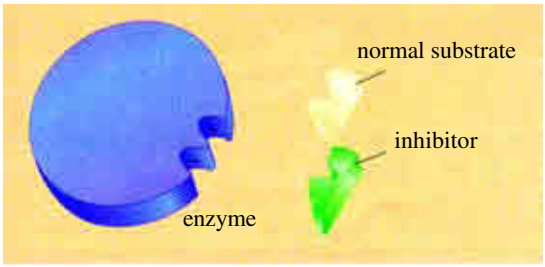


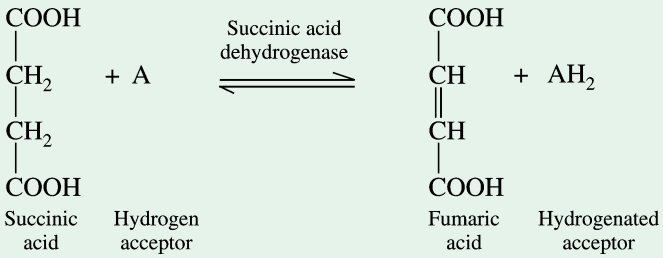
Fig. 18–18. Competitive or substrate analogue inhibition

A competitive inhibitor fits into the active site of the enzyme, preventing the real substrate from gaining access. The inhibitor cannot be converted to the products of the reaction and so the overall rate of reaction is slowed down. If an inhibitor is present in equal concentrations to the substrate, and if both types of molecule bind to the active site equally well, the enzyme can only work at half its normal rate.

Competitive inhibition can be analyzed quantitatively by steady-state kinetics. Because the inhibitor binds reversibly to the enzyme, the competition can be biased to favour the substrate only simply by adding more substrate. When more substrate is present, the probability that an inhibitor molecule will bind is minimized, and the reaction exhibits a normal V_{max}. However, the [s] at which $V_0 = 1/2\ V_{max}$, the K_m will increase in the presence of inhibitor.

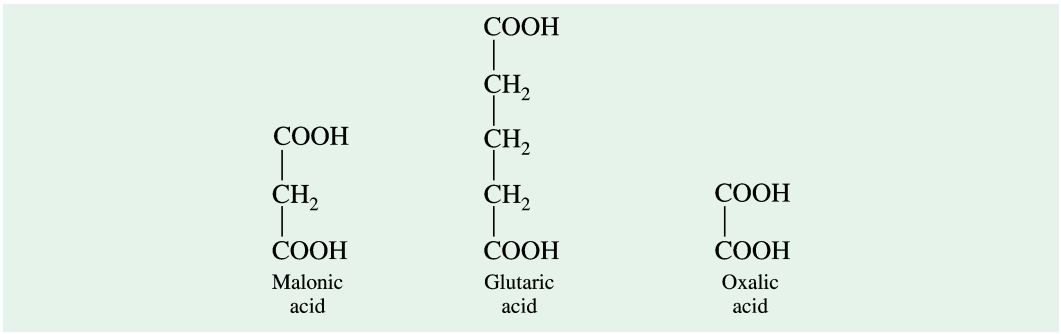
Some well-known examples of competitive inhibition are given below :

1. An enzyme, *succinic acid dehydrogenase* (= *succinodehydrogenase*) catalyzes the conversion of succinic acid to fumaric acid.

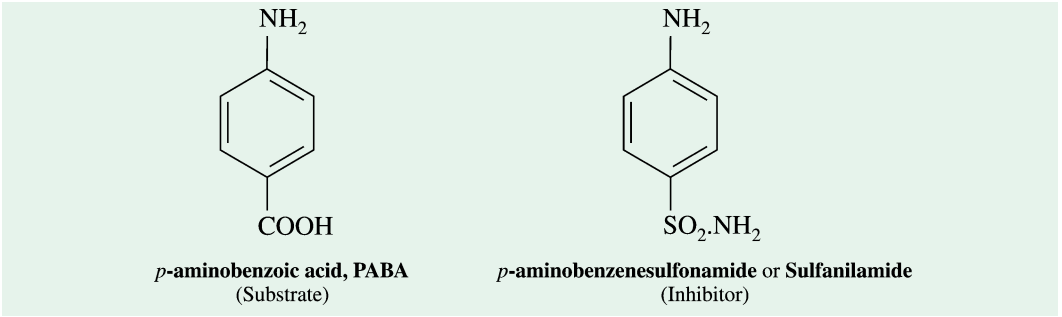


Many organic compounds, which are structurally related to succinic acid, combine with the enzyme, thus inhibiting the reaction. A few inhibitors of this reaction are :

Malonic acid is most efficient of all these inhibitors. When the inhibitor : enzyme ratio is 1 :50, the enzyme is inhibited 50%. Malonic acid differs from succinic acid in having one rather than two methylene groups.



2. Many microorganisms, like bacteria, synthesize the vitamin folic acid from para-aminobenzoic acid (PABA). Sulfanilamide and other sulfa drugs are structural analogues of PABA. Hence,



sulfa drugs act as enzyme inhibitor and occupy the active site of some *bacterial enzymes* catalyzing this reaction. Failure of PABA to combine with the bacterial enzymes at the active site results in blocking off the folic acid synthesis. The resulting deficiency of this vitamin is fatal to these microorganisms. Since man lacks the enzymes necessary for folic acid synthesis from PABA, folic acid is needed as a vitamin in the diet. Thus, the sulfa drugs inhibit growth of the bacteria in man by competing with PABA for the active centres of the bacterial enzymes.

3. Competitive inhibition is used therapeutically to treat patients who have ingested methanol ($CH_3.OH$), a solvent found in gas-line antifreeze. Methanol is converted to formaldehyde by the action of the enzyme alcohol dehydrogenase (E.C. No. 1.1.1.1) . Formaldehyde damages many tissues esp., the optic ones, causing blindness. Ethanol competes effectively with methanol as a substrate for alcohol dehydrogenase. Ethanol, thus, acts as an inhibitor for the substrate methanol and competes with it to occupy the active site of the enzyme. Methanol-poisoning may, thus, be cured by an intravenous infusion of ethanol to the patients so that formaldehyde formation is considerably lowered. Most of the methanol can be extracted harmlessly in the urine.

4. A physiologically important example of competitive inhibition is found in the formation 2, 3- bisphosphoglycerate (BPG) from 1,3-bisphosphoglycerate. *Bis-phosphoglycerate mutase*, the enzyme catalyzing this isomerization reaction, is completely inhibited by even low levels of 2,3-bisphosphoglycerate. In fact, it is not uncommon for an enzyme to be completely inhibited by its own product because of the product's structural resemblance to the substrate. However, increasing the concentration of substrate checks the inhibitory effect.

Michaelis-Menten equation may also be applied to the competitive inhibition of enzymes. Here, besides the normal equation :

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

one must also consider the equilibrium state between the enzyme and the inhibitor, I as follows :

$$E + I \rightleftharpoons EI$$

In the presence of the competitive inhibitor, henceforth, the concentration of the free enzyme would be expressed as :

$$[(E) — (ES) — (EI)]$$

The dissociation of the enzyme-inhibitor compound, K_i would then be defined as :

$$K_i = \frac{[(E) - (ES) - (EI)]\ (1)]}{(EI)}$$

Deriving the Michaelis-Menten equation for this case of inhibition, one obtains as follows :

$$V = \frac{V_m \times (S)\ K_i}{K_m K_i + K_m\ (I) + K_i (S)} \qquad ...(15)$$

Reversal of this equation gives the modified form of Lineweaver-Burk equation :

$$\frac{1}{V} = \frac{K_m K_i + K_m (I) + K_i\ (S)}{V_m \times (S)\ K_i}$$

or

$$\frac{1}{V} = \frac{K_m}{V_m}\left(1 + \frac{(I)}{K_i}\right) \times \frac{1}{(S)} + \frac{1}{V_m} \qquad ...(16)$$

When $\frac{1}{V}$ is plotted against $\frac{1}{(S)}$ (Fig. 18–16), the intercept $\frac{1}{V_m}$ remains the same as in the case of non-inhibited reaction but the slope, which is now $\frac{K_m}{V_m}\left(1 + \frac{(I)}{K_i}\right)$, is increased by the

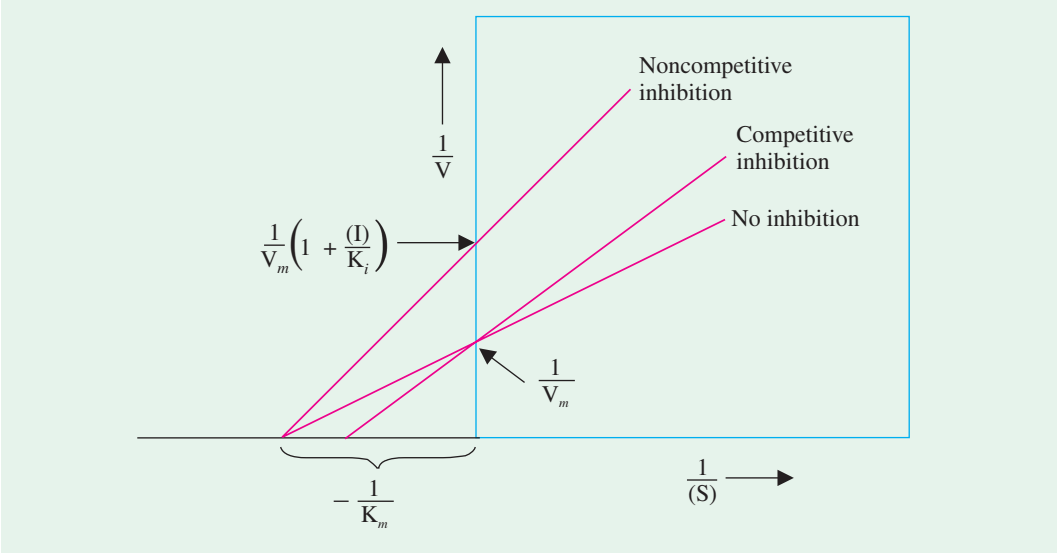


Fig. 18–19. Double reciprocal graph (Lineweaver-Burk plots)

A plot of $\frac{1}{V_m}$ versus $\frac{1}{(S)}$ for an enzyme reaction with (*i*) no inhibitor (*ii*) a competitive inhibitor and (*iii*) a noncompetitive inhibitor.

(Modified from Fairley and Kilgour, 1966)

factor, $1 + \frac{(I)}{K_i}$. Thus, if the substrate concentration is large enough, the effect of the competitive inhibitor can be overcome and V_m may be reached.

The phenomenon of competitive inhibition is of practical value. It may be employed to prevent the growth of one organism in the presence of other, for example: (*a*) bacterial growth inhibited in the

presence of an animal (*b*) growth of the insect inhibited on a fruit tree.

B. Noncompetitive inhibition. Here no competition occurs between the substrate, S and the inhibitor, I (Fig. 18-20). The inhibitor has little or no structural resemblance with the substrate and it binds with the enzyme at a place other than the active site (*i.e.*, at the allosteric site). Since I and S may combine at different sites, formation of both EI and ESI complexes takes place. Both ES and ESI may break down to produce the reaction product (P). It may, however, be noted that in noncompetitive inhibition, the inhibitor and substrate can bind simultaneously to an enzyme molecule since their binding sites are different and hence do not overlap. The enzyme is inactivated when inhibitor is bound, whether or not substrate is also present. Thus, it is apparent that a noncompetitive inhibitor acts by lowering the turnover number rather than by decreasing the proportion of enzyme molecules that are bound to the substrate. Noncompetitive inhibition, in contrast to competitive inhibition, cannot be overcome by increasing substrate concentration. The inhibitor effectively lowers the concentration of active enzyme and hence lowers V_{max}. There is almost no effect on K_m, however.

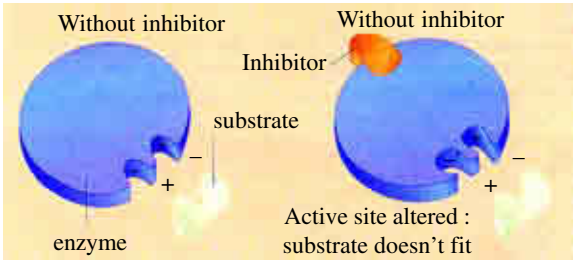


Fig. 18–20. Noncompetitive inhibition

Non-competitive inhibitors attach to enzyme molecules and alter the overall shape, so that the active site cannot function. Although the substrate may still bind, forming an *enzyme–inhibitor substrate* complex, the substrate cannot be turned into product. When the inhibitor molecule is removed, normal function is restored.

Certain noteworthy examples of noncompetitive inhibition are as follows :

1. Various heavy metals ions (Ag^+, Hg^{2+}, Pb^{2+}) inhibit the activity of a variety of enzymes. *Urease,* for example, is highly sensitive to any of these ions in traces. Heavy metals form mercaptides with sulfhydryl (—SH) groups of enzymes :

$$Enz—SH + Ag^+ \rightleftharpoons Enz—S—Ag + H^+$$

The established equilibrium inactivates enzymes that require a —SH group for activity. Because of the reversibility of mercaptide formation, the inhibition can be relieved by removal of the heavy metal ion. In the treatment of lead poisoning, advantage is taken of the metal's affinity for —SH groups. Therefore, the sulfhydryl compounds are administered to interact with the metal in the circulatory system and form mercaptides, which are then excreted.

2. Similarly, cyanide and hydrogen sulfide strongly inhibit the action of iron-containing enzymes like *catalase* and *peroxidase*.

The Lineweaver-Burk equation for this type of inhibition would be :

$$\frac{1}{V} = \left[1 + \frac{(I)}{K_i}\right]\left[\frac{1}{V_m} + \left(\frac{K_m}{V_m}\right) \times \frac{1}{(S)}\right] \quad ...(17)$$

Thus, here both the slope and the intercept are altered, rather increased by the factor $1 + \frac{(I)}{K_i}$ in contrast to the competitive inhibition where only the slope is changed. Moreover, the maximal velocity attained is less than that found in noninhibited case.

C. Uncompetitive inhibition. An uncompetitive inhibitor also binds at an allosteric site (like the noncompetitive inhibitors) but the binding takes place only with the enzyme-substrate (ES) complex, and not the free enzyme molecule.

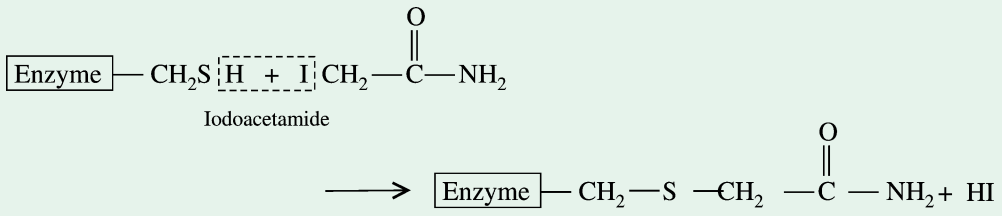
Irreversible Enzyme Inhibition

Although irreversible inhibition was once categorized and tested as noncompetitive inhibition, it is now recognized as a distinct type of inhibition. Irreversible inhibitors are those that combine with or destroy a functional group on the enzyme that is essential for its activity. In fact, an irreversible inhibitor dissociates *very slowly* from its target enzyme because it becomes very tightly bound to its

active site, thus inactivating the enzyme molecule. The bonding between the inhibitor and enzyme may be covalent or noncovalent.

Two common examples of irreversible inhibition are discussed below :

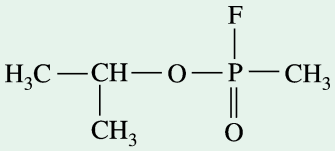
1. Alkylating reagents, such as **iodoacetamide,** irreversibly inhibit the catalytic activity of some enzymes by modifying cysteine and other side chains. Iodoacetamide is a widely-used agent for the detection of sulfhydryl group.



Inactivation of an enzyme (with a critical cysteine residue) by iodoacetamide

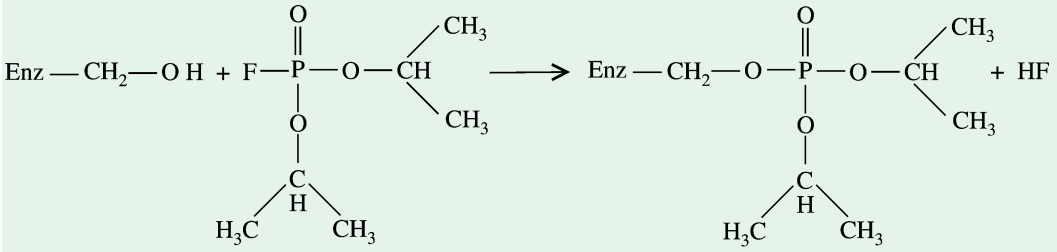
2. Organophosphorus compounds, such as diisopropyl-phosphofluoridate, DIPF, are potent irreversible inhibitors of enzymes that have active seryl residues at their catalytic sites. DIPF is closely

DIPF is also called **diisopropylfl-uorophosphate**, **DIFP** (AL Lehninger, DL Nelson and MM Cox, 1993)



Sarin, a nerve gas

related chemically to nerve gas, whose lethality is due to the inactivation of *acetylcholinesterase*, an enzyme critical for the transmission of nerve impulses. Acetylcholinesterase (Fig. 18-21) cleaves the neurotransmitter acetylcholine, an essential step in normal functioning of nervous system.

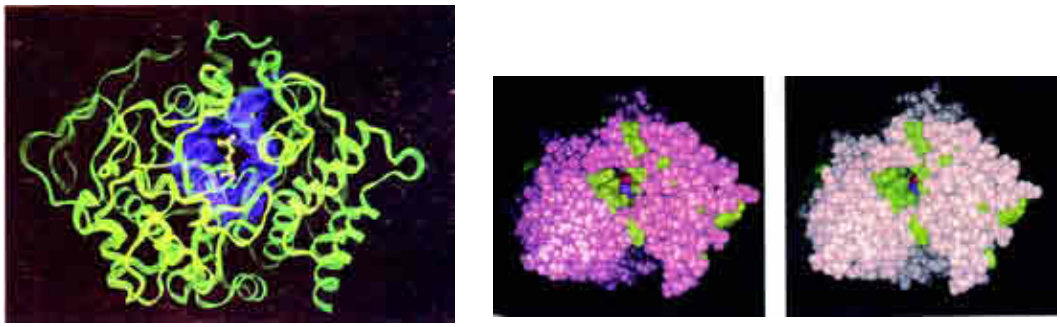


Inactivation of acetylcholinesterase by diisopropylphosphofluoridate, DIPF

A special class of irreversible inhibitors are the **suicide inhibitors.** These compounds are relatively unreactive until they bind to the active site of a specific enzyme. A suicide inhibitor is designed to carry out the first few chemical steps of the normal enzyme reaction. Instead of being transformed into the normal product, however, the inhibitor is converted to a very reactive compound that combines irreversibly with the enzyme. These are also called **mechanism-based inactivators**, because they utilize the normal enzyme reaction mechanism to inactivate the enzyme.

BISUBSTRATE REACTIONS

We have heretofore been concerned with reactions involving enzymes that require only one substrate. Yet, enzymatic reactions involving two substrates and yielding two products account for ~60% of known biochemical reactions.



(A) (B)

Fig. 18–21. The x-ray structure of acetylcholinesterase (AChE)

(A) The ribbon model. The aromatic side chains lining its active site gorge (*purple*) are shown in stick form surrounded by their Van der Waals dot surface. The ACh substrate, which was modeled into the active site (the enzyme was crystallized in its absence), is shown in ball-and-stick form with its atoms gold and its bonds light blue. The entrance to the gorge is at the top of the figure.

(B) Space-filling stereo view looking down into the active site. Aromatic residues are in green, Ser ^{200} is red. Glu ^{199} is cyan, and other residues are grey.

[*Courtesy : (A) Joel Sussman, The Weizmann Institute of Science, Israel and (B) Joel Sussman et al, 1991*].

$$A + B \underset{}{\overset{E}{\rightleftharpoons}} P + Q$$

Almost all of these so-called ***bisubstrate reactions*** are either transferase reactions in which the enzyme catalyzes the transfer of a specific functional group, X, from one of the substrates to the other :

$$P{-}X + B \underset{}{\overset{E}{\rightleftharpoons}} P + B{-}X$$

or oxidation-reduction reactions in which reducing equivalents are transferred between the two substrates. For example, in the peptide hydrolysis reaction catalyzed by trypsin, the peptide carbonyl group with its pendent polypeptide chain is transferred from the peptide nitrogen atom to a water molecule :

$$R_1{-}\overset{\overset{O}{\|}}{C}{-}NH{-}R_2 + H_2O \xrightarrow{\text{Trypsin}} R_1{-}\overset{\overset{O}{\|}}{C}{-}O^- + \overset{+}{H_3}N{-}R_2$$

Similarly, in the alcohol dehydrogenase reaction, a hydride ion is formally transferred from ethanol to NAD^+ :

$$CH_3{-}\overset{\overset{H}{|}}{\underset{\underset{H}{|}}{C}}{-}OH + NAD^+ \xrightarrow[H^+]{\text{Alcohol dehydrogenase}} CH_3{-}\overset{\overset{O}{\|}}{C}H + NADH$$

A. Terminology

In such reactions, the sequence of binding of the substrate to the enzyme molecule may be of different types. Depending on the sequence of binding of the substrate to the enzyme, W.W. Cleland (1967) propounded 4 types of mechanisms and explained them with the help of what are commonly called **'Cleland diagrams'** (Fig. 18–22). Cleland's nomenclatural concept is based on the following 5 *conventions* :

1. The substrates are designated by the letters A, B, C and D in the order that they add to the enzyme.
2. The products are denoted by the letters P, Q, R and S in the order that they leave the enzyme.
3. The enzyme is indicated by the letter E. The intermediary stable forms of enzymes, that are produced and then disappear during the course of reaction, are represented by the letters F, G, H etc.
4. The enzyme-substrate complexes are shown in parenthesis.

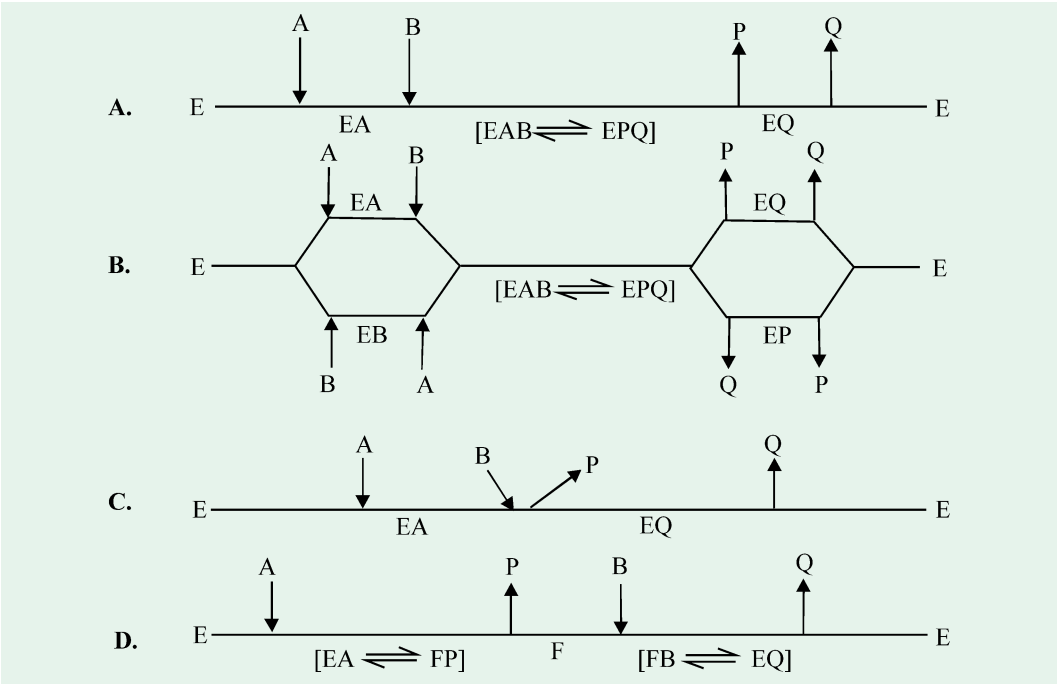


Fig. 18–22. Four substrate binding patterns in bisubstrate enzyme reactions (Cleland diagrams)

A. The ordered sequential mechanism
B. The random sequential mechanism
C. Theorell–Chance mechanism
D. The ping pong mechanism

For describing the enzymatic reactions using Cleland's shorthand notation, certain conventions are followed. The enzyme is represented by a horizontal line and successive additions of substrates and release of products are denoted by vertical arrows. Enzyme forms are placed under the line and rate constants, if given, are to the left of the arrow or on top of the line for forward reactions.

5. The number of reactants and products in a given reaction are specified, in order by the terms *Uni* (one), *Bi* (two), *Ter* (Three), and *Quad* (four). For example, a reaction requiring one substance and yielding three products is designated a *Uni Ter reaction*.

B. Types of Bi Bi reactions

In this section, we shall deal with reactions that require two substrates and yield two products, that is *Bi Bi reactions.* It may, however , be remembered that there are numerous examples of even more complex reactions. Enzyme-catalyzed group-transfer Bi Bi reactions fall under two major mechanistic classifications.

1. Sequential reactions

Reactions in which all substrates combine with the enzyme before a reaction can occur and products be released are known as *sequential reactions.* In such reactions, the group being transferred, X, is directly passed from A (= P-X) to B, yielding P and Q (= B -X). Hence, such reactions are also called **single-displacement reactions.**

Sequential reactions can be further classified into 2 types :

(*a*) those with a compulsory order of substrate addition to the enzyme, which are said to have an **ordered mechanism,** and

(*b*) those with no preference for the order of substrate addition, which are described as having a **random mechanism.**

In the ordered mechanism, the binding of the first substrate is apparently required for the enzyme

to form the binding site for the second substrate, whereas for the random mechanism, both binding sites are present on the free enzyme.

2. Ping pong reactions

Reactions in which one or more products are released before all substrates have been added are known as *ping pong reactions.* In such a reaction, a functional group X of the first substrate A (= P—X) is displaced from the substrate E to yield the first product P and a stable enzyme form F (= E—X) in which X is tightly (often covalently) bound to the enzyme (*ping*). In the second stage of the reaction, X is displaced from the enzyme by the second substrate B to yield the second product Q (= B—X), thereby regenerating the original form of the enzyme, E (*pong*). Such reactions are, hence, also known as **double-displacement reactions.** A notable feature of ping pong Bi Bi reactions is that the substrates A and B do not encounter one another on the surface of the enzyme.

The salient features of the mechanisms underlying these different reaction types are described hereunder :

1. The ordered sequential mechanism. This mechanism envisages that the first substrate (A) binds with the enzyme (E), followed by the second substrate (B). The substrate A which combines first is called the *leading substrate* and the substrate B which combines later is termed the *following substrate.* A ternary complex (EAB) between enzyme and the two substrates is formed. In this complex, both substrates are converted to products (P and Q) and then liberated in the same sequence their corresponding substrate had combined, *i.e.*, the product P is released first followed by Q. Many NAD^+- and $NADP^+$-requiring dehydrogenases follow an ordered Bi Bi mechanism in which the coenzyme is the leading substrate.

2. The random sequential mechanism. In this mechanism, there is no definite sequence of substrate association to the enzyme. They can bind in any order and the products from the ternary complex can also be released in any order. Some dehydrogenases and kinases operate through this mechanism.

3. Theorell–Chance mechanism. It is a variant of the ordered sequential mechanism in which the ternary complex (EAB) is not at all formed. This mechanism operates in the reaction catalyzed by *alcohol dehydrogenase* (E.C. No. 1.1.1.1).

4. The ping pong mechanism. This mechanism envisages that one substrate binds and one product is released before the second substrate can bind to the enzyme and may release the second substrate. Thus, here the enzyme acts as a board and the substrates act as ping pong balls. Many enzymes, including glutamate dehydrogenase (E.C. No. 1.4.1.2), chymotrypsin, transaminases and some flavoenzymes obey ping pong mechanism.

C. Kinetics of Bi Bi Reactions

Steady state kinetic measurements can be utilized to distinguish among the foregoing bisubstrate mechanisms. For doing so, one must first derive their *rate equations.* This can be done in much the same way as for single-substrate-enzymes, *i.e.*, solving a set of simultaneous linear equations consisting of an equation expressing the steady state condition for each kinetically distinct enzyme complex plus one equation representing the conservation condition for the enzyme. This, indeed, is a more complex situation for bisubstrate enzymes than it is for single-substrate or monosubstrate enzymes.

The rate equations for the above-described bisubstrate mechanisms in the absence of products are given below in double reciprocal form.

For ordered Bi Bi reactions :

$$\frac{1}{v_0} = \frac{1}{V_m} + \frac{K_M^A}{V_m[A]} + \frac{K_M^B}{V_m[B]} + \frac{K_S^A K_M^B}{V_m[A][B]} \qquad ...(18)$$

For random Bi Bi reactions :

The rate equation for the *general* random Bi Bi reaction is quite complicated. However, in the special case that both substrates are in *rapid and independent equilibrium* with the enzyme; that is, the EAB–EPQ interconversion is rate determining, the initial rate equation reduces to the following relatively simple form. This mechanism is known as the *rapid equilibrium random Bi Bi mechanism :*

$$\frac{1}{v_0} = \frac{1}{V_m} + \frac{K_S^A K_M^B}{V_m K_S^B [A]} + \frac{K_M^B}{V_m [B]} + \frac{K_S^A K_M^B}{V_m [A][B]} \quad ...(19)$$

For ping pong Bi Bi reactions :

$$\frac{1}{v_0} = \frac{K_M^A}{V_m [A]} + \frac{K_M^B}{V_M [B]} + \frac{1}{V_m} \quad ...(20)$$

Physical Significance of the Bisubstrate Kinetic Parameters

The parameters in the equations describing bisubstrate reactions have meanings similar to those for single-substrate reactions. V_m is the maximal velocity of the enzyme obtained when both A and B are present at saturating concentrations; K_M^A and K_M^B are the respective concentrations of A and B, necessary to achieve $\frac{1}{2}V_m$ in the presence of a saturating concentration of the other; and K_S^A and K_S^B are the respective dissociation constants of A and B from the enzyme, *E*.

ALLOSTERIC ENZYMES

A **modulator** is a metabolite which, when bound to the allosteric site of an enzyme, alters its kinetic characteristics. The modulators for allosteric enzymes may be either stimulatory or inhibitory. A stimulator is often the substrate itself. The regulatory enzymes for which substrate and modulator are identical are called **homotropic.** When the modulator has a structure different than the substrate, the enzyme is called **heterotropic.** Some enzymes have more than one modulators.

The term **allosteric** (*allos*^{G} = other ; *stereos*^{G} = space or site) **site** has been introduced by the two Nobel Laureates, Monod and Jacob to denote an enzyme site, different from the active site, which noncompetitively binds molecules other than the substrate and may influence the enzyme activity.

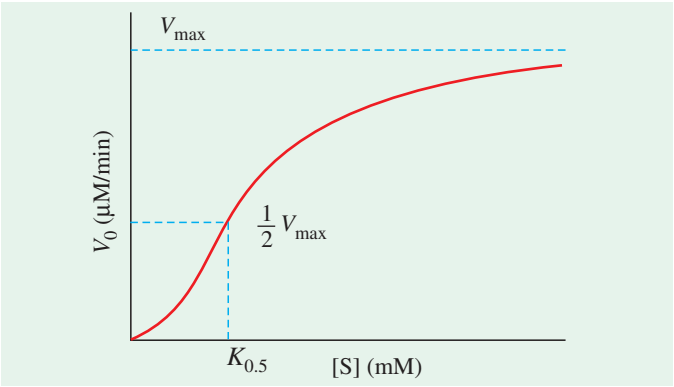


Fig. 18–23. Schematic of an allosteric enzyme's activity by its substrate

Note the sigmoid curve given by a homotropic enzyme, in which the substrate also serves as a positive (stimulatory) modulator. The analogous curve for the nonregulatory enzymes is hyperbolic, as also predicted by the Michaelis-Menten equation. The allosteric enzymes, however, do not obey the Michaelis-Menten kinetics. Also note that a relatively small increase in [S] in the steep part of the curve can cause a very large increase in V_o.

The allosteric enzymes are structurally different from the simple nonregulatory enzymes. Besides the presence of an active or catalytic site, the allosteric enzymes also have one or more regulatory or allosteric sites for binding the modulator. Just as an enzyme's active site is specific for

FRANCOIS JACOB
(Born, 1920)

JACQUES MONOD
(LT, 1910–1976)

Both Jacob and Monod served heroically during World War II, Jacob as member of the Free French Forces, injured seriously in Normandy during August 1944, and Monod as a leader of the Paris Resistance. Monod had already obtained his PhD, Jacob had to wait until 1947 to gain his MD from the University of Paris. Both spent the major parts of their careers at the Pasteur Institute, the famous Paris research centre set up around Louis Pasteur in the late nineteenth century and still one of the most influential European laboratories. Jacob contributed to some of the early work on bacteriophages, with Andre Lwoff and Elie Wollman, before beginning the collaboration with Monod that led to the **operon theory** and the **concept of messenger RNA** in 1961. The two shared the **1965 Nobel Prize** with Lwoff. Both have published important books : *Chance and Necessity* by Monod is a powerful vindication of natural selection, *The Logic of Life* by Jacob an illuminating account of changes in scientific thinking over the past three centuries. Jacob's autobiography. *The Statue Within,* was published in 1987.

its substrate, the allosteric site is specific for its modulator. Enzymes with several modulators generally have different specific binding sites for each. By contrast, in homotropic enzymes, the active site and allosteric site are the same. Most of them have two or more polypeptide chains or subunits, for example, *aspartate transcarbamoylase* (Fig. 18–23) has 12 subunits.

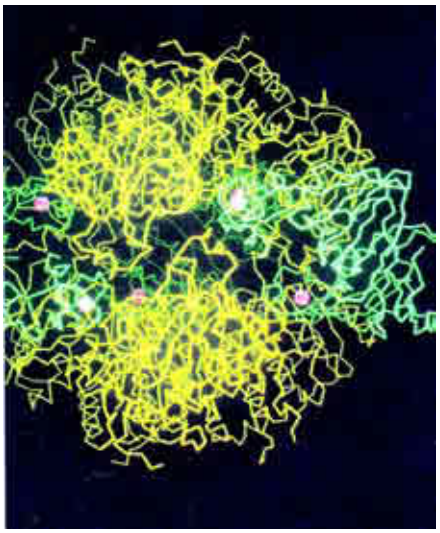


Fig. 18–24. The structure of the enzyme aspartate transcarbamoylase (ATCase) in the T from Catalytic Subunits are shown in yellowed and regulatory subunits in green.

Other differences between nonregulatory enzymes and allosteric enzymes involve kinetic properties. The allosteric enzymes often display sigmoidal plots (Fig. 18–24) of the reaction velocity, V versus substrate concentration, [S], rather than the hyperbolic plots predicted by Michaelis-Menten equation. Recall that the oxygen-binding curve of myoglobin is hyperbolic, whereas that of hemoglobin is sigmoidal. The binding of enzymes to substrates is analogous. In allosteric enzymes, the binding of substrate to one active site can affect the properties of other active sites in the same enzyme molecule. A possible outcome of this interaction between subunits is that the binding of substrate becomes cooperative. In addition, the activity of an allosteric enzyme may be altered by regulatory molecules that are bound to the allosteric sites, just as oxygen binding to hemoglobin is affected by H^+, CO_2 and BPG.

With heterotropic enzymes, however, it is difficult to generalize about the shape of the substrate-saturation curve (Fig. 18–25). An activator may cause the substrate-saturation curve to become more nearly hyperbolic with a decrease in $K_{0.5}$ but no change in V_{max}, thus resulting in an increased reaction velocity at a fixed substrate concentration. On the contrary, a negative modulator (= an inhibitor) may produce a more sigmoid substrate-saturation curve, with an increase in $K_{0.5}$. In other words, allosteric activators shift the substrate-saturation curve to the left (to higher saturation), whereas

allosteric inhibitors shift it to the right (to lower saturation).

[Although one can find a value of [S] on the sigmoid-saturation curve at which V_0 is half-maximal, one cannot put the designation K_m to it because the enzyme does not follow the hyperbolic Michaelis-Menten relationship. Instead, the symbol $[S]_{0.5}$ or $K_{0.5}$ is often used to represent the substrate concentration giving half-maximal velocity of the reaction catalyzed by an allosteric enzyme.]

Mechanism of Kinetic Behaviour of Allosteric Enzymes

Two general models for the interconversion of inactive and active forms of allosteric enzymes have been proposed.

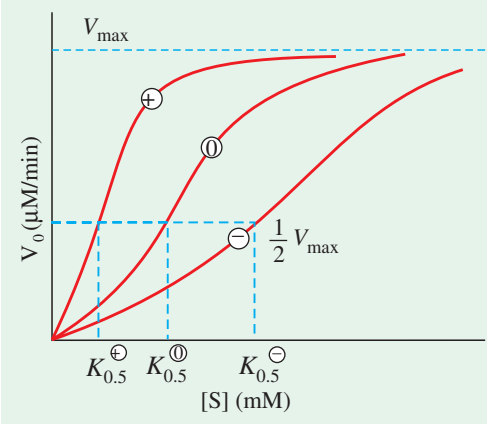


Fig. 18–25. Effects of an activator ⊕, and inhibitor ⊖ and no modulator ⊚ on an allosteric enzyme in which $K_{0.5}$ is modulated without a change in V_{max}

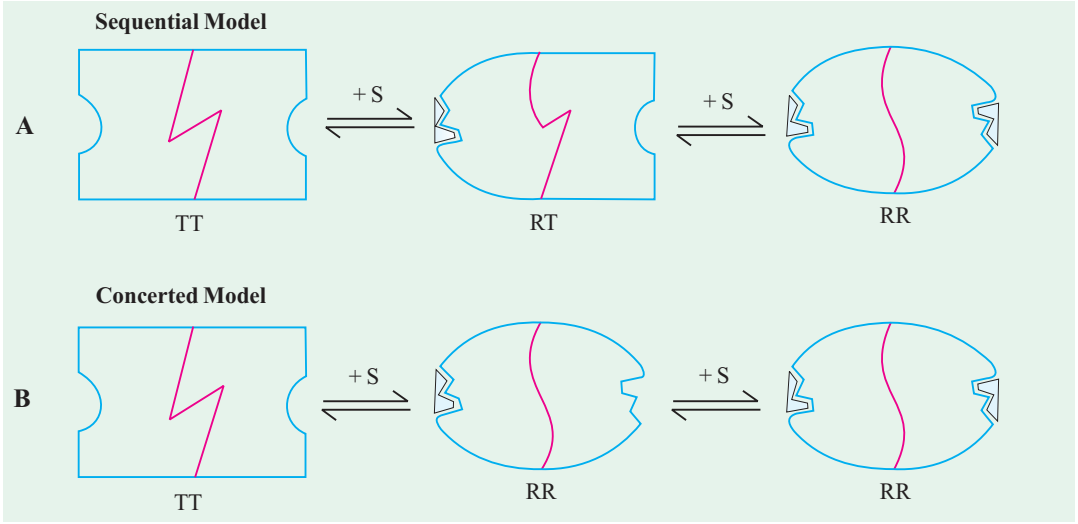


Fig. 18–26. Comparison of the two allosteric models

A. Simple sequential model

B. Concerted or symmetry model

(Adapted from Lubert Stryer, 1995)

A. Simple sequential model

Proposed by Daniel E. Koshland Jr. in 1966, the model envisages that the allosteric enzyme can exist in only two conformational change individually. Binding of substrate increases the probability of the conformational change. A conformational change in one subunit makes a similar change in an adjacent subunit, as well as the binding of a second substrate molecule, more likely. There are more potential intermediate states in this model than in the other (symmetry) model.

Consider an allosteric enzyme consisting of two identical subunits, each containing an active site (Fig. 18–26A). The T (tense) form has low affinity and the R (relaxed) form has high affinity for substrate. In this model, the binding of substrate to one of the subunits induces a T → R transition in that subunit but not in the other. The affinity of the other subunit for substrate is increased because the subunit interface has been altered by the binding of the first substrate molecule.

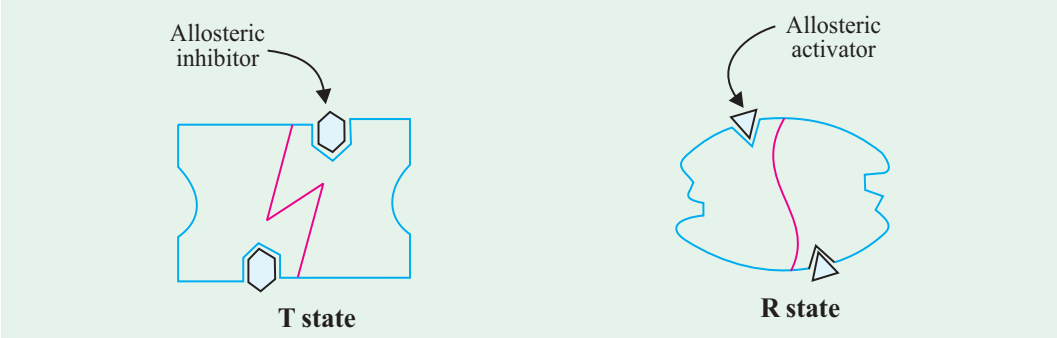


Fig. 18–27. Effect of allosteric activator and allosteric inhibitor on substrate binding

Note that in the connected model, an allosteric inhibitor stabilizes the T state, whereas an allosteric activator stabilizes the R state.

B. Concerted or Symmetry model

Proposed by Jacques Monod and colleagues in 1965, the model envisages that an allosteric enzyme can exist in still two conformations, active and inactive. All subunits are in the active form or all are inactive. Every substrate molecule that binds increases the probability of transition from the inactive to the active state.

In the concerted model, the binding of substrate to one of the subunits increases the probability that both switch from the T to the R form (Fig. 18–26B). Thus, symmetry is conserved in the concerted model but not in the sequential model.

The effects of allosteric activators and inhibitors can be explained quite easily by the concerted model. An allosteric inhibitor binds preferably to the T form, whereas an allosteric activator binds preferentially to the R form (Fig. 18–27). Consequently, *an allosteric inhibitor shifts the $R \rightarrow T$ conformational equilibrium toward T, whereas an allosteric activator shifts it toward R*. The result is that an allosteric activator increases the binding of substrate to the enzyme, whereas an allosteric inhibitor decreases substrate binding.

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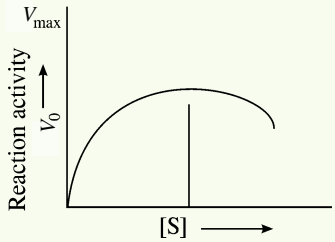
PROBLEMS

1. For enzyme that follows simple Michaelis-Menten kinetics, what is the value of V_{max} if V_0 is equal to 1 µmol/minute at 1/10 K_M?

2. A simple Michaelis-Menten enzyme, in the absence of any inhibitor, displayed the following kinetic behaviour. The expected value of V_{max} is shown on the *y*-axis.



(*a*) Draw a double-reciprocal plot that corresponds to the velocity-versus-substrate curve.

(*b*) Provide an explanation for the kinetics results.

3. The active site of an enzyme usually consists of a pocket on the enzyme surface lined with the amino acid side chains necessary to bind the substrate and catalyze its chemical

transformation. Carboxypeptidase, which sequentially removes the carboxyl-terminal amino acid residues from its peptide substrates, consists of a single chain of 307 amino acids. The two essential catalytic groups in the active site are furnished by Arg^{145} and Glu^{270}.

(*a*) If the carboxypeptidase chain were a perfect α helix, how far apart (in nanometers) would Arg^{145} and Glu^{270} be ?

(*b*) Explain how it is that these two amino acids, so distantly separated in the sequence, can catalyze a reaction occurring in the space of a few tenths of a nanometer.

(*c*) If only these two catalytic groups are involved in the mechanism of hydrolysis, why is it necessary for the enzyme to contain such a large number of amino acid residues ?

4. (*a*) At what substrate concentration will an enzyme having a k_{cat} of 30 s^{-1} and a K_m of 0.005 M show one-quarter of its maximum rate ?

(*b*) Determine the fraction of V_{max} that would be found in each case when $[S] = ½K_m$, 2 K_m, and 10 K_m.

5. The following experimental data were collected during a study of the catalytic activity of an intestinal peptidase capable of hydrolyzing the dipeptide glycylglycine :

$$\text{Glycylglycine} + H_2O \longrightarrow 2 \text{ glycine}$$

[S] (mM)	Product formed (μ mol/min)
1.5	0.21
2.0	0.24
3.0	0.28
4.0	0.33
8.0	0.40
16.0	0.45

From these data, determine by graphical analysis the values of K_m and V_{max} for this enzyme preparation and substrate.

6. Carbonic anhydrase of erythrocytes (M_r 30,000) is among the most active of known enzymes. It catalyzes the reversible hydration of CO_2 :

$$H_2O + CO_2 \rightleftharpoons H_2CO_3$$

which is important in the transport of CO_2 from the tissues to the lungs.

(*a*) If 10 μg of pure carbonic anhydrase catalyzes the hydration of 0.30 g of CO_2 in 1 min at 37°C under optimal conditions, what is the turnover number (k_{cat}) of carbonic anhydrase (in units of min^{-1}) ?

(*b*) From the answer in (*a*), calculate the activation energy of the enzyme-catalyzed reaction (in kJ/mol).

(*c*) If carbonic anhydrase provides a rate enhancement of 10^7, what is the activation energy for the uncatalyzed reaction ?

7. Many enzymes are inhibited irreversibly by heavy-metal ions such as Hg^{2+}, Cu^{2+}, or Ag^+, which can react with essential sulfhydryl groups to form mercaptides :

$$\text{Enz—SH} + Ag^+ \longrightarrow \text{Enz—S—Ag} + H^+$$

The affinity of Ag^+ for sulfhydryl groups is so great that Ag^+ can be used to titrate —SH groups quantitatively. To 10 mL of a solution containing 1.0 mg/mL of a pure enzyme was added just enough $AgNO_3$ to completely inactivate the enzyme. A total of 0.342 μmol of $AgNO_3$ was required. Calculate the *minimum* molecular weight of the enzyme. Why does the value obtained in this way give only the minimum molecular weight ?

8. When enzyme solutions are heated, there is a progressive loss of catalytic activity with time. This loss is the result of the unfolding of the native enzyme molecule to a randomly coiled conformation, because of its increased thermal energy. A solution of the enzyme hexokinase incubated at 45ºC lost 50% of its activity in 12 min, but when hexokinase was incubated at 45ºC in the presence of a very large concentration of one of its substrates, it lost only 3% of its activity. Explain why thermal denaturation of hexokinase was retarded in the presence of one of its substrates.

9. An experiment measuring velocity versus substrate concentration was run, first in the absence of substance A, and then in the presence of substance A. The following data were obtained.

[*S*] (μM)	*Velocity in Absence of A* ($\mu mol\ min^{-1}$)	*Velocity in Presence of A* ($\mu mol\ min^{-1}$)
2.5	0.32	0.20
3.3	0.40	0.26
5.0	0.52	0.36
10.0	0.69	0.56

Is substance A an activator or an inhibitor ? If it is an inhibitor, what kind of inhibitor is it ?

10. For the experiment in Question 9, calculate the K_m and V_{max} both in the absence and in the presence of substance A. Are these results consistent with your answer for Question 9 ?