

OUTLINES OF BIOCHEMISTRY

FOURTH EDITION

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Enzymes

This chapter introduces the student to the basic properties of the enzyme. Since all metabolic reactions are catalyzed by enzymes, the student should study this chapter with care. The chapters that follow will employ terms such as K_m , V_{max} , competitive, noncompetitive, uncompetitive inhibition, allosteric enzymes, regulatory enzymes, oligomeric enzymes, active centers, etc. These terms are all defined in this chapter.

Purpose

One of the unique characteristics of a living cell is its ability to permit complex reactions to proceed rapidly at the temperature of the surrounding environment. In the absence of the cell these reactions would proceed too slowly. The complex metabolic machinery so fundamental to a cell could not exist under such sluggish conditions. The principal agents which participate in the remarkable transformations in the cell belong to a group of proteins named enzymes.

**7.1
Introduction**

An enzyme is a protein that is synthesized in a living cell and catalyzes or speeds up a thermodynamically possible reaction so that the rate of the reaction is compatible with the biochemical process essential for the maintenance of a cell. The enzyme in no way modifies the equilibrium constant or the ΔG of a reaction. Being a protein, an enzyme loses its catalytic properties if subjected to agents like heat, strong acids or bases, organic solvents, or other conditions which denature the protein.

The high specificity of the catalytic function of an enzyme is due to its protein nature; that is, the highly complex structure of the enzyme protein, can provide both the environment for a particular reaction mechanism and the template function to recognize a limited set of substrates. That region of the protein which participates directly in the conversion of substrate to product is called the active site. Much progress has been made in recent

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years in defining and identifying the active sites of a number of proteins but much is still unknown concerning the unique properties of this region which enables a chemical reaction to take place efficiently, effectively and at a temperature compatible to the cell. Because of enzyme specificity, literally thousands of enzymes are required with each enzyme catalyzing only one reaction or a group of closely related reactions, for example, kinase. Thus, the study of enzyme chemistry is an essential prerequisite to an understanding of the regulation of enzyme activity and in turn the mechanisms of cellular growth and reproduction.

Let us now describe the properties of enzymes.

7.2 Effect of Enzyme Concentration and Substrate Concentration

As is true for any catalyst, the rate of an enzyme-catalyzed reaction depends directly on the concentration of the enzyme. Figure 7-1 depicts the relation between the rate of a reaction and increasing enzyme concentration in the presence of an excess of the compound which is being transformed (also called the substrate).

With a fixed concentration of enzyme and with increasing substrate concentration, a second important relationship is observed. A typical curve is shown in Figure 7-2. Let us discuss the implications of this curve in more detail.

With fixed enzyme concentration, an increase of substrate will result at first in a very rapid rise in velocity or reaction rate. As the substrate concentration continues to increase, however, the increase in the rate of reaction begins to slow down until, with a large substrate concentration, no further change in velocity is observed.

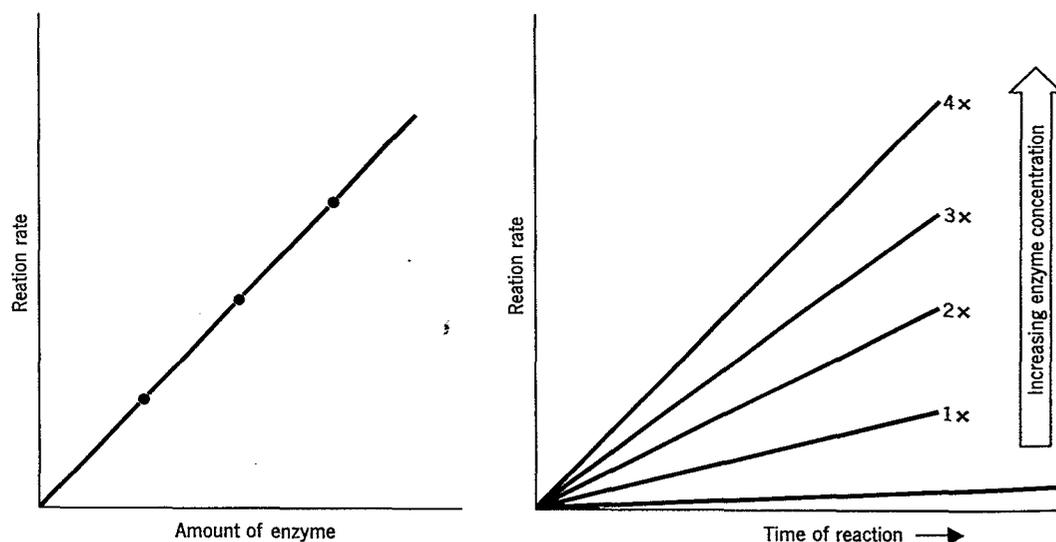


Figure 7-1

Effect of enzyme concentration on reaction rate, assuming that substrate concentration is in saturating amounts.

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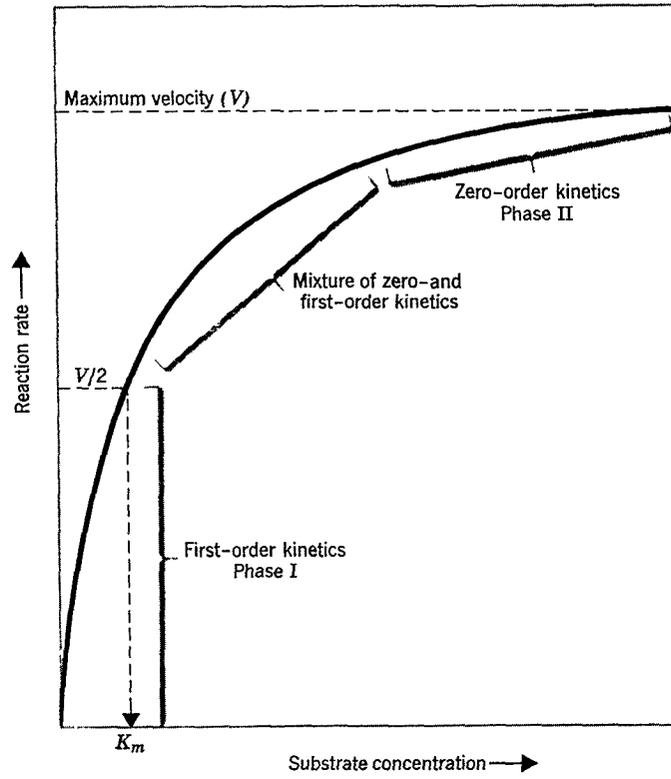


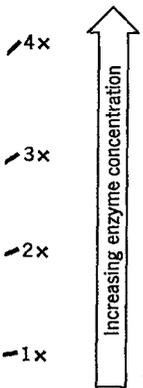
Figure 7-2
Effect of substrate concentration on reaction rate, assum-
ing that enzyme concentration is constant.

Michaelis and others in the early part of this century reasoned correctly that an enzyme-catalyzed reaction at varying substrate concentrations is diphasic; that is, at low substrate concentrations the active sites on the enzyme molecules are not saturated by substrate and thus the enzyme rate varies with substrate concentration (phase I). As the number of substrate molecules increases, the sites are covered to a greater degree until at saturation no more sites are available, the enzyme is working at full capacity and now the rate is independent of substrate concentration (phase II). This relationship is shown in Figure 7-3.

The mathematical equation that defines the quantitative relationship between the rate of an enzyme reaction and the substrate concentration and thus fulfills the requirement of the rectangular hyperbolic curve (Figure 7-2) is the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{7-1}$$

In this equation v is the observed velocity at given substrate concentration $[S]$; K_m is the Michaelis constant expressed in units of concentration (mole/



at substrate

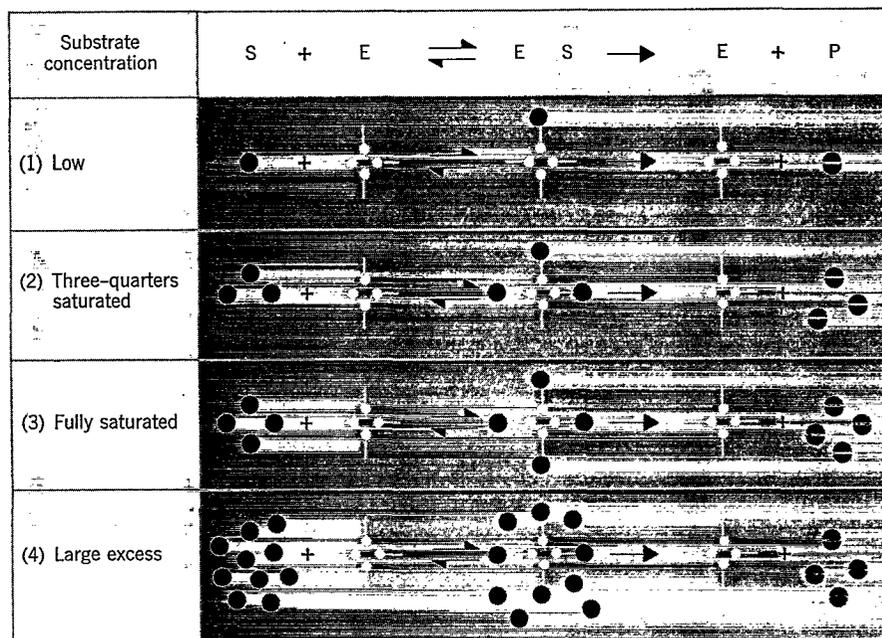


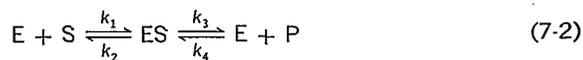
Figure 7-3

Diagrammatic demonstration of effect of substrate concentration on saturation of active sites of enzyme molecules. Note that for a unit time interval, cases 3 and 4 give the same amount of P (product) despite the large excess of substrate in case 4.

liter); and V_{\max} is the maximum velocity at saturating concentration of substrate.

Equation 7-1 is readily derived employing the Briggs-Haldane assumption of steady-state kinetics by a consideration of the following steps:

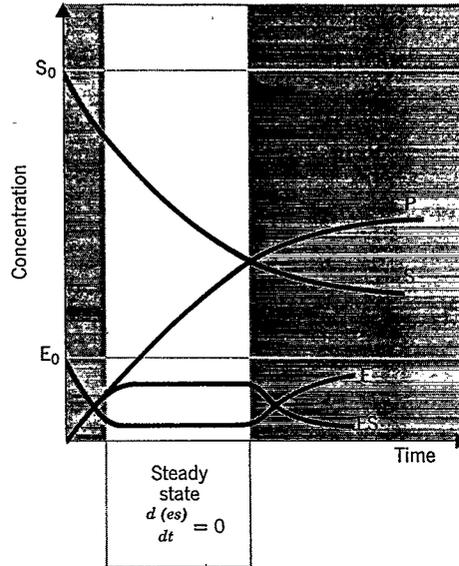
- (1) A typical enzyme-catalyzed reaction involves the reversible formation of an enzyme-substrate complex (ES) which eventually breaks down to form the enzyme, E, again and the product, P. This is represented in equation 7-2:



where k_1 , k_2 , k_3 , and k_4 are the rate constants for each given reaction.

- (2) A few milliseconds after the enzyme and substrate have been mixed, a concentration of ES builds up and does not change as long as S is in large excess and $k_1 \gg k_3$. This condition is called the steady state of the reaction, since the rate of decomposition of ES just balances the rate

of formation. Recognizing that the rate of formation of ES is equal to the rate of decomposition of ES, we can write



Rate of formation of [ES] = Rate of decomposition of [ES]

$$k_1[E][S] + k_4[E][P] = k_2[ES] + k_3[ES] \tag{7-3}$$

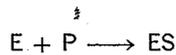
and therefore,

$$[E](k_1[S] + k_4[P]) = [ES](k_2 + k_3) \tag{7-4}$$

$$\frac{[ES]}{[E]} = \frac{k_1[S] + k_4[P]}{k_2 + k_3}$$

$$\frac{[ES]}{[E]} = \frac{k_1[S]}{k_2 + k_3} + \frac{k_4[P]}{k_2 + k_3} \tag{7-5}$$

- (3) We can simplify this equation by considering that since we are examining equation 7-2 at an early stage of the enzyme-catalyzed reaction, P will be very small and hence the rate of formation of ES by the reaction



will be extremely low. Thus, the term $k_4[P]/(k_2 + k_3)$ can be ignored, and equation 7-5 simplifies to

$$\frac{[ES]}{[E]} = \frac{k_1[S]}{k_2 + k_3} \tag{7-6}$$

The three constants k_1 , k_2 , and k_3 can be combined into a single constant, K_m , by the relationship

$$\frac{k_2 + k_3}{k_1} = K_m \tag{7-7}$$

and thus equation 7-6 can be further simplified to

$$\frac{[E]}{[ES]} = \frac{K_m}{[S]} \quad (7-8)$$

- (4) We are now faced with the problem of converting $[E]$ and $[ES]$ into easily measurable values. We can resolve this problem if we consider that the total enzyme concentration $[E]_t$ in the reaction consists of the enzyme, $[E]$, which is free plus that which is combined with substrate, $[ES]$. The free enzyme concentration $[E]$ therefore is $[E]_t - [ES]$ and

$$\begin{aligned} \frac{[E]}{[ES]} &= \frac{[E]_t - [ES]}{[ES]} = \frac{[E]_t}{[ES]} - 1 \\ \frac{[E]_t}{[ES]} - 1 &= \frac{K_m}{[S]} \\ \frac{[E]_t}{[ES]} &= \frac{K_m}{[S]} + 1 \end{aligned} \quad (7-9)$$

Since these terms still cannot be readily determined by the usual techniques available, we must resort to the following relationships: The maximum initial velocity (V_{max}) is attained when the total enzyme $[E]_t$ is completely complexed with saturating amounts of S or

$$V_{max} = k[E]_t \quad (7-10)$$

Moreover, the initial velocity (v) is equal to the concentration of enzyme present as the ES complex at a given concentration of S, or

$$v = k[ES] \quad \text{and thus} \quad \frac{V_{max}}{v} = \frac{[E]_t}{[ES]} \quad (7-11)$$

Finally, the ratio V_{max}/v can now be substituted for $[E]_t/[ES]$ to yield

$$\frac{V_{max}}{v} = \frac{K_m}{[S]} + 1 \quad (7-12)$$

Inverting and rearranging, we obtain

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (7-13)$$

The constant K_m is important since it provides a valuable clue to the mode of action of an enzyme-catalyzed reaction.

Thus, if we permit $[S]$ to be very large, K_m becomes insignificant and equation 7-1 reduces to

$$v = V_{max}$$

or a zero-order reaction in which v is independent of substrate concentration. If we select $v = \frac{1}{2} V_{max}$, equation 7-1 can be written as

$$\begin{aligned} \frac{V_{max}}{2} &= \frac{V_{max}[S]}{K_m + [S]} \\ K_m + [S] &= 2[S] \\ K_m &= [S] \end{aligned}$$

In agreement with the experimental curve depicted in Figure 7-2, the dimensions of K_m are expressed in moles per liter, a concentration expression.

If, however, K_m is large compared to $[S]$, equation 7-1 becomes

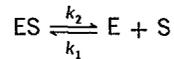
(7-8)

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

That is, v depends on S and the reaction is first-order. These conditions of first-order and zero-order kinetics are indicated in Figure 7-2, and thus equation 7-1 fulfills the requirement of a simple enzyme-catalyzed reaction. We shall soon see, however, that enzyme kinetics can be somewhat more complex when we discuss the kinetics of regulatory enzymes later in this chapter (Section 7.9.1).

Frequently, K_m has been loosely defined as the dissociation constant of an enzyme-catalyzed reaction. Since the simple reaction

(7-9)



is defined by

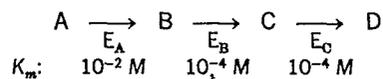
$$K_s = \frac{[E][S]}{[ES]} = \frac{k_2}{k_1}$$

and since K_m is defined as $(k_2 + k_3)/k_1$, K_m will always be equal to or greater than K_s , the dissociation constant. Since $1/K_s$ is the affinity constant or k_1/k_2 , $1/K_m$ will also be equal to or less than the affinity constant of the reaction.

Another important and quite practical consideration is the conclusion that the observed velocity (v) is equal to the maximum velocity (V_{\max}) when $[S] \geq 100K_m$, or zero-order kinetics, and that $v = k[S]$, or first-order kinetics, when $S \leq 0.01K_m$. In setting up experimental conditions for testing enzyme activity, one attempts to operate at saturating or zero-order kinetics, since under these conditions, the enzyme activity is directly proportional to enzyme concentration and independent of substrate concentration.

K_m values are of some use in predicting rate limiting steps in a biochemical pathway such as:

(7-13)



In the conversion of A to D, E_A , E_B and E_C are involved. It is apparent that if the concentration of A is at $10^{-4} M$ in the cell, E_A will be catalyzing reaction $A \rightarrow B$ at a very low rate and would thus be the step that regulates $A \rightarrow D$ conversion. It should be emphasized, however, that K_m values depend on pH, temperature ionic strength of the milieu. Since these parameters are impossible to determine in the cell, K_m values obtained with highly purified enzyme under carefully defined conditions, may bear no relationship to actual K_m values of enzymes functioning in the cell. However, the relations between K_m values in a metabolic sequence may give information about where in the sequence the rate limiting step is located even when measured under in vitro conditions. Thus, this kinetic parameter is an important

constant for an enzyme protein and values for a number of enzymes are listed in Table 7-1.

Important terms such as enzyme units, specific activity, and catalytic center activity or turnover number are defined in Table 7-2.

The terms, K_m and V_{max} , are important values that must be carefully determined. While Figure 7.2 depicts a very simple procedure for obtaining rough approximations of these values, a number of other procedures have been described in the literature. Probably, the method most employed by

Table 7-1
Kinetic Parameters of Some Enzymes

Enzyme	Substrate	K_m (μ/l)	K_i (μ/l)	Inhibitor	Type
Triose phosphate dehydrogenase (rabbit muscle)	D-Glyceraldehyde 3 phosphate	9×10^{-5}	3×10^{-6}	1,3 Diphosphoglycerate	C
			2×10^{-7}	D-Threose 2,4 diphosphate	NC
Succinic dehydrogenase (bovine heart)	Succinate	1.3×10^{-3}	4.1×10^{-5}	Malonate	C
Alcohol dehydrogenase (yeast)	Ethanol	1.3×10^{-2}	6.7×10^{-4}	Acetaldehyde	NC
Glucose-6-phosphatase (rat liver)	Glucose-6-phosphate	4.2×10^{-4}	6×10^{-3}	Citrate	C
Ribulose diphosphate carboxylase (spinach)	Ribulose di-phosphate	1.2×10^{-4}	4.2×10^{-3}	Pi	C
	HCO_3^-	2.2×10^{-2}	9.5×10^{-3}	3 Phosphoglyceric acid	C
Fructose 1,6 diphosphate aldolase (yeast)	Fructose 1-6 diphosphate	3×10^{-4}	2×10^{-4}	L-Sorbose-1- PO_4	C
Succinyl CoA synthetase (pig heart)	Succinate	5×10^{-4}	2×10^{-5}	Succinyl CoA	NC
	CoA	5×10^{-6}	7×10^{-3}	Pi	UC

^a C = competitive
NC = noncompetitive
UC = uncompetitive

Table 7-2
Important Terms in Enzymology

1. Enzyme unit—Amount of enzyme which will catalyze the transformation of 1 μ mole of substrate per minute under defined conditions
2. Specific activity—Units of enzyme per milligram of protein
3. Catalytic center activity—Number of molecules of substrate transformed per minute per catalytic center (a newer term for turnover numbers)

enzyme chemists is the so-called reciprocal Lineweaver-Burke equation which involves taking the reciprocal of both sides of equation 7-1.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \left(\frac{1}{[S]} \right) + \frac{1}{V_{max}} \quad (7-13)$$

which is equivalent to the straight-line equation

$$y = ax + b$$

If now a double reciprocal plot is made with $1/v$ values on the ordinate and $1/[S]$ values on the abscissa, a straight-line relation exists from which K_m can be easily evaluated (see Figure 7-4).

In the previous section, a kinetic equation was derived to describe the enzymic conversion of one substrate to one or more products. However, most enzymes catalyze reactions which involve two or more substrates. The ques-

7.3
Multi-substrate Reaction

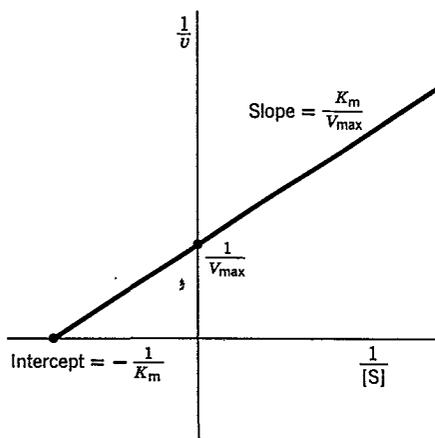


Figure 7-4
A typical Lineweaver-Burk plot of equation 7-13. Lines are extended to $1/v = 0$ to obtain greater accuracy in determining the constants.

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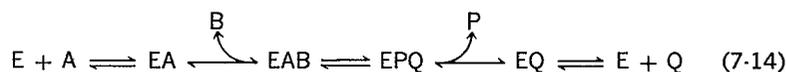
tion arises as to the precise sequence of substrates binding to the enzyme and products being released from the enzyme.

Kineticists have recognized three general mechanisms which describe multi-substrate enzyme systems. Two of these mechanisms, termed *ordered* and *random*, imply that all substrates must be added to the enzyme before any products can be released. The third mechanism, called *ping pong*, states that one or more products may be released from the enzyme before all the substrates have been added to the enzyme.

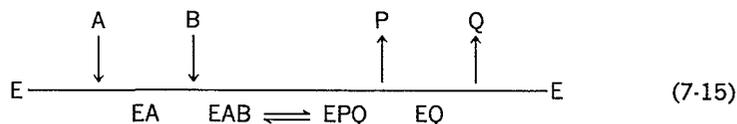
Kineticists have a short-hand description of these multi-substrate reactions which we shall now describe. All substrates, if not specifically stated, are designated as A, B, C, and D and all products as P, Q, R, etc. Different forms of the enzyme are called E, F, G, etc., with E as the first form of the enzymes. The use of these notations will become clear in the following discussion.

7.3.1 Ordered Mechanism. In this mechanism, there is a precise order by which substrates associate with the active sites of an enzyme and the sequence by which the products are released.

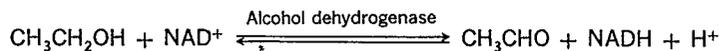
The reaction:



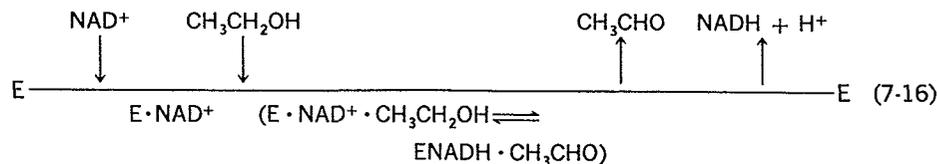
states that only A can first complex with enzyme E and only then can B form the complex EAB. Catalysis occurs, first P and then Q are released in that order. The short-hand notation of this reaction would be:



and this reaction would be called an Ordered Bi, Bi mechanism. The term, Bi, indicates two substrates or products, Uni would indicate a single substrate or product and Ter, three substrates or products. A good example would be the reaction

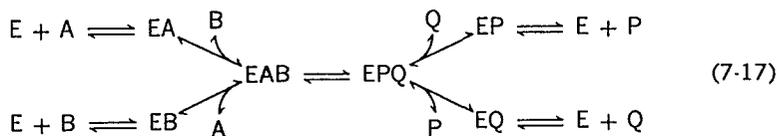


Kinetic analysis revealed the following mechanism:

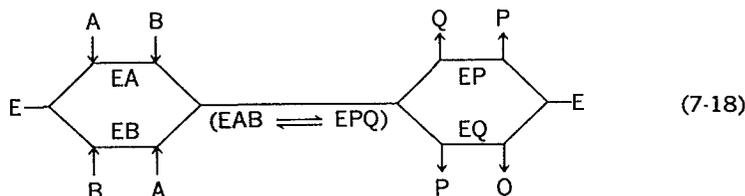


7.3.2 Random Mechanism. When substrates A and B add to an enzyme and products P and Q are released in a random fashion, such a sequence is designated as a random mechanism.

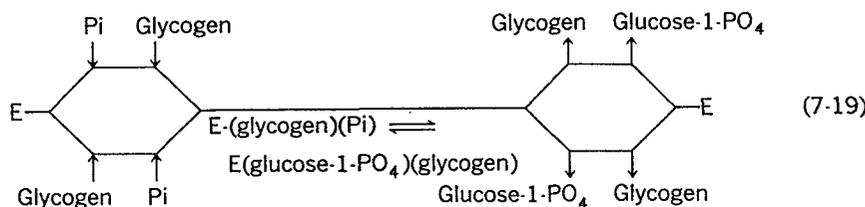
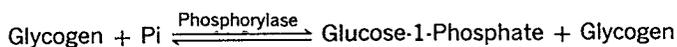
Thus a general reaction would be:



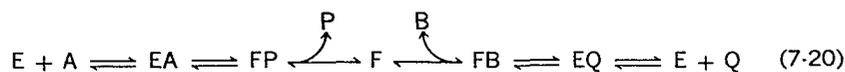
The short-hand notation would be:



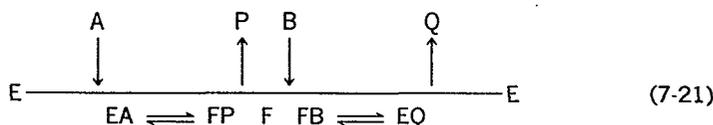
and the reaction would be a Random Bi, Bi mechanism. A good example would be:



7.3.3 Ping Pong Mechanism. A typical reaction sequence is depicted as:

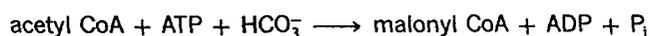


In this sequence, the enzyme complexes formed are EA, FP, FB and EQ with A being first converted to P and then B to Q, F designating a modified enzyme (i.e. X-enzyme where X might be a phosphorylated, carboxylated or other functional group attached to the enzyme transiently). F combines with B with a subsequent transfer of X to B to form Q, the product, with a simultaneous regeneration of E. This sequence is depicted as:

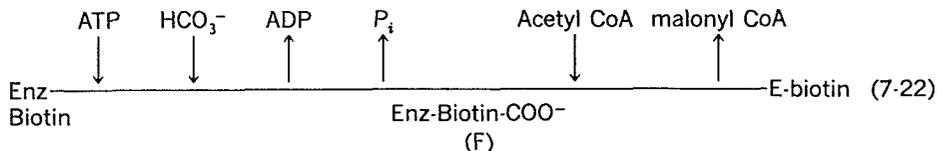


or a Bi, Bi ping pong mechanism.

An example is the rat liver acetyl CoA carboxylase which catalyzes the overall reaction:



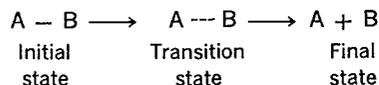
as a Bi, Bi, uni, uni ping pong mechanism:



The procedures employed by the enzyme chemist to determine precisely the order of additions in multisubstrate systems include detailed kinetic analysis of the reaction in terms of equilibrium constants of substrates and cofactors, V_{\max} , product inhibition kinetics, binding determinations for substrates, etc. References to procedures to carry out these studies are found at the end of this chapter.

7.4 Effect of Temperature

A chemical transformation such as:



involves the activation of a population of A - B molecules to an energy-rich state, called the transition state. When reacted, the bond holding A and B will be so weakened that it will break leading to the formation of products A and B. The rate of a reaction will thus be proportional to the concentration of the transition-state species. The concentration of the transition-state species, in turn, depends on the critical thermal kinetic energy required to produce transition-state species of the reacting molecules. The important feature of an enzyme-catalyzed reaction is that an enzyme lowers the activation energy. By interacting with the substrate A-B in a manner that requires less energy, the transition state level is more readily attained with a result that more molecules will react. This concept is diagrammed in Figure 7-5. Note that regardless of the route of reaction both the catalyzed and non-catalyzed reaction have the same ΔG of reaction. Thus we see that an enzyme does not alter the ΔG or equilibrium constant of a reaction but lowers the activation energy which molecule A must attain before it can undergo change.

The familiar Arrhenius equation relates the specific reaction rate constant, k , to temperature:

$$\log k = \log A - E_A/2.3 RT \quad (7.23)$$

where A is a proportionality constant, E_A is the activation energy, R is the gas constant, and T the absolute temperature. It has been observed that most chemical reactions at 37° have E_A values at 15,000-20,000 calories per mole whereas many enzymically catalyzed reactions have E_A values ranging from 2000-8000 calories per mole. We can therefore calculate the differences in the rate constants of a chemical reaction proceeding at 37° in the absence and presence of an enzyme appropriate for that reaction.

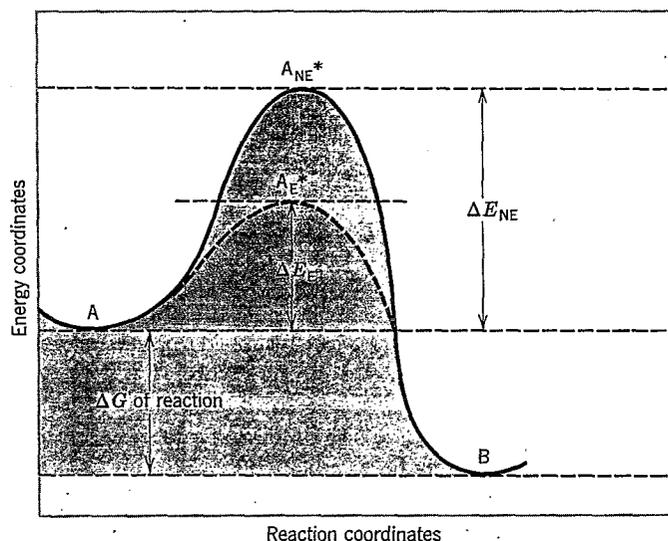


Figure 7-5

A diagram showing the energy barriers of a reaction $A \rightarrow B$: A_{NE}^* indicates the activated complex in a non-enzymic reaction; A_E^* shows the activated complex in an enzyme-catalyzed reaction; A is the initial substrate; B is the product; ΔE_{NE} is the energy of activation for non-enzymic reaction; ΔE_E is the energy for the enzymic reaction; ΔG is the difference in free energy in $A \rightarrow B$.

Thus:

$$\text{Chemical reaction: } \log k_c = \log A - \frac{20,000}{1,354}$$

$$\text{Enzymic reaction: } \log k_e = \log A - \frac{6,000}{1,354}$$

$$\log \frac{k_e}{k_c} = \frac{+20,000 - 6,000}{1,354} = \frac{14,000}{\sim 1400} = 10$$

$$\frac{k_e}{k_c} = 10^{10}$$

thus an enzyme-catalyzed reaction proceeds at a tremendously faster rate than would the same noncatalyzed reaction.

Of course, a noncatalyzed reaction rate can be greatly increased by raising the temperature of the environment. The student can readily appreciate the observation that such a condition would be highly unfavorable in a living cell. Indeed, enzymes are very sensitive to elevated temperatures. Because of the protein nature of an enzyme thermal denaturation of the enzyme protein with increasing temperatures will decrease the effective concentration of an enzyme and consequently decrease the reaction rate. Up to perhaps 45°C

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we calculate the
activation energy at 37°C
for that reaction.

the predominant effect will be an increase in reaction rate as predicted by chemical kinetic theory. Above 45°C an opposing factor, namely thermal denaturation will become increasingly important, however, until at 55°C rapid denaturation will destroy the catalytic function of the enzyme protein. The dual effects of a temperature-enzyme reaction relationship are depicted in Figure 7-6.

7.5 Effect of pH

Since enzymes are proteins, pH changes will profoundly affect the ionic character of the amino and carboxylic acid groups on the protein and will therefore markedly affect the catalytic site and conformation of an enzyme. In addition to the purely ionic effects, low or high pH values can cause considerable denaturation and hence inactivation of the enzyme protein. Moreover, since many substrates are ionic in character (e.g., ATP, NAD⁺, amino acids, and CoASH) the active site of an enzyme may require particular ionic species for optimum activity.

These effects are probably the main determinants of a typical enzyme

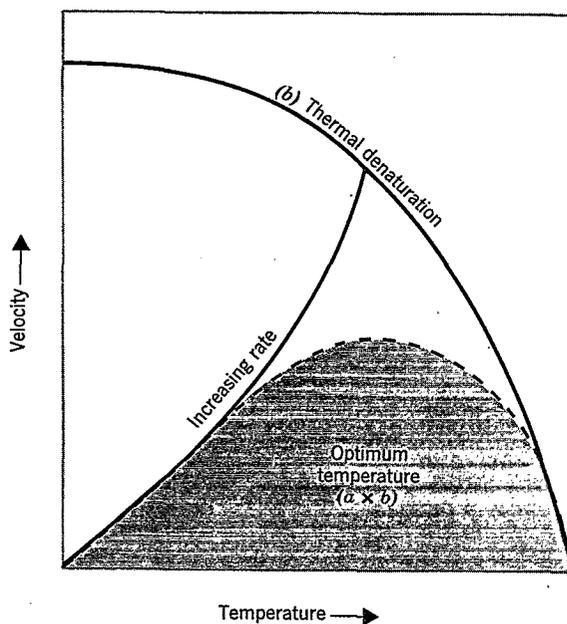


Figure 7-6

Effect of temperature on reaction rate of an enzyme-catalyzed reaction: (a) represents the increasing rate of a reaction as a function of temperature; (b) represents the decreasing rate as a function of thermal denaturation of the enzyme. The dashed line curve represents the combination of $(a \times b)$.

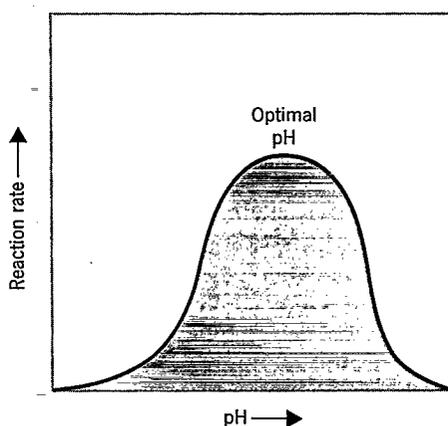


Figure 7-7
Effect of pH on an enzyme-catalyzed reaction.

activity-pH relation. Thus a bell-shaped curve obtains with a relatively small plateau and with sharply decreasing rates on either side as indicated in Figure 7-7. The plateau is usually called the *optimal* pH point.

In enzyme studies it becomes extremely important to determine early in the investigation the optimal pH and its plateau range. The reaction mixture must then be carefully controlled with buffers of suitable buffering capacity.

In the milieu of the cell the control of the pH in various parts of the cell becomes important since a marked shift in enzyme rates will result if pH stability is not maintained. This would result in major disturbances in the closely geared catabolic and anabolic systems of the cell. Obviously, then, it would be of great value in understanding the regulation of cellular metabolism if we had better knowledge of how pH is controlled or modified in the cellular geography.

Although much is now known about the physical, chemical, and structural aspects of enzymes, the mystery of the enormous catalytic power of an enzyme remains unresolved. Once it was believed that the identification and localization of the amino acid residues associated with a catalytic site would explain the catalytic activity of any enzyme. Now biochemists realize that this approach, while still valid, is somewhat naive. In recent years enzyme chemists have designed ingenious reagents to probe and identify the active site of enzyme and in fact, at present, highly sophisticated physical techniques such as nuclear magnetic resonance spectrometry and electron spin resonance spectrometry as well as high-resolution x-ray crystallography have provided the enzyme chemist with data useful for the development of answers to the mystery of the catalytic power of a protein. As a result, the student can readily tap a large literature, where by the skillful use of physical organic principles, an enzyme chemist can "explain" the events which convert a

7.6 Why Are Enzymes Catalysts?