bands in each compound would tend to become equal as the temperature is raised. This does indeed occur as shown in Table II, thus lending strong support to the above conclusions. The samples were run as described except that hexachlorobutadiene was used as a solvent. Since the dielectric properties of this solvent are similar to those of carbon tetrachloride, the O–H spectra obtained from both solvents are nearly identical, but the temperature can be extended well beyond the liquid range of carbon tetrachloride with hexachlorobutadiene.

Finally, as will be shown in a future publication, the association bands of *o*-halophenols are approximately the same width as the associated bands of some compounds containing O–H—N or O–H—O hydrogen bonds. In these latter compounds, the interacting distances and rotational configurations must be just as restricted as in the *o*-halophenols.

TABLE II

Halogen substituents 2	6	T, °C.	Absorbance ratio (peak)
F	Cl	36	0.61
		139	.68
F	Br	33	.68
		125	.74
Cl	I	35	2.73
		139	2.08
Br	I	29	1.9
		138	1.5

This means that for these compounds, the ΔS term involved in formation of the hydrogen bond must be nearly zero, as it is for the halophenols. This is the principal reason for the narrowness of these types of bands.

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Multiple Intermediates in Steady State Enzyme Kinetics.[1,2] I. The Mechanism Involving a Single Substrate and Product

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The method of Christiansen has been employed to derive the general steady-state rate equation for an enzymatically catalyzed reversible reaction involving a single substrate and a single product but with an arbitrary number of intermediates. The observable kinetic parameters (Michaelis constants and maximum velocities) define lower limits to the rate constants for the unimolecular and bimolecular steps. The magnitude of the latter for a number of enzyme systems suggests that a diffusion controlled combination of enzyme and substrate is being observed. The kinetic behavior of the intermediates during the steady-state period is examined. A particular model for the *p*H variation of the rate as applied to the *n*-intermediate system is also considered.

Introduction

In studies of the kinetics of enzymatic reactions under steady-state conditions, it is generally impossible to assign values for the rate constants for specific steps in a mechanism which involves even a reasonable number of intermediates. For a reversible system involving a single substrate and a single product which can be described in terms of one intermediate, the four measurable kinetic parameters (two Michaelis constants and two maximum velocities) suffice to determine the four rate constants. However, such a mechanism if not requiring revision by studies of the influence of other variables, *e.g.*, *p*H, generally falls far short of providing an adequate description of the chemical steps involved.

A number of years ago Christiansen[3] proposed a method for dealing in a straightforward fashion with the steady-state kinetics of complex reaction schemes. Use has been made of this approach in treating multi-barrier kinetic processes by Eyring and associates.[4] It has been applied to sequences of enzymatic reactions by Christiansen,[5] Hearon[6] and Lumry.[7] Most recently Hearon has pointed out some of the complexities which may have to be taken into consideration in dealing with the effect of inhibitors on the kinetics of complex enzymatic mechanisms.[8]

It is the purpose of this paper to develop a general steady-state rate equation by the use of Christiansen's method for an enzymatically catalyzed reversible reaction involving a single substrate and product but with an arbitrary number of intermediates. The resulting expression permits a ready definition of Michaelis constants and maximum velocities. The equations for these parameters are put in such a form that certain general arguments can be made concerning their relation to the actual rate constants in the mechanism. Moreover, such an approach clearly indicates which conclusions commonly drawn from

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(2) Presented in part before the 135th National Meeting of the American Chemical Society, Boston, April 5–10, 1959.

(3) J. A. Christiansen, *Z. physik. Chem.*, **28B**, 303 (1935); **33B**, 145 (1936).

(4) B. J. Zwolinski, H. Eyring and C. E. Reese, *J. Phys. Colloid Chem.*, **53**, 1426 (1949); R. B. Parlin and H. Eyring in "Ion Transport Across Membranes," ed. by H. T. Clarke, Academic Press, Inc., New York, N. Y., 1954, p. 103.

(5) J. A. Christiansen, *Acta Chem. Scand.*, **3**, 493 (1949).

(6) J. Z. Hearon, *Physiol. Rev.*, **32**, 499 (1952).

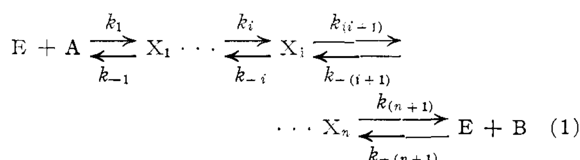
(7) R. Lumry, *Disc. Faraday Soc.*, **20**, 257 (1955).

(8) J. Z. Hearon, S. A. Bernhard, S. L. Friess, D. F. Botts and M. F. Morales in "The Enzymes," Vol. I, 2nd Ed., Academic Press, Inc., New York, N. Y., 1959, pp. 89–108.

considerations of mechanisms of a simple character are of general significance. While the language is that of enzyme kinetics, it is evident that many of these considerations will apply to heterogeneous reactions which follow Langmuir kinetics and more generally to catalytic reactions which admit the possibility of saturation of the catalytic species.

Of alternative methods for developing the general steady-state equations of enzyme kinetics,[9] mention should be made of the approach of King and Altman.[10] Their schematic method permits the expression of the rate equations in a form which displays the dependence on specific rate constants in a particularly simple fashion.

The Steady-state Equations.—Let us consider a reaction involving the reversible interconversion of molecules A and B catalyzed by an enzyme E where the mechanism involves a sequence of n intermediates. This reaction sequence may be represented as follows



$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \cdots \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} X_i \underset{k_{-(i+1)}}{\overset{k_{(i+1)}}{\rightleftharpoons}} \cdots X_n \underset{k_{-(n+1)}}{\overset{k_{(n+1)}}{\rightleftharpoons}} E + B \quad (1)$$

where the k_i's are rate constants in the forward path and k_{-i}'s are rate constants in the reverse path. The condition of conservation of enzymatic sites must apply so

$$\sum_{i=1}^{n}(X_i) + (E) = (E)_0 \quad (2a)$$

where the (X_i) is the concentration of intermediates, (E) is the concentration of free enzyme sites, and $(E)_0$ is the total concentration of enzyme sites. The condition of conservation of substrate and product can be written as

$$\sum_{i=1}^{n}(X_i) + (A) + (B) = (A)_0 \quad (2b)$$

where $(A)_0$ is the total concentration of substrate and product.

The kinetics of this reaction scheme may be represented after the fashion of Christiansen[3]

$$\frac{-d(A)}{dt} \equiv v_1 = k_1(E)(A) - k_{-1}(X_1) \quad (3)$$
$$v_2 = k_2(X_1) - k_{-2}(X_2)$$
$$\vdots$$
$$v_i = k_i(X_{i-1}) - k_{-i}(X_i)$$
$$v_{i+1} = k_{(i+1)}(X_i) - k_{-(i+1)}(X_{i+1})$$
$$\vdots$$
$$v_n = k_n(X_{n-1}) - k_{-n}(X_n)$$
$$\frac{d(B)}{dt} \equiv v_{n+1} = k_{n+1}(X_n) - k_{-(n+1)}(E)(B)$$

Now it is readily apparent that the rate of appearance of the ith intermediate can be written as

$$\frac{d(X_i)}{dt} = [k_i(X_{i-1}) - k_{-i}(X_i)] - [k_{(i+1)}(X_i) - k_{-(i+1)}(X_{i+1})] \quad (4)$$
$$= v_i - v_{i+1}$$

(9) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, p. 111.

(10) E. L. King and C. Altman, *J. Phys. Chem.*, **60**, 1375 (1956).

The steady-state condition for this sequence is defined by setting $d(X_i)/dt$ equal to zero, *i.e.*, under steady-state conditions

$$v_1 = v_2 = \cdots v_i = v_{i+1} = \cdots = v_n = v_{n+1} \equiv v \quad (5)$$

In more physical terms, the net rate of appearance of each intermediate $d(X_i)/dt$, where (X_i) is the concentration of a substance in a free energy "valley," must be small in comparison to the rate of passage of material over the free energy barrier on either side of this "valley" for the steady-state approximation to be applicable. For $|d(X_i)/dt| << |v_i|$, then equation 5 follows from equation 4 applied to each intermediate.

Equation 5 combined with the definitions of the v_i in equation 3 and the condition of conservation of enzymatic sites in equation 2a suffices to define the over-all rate of reaction v in terms of the concentrations of total enzymatic sites $(E)_0$, the concentrations of the reactant (A) and the product (B). This may be seen readily in the following way. We first obtain an expression for the steady-state concentration of the ith intermediate by simply adding the $n - i$ equations for $v_{i+1} \cdots v_{n+1}$ multiplied by the appropriate factors to eliminate the concentrations of all the intermediates save the ith as

$$k_{(i+1)}(X_i) - k_{-(n+1)}(E)(B)\prod_{r=i}^{n-1}\frac{k_{-(r+1)}}{k_{(r+2)}} = v_{i+1} + \cdots + \prod_{r=i}^{s}\frac{k_{-(r+1)}}{k_{(r+2)}} + \cdots + v_{n+1}\prod_{r=i}^{n-1}\frac{k_{-(r+1)}}{k_{(r+2)}} = v\left[1 + \sum_{s=i}^{n-1}\prod_{r=i}^{s}\frac{k_{-(r+1)}}{k_{(r+2)}}\right] \quad (6a)$$

The last equality follows from the steady-state condition of equation 5. Equation 6a can be solved for (X_i) to yield

$$(X_i) = v\sum_{s=i}^{n}\frac{K_i^s}{k_{(s+1)}} + (E)(B)K_i^{n+1} \quad (6b)$$

where

$$K_i^s \equiv \prod_{r=i+1}^{s}\frac{k_{-r}}{k_r} \equiv \frac{(X_i)_{eq}}{(X_s)_{eq}}$$

for $n + 1 > s > i$. For $n + 1 > i > s$, K_i^s is defined as equal to $1/K_s^i$ while $K_i^i = 1$. When v equals zero, the system is at equilibrium and $(X_i)_{eq}/(E)_{eq}(B)_{eq} = K_i^{n+1}$ as must be the case. For $i = 1$, equation 6b becomes

$$(X_1) = \sum_{s=1}^{n}\frac{K_1^s}{k_{(s+1)}} + (E)(B)K_1^{n+1} \quad (6c)$$

Another relation for (X_1) in terms of (E), (A) and v can be obtained from the first expression of the set of equations 3, namely

$$(X_1) = \frac{k_1}{k_{-1}}(E)(A) - \frac{v}{k_{-1}} \quad (7)$$

Eliminating (X_1) from equations 6c and 7 yields an expression for the concentration of free enzymatic sites (E)

$$(E) = \frac{v\sum_{s=0}^{n}\frac{K_0^s}{k_{(s+1)}}}{(A) - (B)K_0^{n+1}} \quad (8)$$

where

$$K_0^{n+1} = \prod_{r=1}^{n+1} \frac{k_{-r}}{k_r}$$

The conservation of enzymatic sites expressed by equation 2a coupled with equation 6b provides a relation between (E), $(E)_0$ and v.

$$v \sum_{i=1}^{n} \sum_{s=i}^{n} \frac{K_i^s}{k_{(s+1)}} + (E)(B) \sum_{i=1}^{n} K_i^{n+1} + (E) = (E)_0 \quad (9)$$

Equations 8 and 9 can be combined to give the over-all steady-state rate of reaction (v) in terms of (A), (B), $(E)_0$, and aggregates of the rate constants.

$$v = \frac{(E)_0\,[(A) - (B)K_0^{n+1}]}{\sum_{s=0}^{n} \frac{K_0^s}{k_{(s+1)}} + (A)\left[\sum_{i=1}^{n}\sum_{s=i}^{n}\frac{K_i^s}{k_{(s+1)}}\right] + (B)\left[K_0^{n+1}\sum_{i=1}^{n}\sum_{s=0}^{i-1}\frac{K_i^s}{k_{(s+1)}}\right]} \quad (10)$$

Equation 10 can obviously be written in the Michaelis–Menten form for an enzymatically catalyzed reversible reaction, *i.e.*

$$v = \frac{\frac{V_A}{K_A}(A) - \frac{V_B}{K_B}(B)}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B}} \quad (11)$$

where the maximum velocities (V_A and V_B) and Michaelis constants (K_A and K_B) are defined as

$$V_A = \frac{(E)_0}{\sum_{i=1}^{n}\sum_{s=i}^{n}\frac{K_i^s}{k_{(s+1)}}} \quad (12a)$$

$$K_A = \frac{\sum_{s=0}^{n}\frac{K_0^s}{k_{(s+1)}}}{\sum_{i=1}^{n}\sum_{s=i}^{n}\frac{K_i^s}{k_{(s+1)}}} \quad (12b)$$

$$V_B = \frac{(E)_0}{\sum_{i=1}^{n}\sum_{s=0}^{i-1}\frac{K_i^s}{k_{(s+1)}}} \quad (12c)$$

$$K_B = \frac{\sum_{s=0}^{n}\frac{K_0^s}{k_{(s+1)}}}{K_0^{n+1}\sum_{i=1}^{n}\sum_{s=0}^{i-1}\frac{K_i^s}{k_{(s+1)}}} \quad (12d)$$

It is immediately apparent that when $v = 0$

$$\frac{(B)_{eq}}{(A)_{eq}} = \frac{V_A K_B}{V_B K_A} = \frac{1}{K_0^{n+1}} \quad (13)$$

—a relationship between the kinetic parameters and the over-all equilibrium constant usually attributed to Haldane.[11]

The question often has been raised as to the conditions under which the Michaelis constants can be regarded as equilibrium constants, *i.e.*, the existence of a quasi-equilibrium.[12] From equation 12b it can be seen that the sums in both numerator and denominator must each contain a term which far outweighs all the other terms and moreover corresponds to the same value of $s = r$, *i.e.*

$$K_A \cong \frac{\frac{K_0^r}{k_{(r+1)}}}{\frac{K_h^r}{k_{(r+1)}}} = K_0^r K_r^h = K_0^h \equiv \frac{(E)_{eq}(A)_{eq}}{(X_h)_{eq}} \quad r \geqslant h$$

Since equation 12d has the same summation in the numerator as equation 12b, the condition that K_B become approximately equal to an equilibrium constant yields

$$K_B \cong \frac{1}{K_0^{n+1}} \frac{\frac{K_0^r}{k_{(r+1)}}}{\frac{K_j^r}{k_{(r+1)}}} = K^j{}_{n+1} \equiv \frac{(E)_{eq}(B)_{eq}}{(X_j)_{eq}} \quad j > r$$

It follows from the requirement that $h \leq r < j$ that K_A and K_B can only reduce to equilibrium constants for intermediates (X_h) and (X_j), respectively, such that $h < j$. This result has the obvious consequence that for a single intermediate mechanism only one of the two Michaelis constants can approximate an equilibrium constant.

Lower Limits on the Rate Constants.—Any study of the reaction represented by the scheme in equation 1 conducted under conditions where the steady-state relation of equation 5 applies yields information on only the four kinetic parameters in equation 11, only three of these parameters being independent by virtue of equation 13. However, it is possible to place lower limits on the rate constants for a mechanism like the above. In particular, it can be seen from equation 12a that retaining just one of the positive terms in the denominator yields the inequality

$$V_A \leq \frac{(E)_0}{\frac{K_i^i}{k_{(i+1)}}}$$

or

$$k_{(i+1)} \geq \frac{V_A}{(E)_0} \qquad i \neq 0 \quad (14a)$$

inasmuch as $K_i^i \equiv 1$. That is, $V_A/(E)_0$ is the lower limit of any *unimolecular* rate constant for the forward path. The equality sign holds when there is a *single* intermediate. Similarly for the reverse path

$$k_{-i} \geq \frac{V_B}{(E)_0} \qquad i \neq n+1 \quad (14b)$$

as can be seen from equation 12c or from the symmetry of the mechanism in equation 1. The above inequalities simply state that a maximum velocity, *e.g.*, $V_A/(E)_0$, cannot exceed in magnitude any unimolecular rate constant for the conversion of one intermediate to the next in the sequence for the reaction proceeding in a given direction, *e.g.*, A → B. It should be noted that neither V_A nor V_B contains either k_1 or $k_{-(n+1)}$, the two bimolecular rate constants in the sequence. This is to be expected physically because the maximum velocities represent rates which are attained only when there is total saturation of the enzymatic sites, and hence when the over-all rate of reaction is independent of the concentrations of A and B. Also V_A does not contain k_{-1} and V_B does not contain $k_{(n+1)}$ which are the rate constants for

(11) J. B. S. Haldane, "Enzymes," Longmans, Green, London, 1930.

(12) Reference 8, p. 57.

the dissociation of the first intermediate in the forward (X_1) and the reverse paths (X_n).

In a similar way it is possible to place lower limits on the two bimolecular rate constants, k_1 and $k_{-(n+1)}$. From equations 12a, 12b and 12c it can be seen that

$$\frac{V_A + V_B}{(E)_0 K_A} = \frac{\sum_{i=1}^{n} \sum_{s=0}^{n} \frac{K_i^s}{k_{(s+1)}}}{\frac{1}{k_1}\left[1 + \sum_{s=1}^{n} \frac{k_{-1} K_1^s}{k_{(s+1)}}\right]\left[\sum_{i=1}^{n} \sum_{s=0}^{i-1} \frac{K_i^s}{k_{(s+1)}}\right]} \quad (15a)$$

After some algebraic manipulation, we obtain

$$k_1 = \frac{V_A + V_B}{(E)_0 K_A}\left\{1 + \frac{\sum_{i=2}^{n}\left[\sum_{s=1}^{i=1} \frac{K_i^s}{k_{(s+1)}} + \left(\sum_{s=1}^{n} \frac{K_i^s}{k_{(s+1)}}\right)\left(\sum_{s=1}^{i-1} \frac{k_{-1}K_1^s}{k_{(s+1)}}\right)\right]}{\sum_{i=1}^{n} \sum_{s=0}^{n} \frac{K_i^s}{k_{(s+1)}}}\right\} \quad (15b)$$

As the numerator of the fraction in braces is greater than or equal to zero, it follows that

$$k_1 \geq \frac{V_A + V_B}{(E)_0 K_A} \quad (16a)$$

with the equality sign holding for the one intermediate mechanism. The symmetry of the mechanism ensures that

$$k_{-(n+1)} \geq \frac{V_A + V_B}{(E)_0 K_B} \quad (16b)$$

Essentially these lower limits have been given for a two intermediate mechanism by a different type of argument.[18]

The lower limits on the unimolecular rate constants place upper limits on the duration of the transient state period for the production of intermediates in the system. This derives from the fact that the transient terms in the approximate solution of the differential equations for the system are of the form $e^{-t/\tau}$ where τ is a measure of the length of the transient state period. For a two intermediate mechanism there are two transient terms of this form for the appearance of product.[14-16]

At high substrate concentration the more slowly decaying of these exponential terms is given by

$$\tau = \frac{1}{k_2 + k_{-2} + k_3} < \frac{(E)_0}{2V_A + V_B}$$

Of perhaps more interest are the lower limits on the bimolecular rate constants. It has previously been pointed out on the basis of a model for diffusion into a hemispherical sink that the diffusion controlled combination of a small substrate molecule (with a diffusion coefficient $D_1 \sim 10^{-5}$ cm.^{2} sec.$^{-1}$) with a larger enzyme molecule ($D_2 \sim 10^{-7}$ cm.^{2} sec.$^{-1}$) would occur with a bimolecular rate constant of 10^8 to 10^{10} M^{-1} sec.$^{-1}$.[17] These values can be regarded as theoretical upper limits to the bimolecular rate constants.

(13) R. A. Alberty and W. H. Peirce, THIS JOURNAL, **79**, 1526 (1957).

(14) H. Gutfreund, *Disc. Faraday Soc.*, **20**, 167 (1955).

(15) L. Ouellet and K. J. Laidler, *Can. J. Chem.*, **34**, 146 (1956).

(16) Reference 8, p. 117.

(17) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, **62**, 154 (1958).

In Table I are gathered estimated lower limits for the bimolecular rate constants for several rather different enzyme systems. As it has been possible to study few such reactions in the forward and reverse directions, most of the lower limits are based on the inequality

$$k_1 > \frac{V_A}{(E)_0 K_A} \quad (16c)$$

which follows from (16a). The fact that most of the values quoted are within an order of magnitude of the diffusional upper limit even for reactions involving macromolecular substrates ($D_1 \sim 10^{-6}$ to 10^{-7} cm.^{2} sec.$^{-1}$) such as (a) and (d) is of considerable significance. This suggests for these systems with a chemical mechanism involving a small number of intermediates the first step may be regarded as a diffusion-controlled combination of substrate and enzyme. It is of considerable interest that such inferences about the rapidity of combination of enzyme and substrate can be made from data obtained during the steady-state period of the reaction.

TABLE I

LOWER LIMITS ON BIMOLECULAR RATE CONSTANTS

	Enzyme	Substrate	Minimum value (M^{-1} sec.$^{-1}$)	Ref.
(a)	β-Amylase	Amylose	5.8×10^{7} [g]	[a]
(b)	Urease	Urea	5.0×10^{6} [g]	[b]
(c)	Adenosine triphosphatase	Adenosine triphosphate	8×10^{6} [g]	[c]
(d)	Cytochrome c reductase	Cytochrome c	9×10^{7} [g]	[d]
(e)	Acetylcholinesterase	Acetylcholine	$\sim 10^{9}$ [g]	[e]
(f)	Fumarase			
	(1) Phosphate	Fumarate	4.2×10^{7} [h]	[f]
	pH 7.0	L-Malate	1.6×10^{7} [h]	
	(2) "Tris" acetate	Fumarate	6.5×10^{8} [h]	
	pH 7.0	L-Malate	1.6×10^{8} [h]	

[a] J. M. Bailey and D. French, *J. Biol. Chem.*, **226**, 1 (1957). These authors quote a somewhat lower value for k_1 than the above limit based on an assumed non-random degradation process. The above cited lower limit applies to a random degradation process. This problem will be discussed more fully elsewhere by one of us (L. P.). [b] M. C. Wall and K. J. Laidler, *Arch. Biochem. Biophys.*, **43**, 299 (1953). [c] L. Ouellet, K. J. Laidler and M. Morales, *ibid.*, **39**, 37 (1952). [d] T. R. Hogness in "A Symposium on Respiratory Enzymes," University of Wisconsin Press, Madison, 1942, p. 134. [e] D. Nachmansohn and I. B. Wilson in "Advances in Enzymology," Vol. XII, Interscience Publishers, Inc., New York, N. Y. 1951, p. 259. [f] C. Frieden, R. G. Wolfe and R. A. Alberty, THIS JOURNAL, **79**, 1523 (1957). Considerably larger values for the lower limits ($\sim 10^9$ to 10^{10} M^{-1} sec.$^{-1}$) in the "Tris" buffer system can be given in terms of the pH independent kinetic parameters defined below (see ref. 13). [g] From (16c). [h] From (16a).

In order to obtain values for the lower limits on the rate constants the "value" of $(E)_0$ must be known. Most often the only estimate of $(E)_0$ available is the molar concentration of the enzyme. Use of such an estimate ignores the possibility of multiple sites in the enzyme and tends to give a spuriously high value for the lower limit. This is possibly the case in systems (b), (c) and (f) in Table I. Determinations of the number of active sites are available for a number of enzyme

systems, however, and additional estimates should become available as these systems are more thoroughly studied. It should be realized that the above treatment applies to enzymes with multiple sites only if they are independent. Use of a value for $(E)_0$ expressed in terms of the molar concentration of sites still involves the assumption that the stoichiometric concentration of sites represents active enzyme. This assumption is difficult to circumvent in enzymatic assays and may lead to a spuriously low limit if an appreciable fraction of the enzyme is unreactive.

Lastly the influence of solution environment on the kinetic parameters may be quite significant. As can be seen from Table I, the lower limits for the two bimolecular rate constants in the fumarase system are more than an order of magnitude smaller in sodium phosphate than in "tris" acetate at the same *p*H. This difference can reasonably be accounted for if the phosphate with a double negative charge clings to the enzymatic site more tenaciously than the acetate anion with a single negative charge. Such an interaction manifests itself by increasing the Michaelis constant in phosphate over "tris." It seems likely from studies of the effect of the variation of ionic strength on the kinetic parameters that there is an important electrostatic contribution to the interaction of the substrate anions with the enzyme.[17,18] The nature of the buffer ions may thus greatly influence the apparent rate constants.

The Kinetic Behavior of Intermediates.—During the steady-state period of a reversible enzymatic reaction, the concentration of an intermediate may obviously either decline or rise to its equilibrium value. The first possibility corresponds to the existence of a maximum in the concentration of an intermediate during the transient period of the reaction. Before examining the temporal behavior of the intermediates, it is of interest to consider the kinetic behavior of the free enzyme concentration in the post transient state period. From equations 8, 11, 12a and 12b, we have the familiar expression for the free enzyme concentration

$$(E) = \frac{(E)_0}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B}} \qquad (17)$$

It follows from the conservation equations 2a and 2b and the above expression that

$$\frac{d(E)}{d(B)} = \frac{\left(\frac{1}{K_A} - \frac{1}{K_B}\right)(E)_0}{\left\{\left[1 + \frac{(A)}{K_A} + \frac{(B)}{K_B}\right]^2 + \frac{(E)_0}{K_A}\right\}} \cong \frac{\left(\frac{1}{K_A} - \frac{1}{K_B}\right)(E)_0}{\left[1 + \frac{(A)}{K_A} + \frac{(B)}{K_B}\right]^2} \qquad (18)$$

where the second equality holds when $(E)_0 << (A)_0$. Of course, (E) cannot have gone through a maximum inasmuch as initially it was equal to $(E)_0$, and ultimately it must approach a value $(E)_{eq} < (E)_0$. However, it may decline to this value with increasing time (or (B)) if $K_A > K_B$ or rise to its equilibrium value if $K_B > K_A$. The latter situation corresponds to the existence of a minimum in the time course of its concentration. The behavior of the free enzyme concentration with increasing time for the reaction proceeding in the direction of A → B is schematically illustrated in Fig. 1. If $K_A = K_B$, then $d(E)/d(B) = 0$

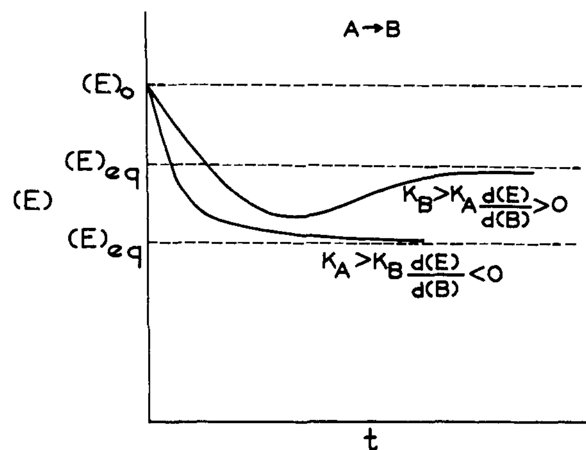


Fig. 1.—Schematic representation of the time course of the free enzyme concentration.

and the steady-state value of (E) is identical to its equilibrium value as can be seen from equations 2a, 2b and 17

$$(E) = \frac{\{[K_A + (A)_0 - (E)_0]^2 + 4(E)_0K_A\}^{1/2} - [K_A + (A)_0 - (E)_0]}{2} \cong \frac{(E)_0K_A}{(A)_0 + K_A} \qquad (19)$$

where the second relation is obtained when $(A)_0 >> (E)_0$ by binomial expansion of the square root. This latter condition coupled with the equality of the Michaelis constants makes equation 11 pseudo-first order.

It is obvious from equation 2a that the time course of (E) determines the time course of the total concentration of intermediates $\sum_{i=1}^{n}(X_i)$. For example, a minimum in (E) implies a maximum in $\sum_{i=1}^{n}(X_i)$. Also it follows from the fact that $d(E)/d(A)$ is opposite in sign to $d(E)/d(B)$ that a minimum for (E) in the forward direction (A → B) implies the *absence* of a minimum in the reverse direction (B → A).

For a mechanism involving a single intermediate, equality of K_A and K_B requires that the two bimolecular rate constants be equal, *i.e.*, $k_1 = k_{-2}$. In this case the differential equations for the appearance of intermediate and product are integrable without approximation.[19] Calculations show that the condition $(A)_0 >> (E)_0$ is sufficient to ensure that the intermediate and free enzyme concentrations effectively attain their equilibrium values early in the reaction, and the steady-state approximation can be employed with confidence after this brief transient period.[19]

(18) G. G. Hammes and R. A. Alberty, *J. Phys. Chem.*, **63**, 274 (1959).

(19) W. G. Miller and R. A. Alberty, THIS JOURNAL, **80**, 5146 (1958).

The time dependence of the concentration of intermediates during the steady-state period is somewhat more complicated. From equations 6b, 11 and 17 we have an expression for the concentration of the ith intermediate

$$(X_i) = \frac{\frac{V_A}{K_A}\left\{[(A) - (B)K_0^{n+1}]\sum_{s=i}^{n}\frac{K_i^s}{k_{(s+1)}} + (B)K_i^{n+1}\sum_{s=0}^{n}\frac{K_0^s}{k_{(s+1)}}\right\}}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B}} \quad (20)$$

The behavior of (X_i) during the steady-state period can be most easily examined by considering the sign of the derivative

$$\frac{d(X_i)}{d(B)} = -\frac{\frac{V_A}{K_A}(K_i^{n+1})\left\{\left[1 + \frac{(A)_0}{K_B}\right]\sum_{s=i}^{n}\frac{K^s_{n+1}}{k_{(s+1)}} - \left[1 + \frac{(A)_0}{K_A}\right]\sum_{s=0}^{i-1}\frac{K_0^s}{k_{(s+1)}}\right\}}{\left[1 + \frac{(A)}{K_A} + \frac{(B)}{K_B}\right]^2} \quad (21)$$

where it has been assumed that $(A)_0 >> (E)_0$.

For (X_i) to decrease during the steady-state period $d(X_i)/d(B) < 0$ or the quantity in braces in equation 21 must be greater than zero, *i.e.*

$$\frac{\sum_{s=i}^{n}\frac{K^s_{n+1}}{k_{(s+1)}}}{\sum_{s=0}^{i-1}\frac{K_0^s}{k_{(s+1)}}} > \frac{1 + \frac{(A)_0}{K_A}}{1 + \frac{(A)_0}{K_B}} \quad (22a)$$

The condition that the derivative in equation 21 be less than zero corresponds to the concentration of the ith intermediate having gone through a maximum in the transient period. For $d(X_i)/d(B) > 0$, (X_i) increases monotonically from its initial value of zero to its final equilibrium value. These two possible modes of behavior of the concentration of an intermediate with increasing time for the reaction proceeding in the direction of $A \rightarrow B$ are schematically represented in Fig. 2.

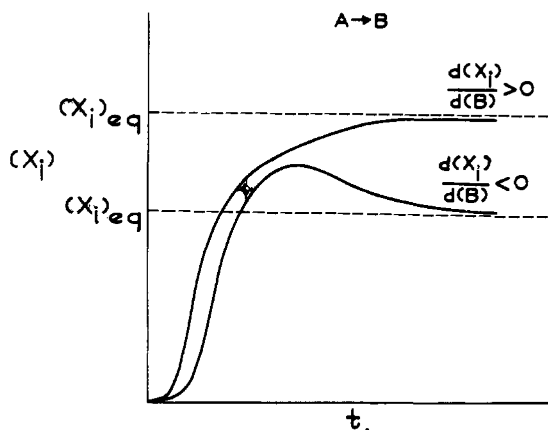


Fig. 2.—Schematic representation of the time course of the concentration of an intermediate.

From the definition of the sums appearing in the above inequality, it is immediately apparent that

$$\frac{\sum_{s=h}^{n}\frac{K^s_{n+1}}{k_{(s+1)}}}{\sum_{s=0}^{h-1}\frac{K_0^s}{k_{(s+1)}}} > \frac{\sum_{s=i}^{n}\frac{K^s_{n+1}}{k_{(s+1)}}}{\sum_{s=0}^{i-1}\frac{K_0^s}{k_{(s+1)}}} \quad (22b)$$

where $h < i$. Hence, if the concentration of the ith intermediate goes through a maximum as the reaction proceeds in the direction of $A \rightarrow B$, the concentration of the hth intermediate will go through a maximum also. In other words, *the inequality (22a) will be satisfied by all intermediates preceding the ith in the reaction sequence and all will exhibit concentration maxima in the direction* $A \rightarrow B$.

If the inequality (22a) applies to the nth intermediate, then all n intermediates go through concentration maxima. This is a *sufficient but not a necessary condition* (save for a single intermediate mechanism) for $K_A < K_B$ and for the concentration of the free enzyme (E) to exhibit a concentration minimum. In this extreme case, inequality (22a) becomes

$$\frac{\sum_{s=i}^{n}\frac{K^s_{n+1}}{k_{(s+1)}}}{\sum_{s=0}^{i-1}\frac{K_0^s}{k_{(s+1)}}} > \frac{1 + \frac{(A)_0}{K_A}}{1 + \frac{(A)_0}{K_B}} > 1 \quad i = 1 \ldots n$$

For a system involving a *single* intermediate ($i = n = 1$) the inequality (22a) reduces to the requirement that $k_1 > k_{-2}$ or $K_A < K_B$ which is to be expected from the earlier remarks concerning the temporal behavior of (E).

Reversing the inequality 22a defines the condition that $d(X_i)/d(B) > 0$ in which case (X_i) increases monotonically to its equilibrium value. By the same argument as presented above it can be seen readily that if (X_i) does not go through a maximum then the (X_j) will not exhibit maxima for all $j > i$ in the direction $A \rightarrow B$.

For several enzymes reaction conditions may be found where $K_A = K_B$ usually by operating at a particular pH.[19] As has been remarked, the equality of the Michaelis constants is a necessary and sufficient condition for the free enzyme concentration (E) to be effectively at its equilibrium value after the transient period. For a single intermediate sequence this also obviously applies to the concentration of the intermediate (X_i). When $K_A = K_B$, inequality 22a becomes

$$\frac{\sum_{s=i}^{n}\frac{K^s_{n+1}}{k_{(s+1)}}}{\sum_{s=0}^{i-1}\frac{K_0^s}{k_{(s+1)}}} > 1 \qquad n > 1 \quad (22c)$$

i.e., the condition for the existence of a maximum in the concentration of the ith intermediate is independent of the total substrate concentration.

When $d(X_i)/d(B) = 0$, the concentration of the ith intermediate must be effectively at its equilibrium value in the steady-state period of the reaction, *i.e.*, equation (23a) therefore is derivable. In this circumstance inequality (22a) becomes an equality. From the definition of the sums appearing in (22a) it is apparent that if $d(X_i)/d(B) =$

$$(X_i) = (X_i)_{eq} = \frac{(E)_0(A)_0K_i^{n+1}}{1 + K_0^{n+1} + (A)_0\left(\frac{K_0^{n+1}}{K_A} + \frac{1}{K_B}\right)} = \frac{(E)_0(A)_0K_i^{n+1}}{1 + K_0^{n+1} + (A)_0 \sum_{j=1}^{n} K_j^{n+1}} \quad (23)$$

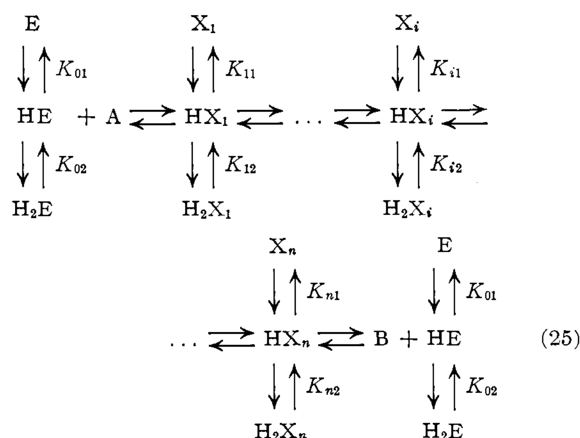
0, then $d(X_h)/d(B) < 0$ for $h < i$ and $d(X_j)/d(B) > 0$ for $j > i$. Hence the concentrations of all intermediates preceding the ith exhibit maxima while none succeeding it do. The condition that $d(X_i)/d(B)$ be identically zero is of course artificial. However, it leads to the physically obvious conclusion by consideration of the sums appearing in (22a) that if the ith intermediate is at its equilibrium concentration, then the intermediates close to it in the sequence may be correspondingly close to their equilibrium concentrations as well.

It is of some interest to point out that if $K_i^{n+1} = 0$ for all i then from equation 6b

$$(X_i) = v \sum_{s=i}^{n} \frac{K_i^s}{k_{(s+1)}} \quad (24)$$

Hence, the ratio of the concentrations of any two intermediates during the steady-state period is constant. One way of achieving this condition is for $k_{-(n+1)} = 0$. This is a common assumption in treating the kinetics of so-called *irreversible* enzyme catalyzed reactions. Irreversibility of course requires only that $K_0^{n+1} << 1$ which may be satisfied without K_i^{n+1} being zero for any intermediate (i). Also, as has been pointed out by several workers,[20-22] $K_0^{n+1} << 1$ is not sufficient to ensure that $K_A << K_B$ and permit the neglect of the product term in the denominator of equation 11. More explicitly K_A and K_B may be of the same order of magnitude for an irreversible reaction ($K_0^{n+1} << 1$) if $V_B << V_A$. In point of fact the product inhibition constants of enzymatically catalyzed reactions may be viewed as Michaelis constants for the reverse reaction.

The Effect of *p*H on the Kinetics.—The measurable parameters of many enzyme catalyzed reactions (Michaelis constants and maximum velocities) exhibit a marked dependence on *p*H and often on the nature of the buffer ions. In order to discuss the effect of *p*H on the kinetics of the reaction sequence (1), we modify this scheme in the conventional way for a single intermediate mechanism,[23-26] as is shown in equation sequence (25) where the K's with double subscripts refer to acid dissociation constants. The above reaction scheme assumes that there is only *one* state of ionization of the "active site" which is capable of catalyzing the interconversion of A and B. This has been indicated above as the state with one bound proton. In actuality little is known about the "active site" of enzymes and their state of ionization for optimal activity. However, inclusion of three possible ionic forms or two proton dissociation steps is *sufficient* to account for the bell shaped curves often found for maximal velocities plotted against *p*H for a number of enzyme reactions.



$$\begin{array}{ccc} X_n & & E \\ \downarrow\uparrow K_{n1} & & \downarrow\uparrow K_{01} \\ \ldots \rightleftarrows HX_n & \rightleftarrows & B + HE \\ \downarrow\uparrow K_{n2} & & \downarrow\uparrow K_{02} \\ H_2X_n & & H_2E \end{array} \quad (25)$$

The total concentration of the ith intermediate $(X_i)_T$ in all its forms is given by

$$(X_i)_T = (HX_i)\left[1 + \frac{K_{i1}}{(H^+)} + \frac{(H^+)}{K_{i2}}\right] \equiv (HX_i)f_i \quad (26)$$

This expression follows from the steady state relation

$$\frac{d(X_i)}{dt} = \frac{d(H_2X_i)}{dt} = 0$$

which of course is identical to the result obtained by considering the species X_i, HX_i, and H_2X_i to be in ionic equilibrium. Additional ionic species simply add further terms in f_i depending on higher powers of (H^+) and reciprocal (H^+).

Applying the complete steady-state treatment to this sequence[27] yields an equation of the same form as (11) with the following definitions for the kinetic observables

$$V_A = \frac{(E)_0}{\sum_{i=1}^{n} \sum_{s=i}^{n} \frac{K_i^s f_i}{k_{(s+1)}}} = \frac{V_A'}{\left[1 + \frac{K_{A1}}{(H^+)} + \frac{(H^+)}{K_{A2}}\right]} \quad (27a)$$

$$K_A = \frac{f_0 \sum_{s=0}^{n} \frac{K_0^s}{k_{(s+1)}}}{\sum_{i=1}^{n} \sum_{s=i}^{n} \frac{K_i^s f_i}{k_{(s+1)}}} = \frac{K_A'\left[1 + \frac{K_{01}}{(H^+)} + \frac{(H^+)}{K_{02}}\right]}{1 + \frac{K_{A1}}{(H^+)} + \frac{(H^+)}{K_{A2}}} \quad (27n)$$

$$V_B = \frac{(E)_0}{\sum_{i=1}^{n} \sum_{s=0}^{i-1} \frac{K_i^s f_i}{k_{(s+1)}}} = \frac{V_B'}{1 + \frac{K_{B1}}{(H^+)} + \frac{(H^+)}{K_{B2}}} \quad (27c)$$

$$K_B = \frac{f_0 \sum_{s=0}^{n} \frac{K_0^s}{k_{(s+1)}}}{K_0^{n+1} \sum_{i=1}^{n} \sum_{s=0}^{i-1} \frac{K_i^s f_i}{k_{(s+1)}}} = \frac{K_B'\left[1 + \frac{K_{01}}{(H^+)} + \frac{(H^+)}{K_{02}}\right]}{1 + \frac{K_{B1}}{(H^+)} = \frac{(H^+)}{K_{B2}}} \quad (27d)$$

The so-called *p*H independent variables designated by primes, *e.g.*, V_A' are identical to the quantities defined in equation 12. Accepting the above

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model for pH effects, permits definition of pH independent lower limits for the rate constants in terms of these variables. The Haldane relation, equation 13, is satisfied by both the primed and unprimed variables.

Four "acid dissociation constants" are defined in the above equations, *e.g.*

$$K_{A1} = \frac{\sum_{i=1}^{n} \sum_{s=i}^{n} \frac{K_i^s K_{i1}}{k_{(s+1)}}}{\sum_{i=1}^{n} \sum_{s=i}^{n} \frac{K_i^s}{k_{(s+1)}}}$$

These quantities are usually readily obtainable from an analysis of the pH dependence of the maximum velocities in the forward and reverse direction (see ref. 23–26). However, it is only in the case of a system described in terms of a single intermediate that these parameters can be identified with dissociation constants. When $i = n = 1$, $K_{A1} = K_{B1} = K_{11}$ and $K_{A2} = K_{B2} = K_{12}$. In general these parameters will be complex aggregates of rate constants and dissociation constants.

It is evident from equations 27 that plots of either V_A/K_A or V_B/K_B *vs.* pH yield the dissociation constants for the free enzyme, *i.e.*, K_{01} and K_{02} irrespective of the number of intermediates in the system. *Hence for an enzyme which catalyzes the reaction of several substrates the pH dependence of* V_A/K_A *should be the same provided that the substrates all interact with the same groups in the enzyme.* This is very clear from studies by Smith and coworkers of the hydrolysis of a number of synthetic substrates by the enzyme papain. Plots of the quantity $V_A/(E)_0K_A$ *vs.* pH yield values of the pK's of the two dissociable groups which are identical for both charged and neutral substrates while the pH dependence of $V_A/(E)_0$ may be markedly different.[28,29] Smith identifies $V_A/K_A(E)_0$ with the bimolecular rate constant for a one intermediate hydrolytic mechanism.[30] From the preceding discussion this quantity is always a lower limit for such a constant, but studies of its variation with pH are of course consonant with any interpretation of its significance. For an enzyme which reacts with many substrates, it is tempting to propose the superimposability of V_A/K_A or V_B/K_B *vs.* pH plots for the various substrates as a diagnostic test for the involvement of the same *acidic* or *basic* groups in all its reactions.

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MADISON, WISCONSIN

[CONTRIBUTION FROM THE CHEMICAL LABORATORY, FACULTY OF SCIENCE, TOKYO UNIVERSITY]

Infrared Spectra of Acetylglycine N-Methylamide and the Assignment

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The infrared spectra of acetylglycine N-methylamide and its N-deuterated compound have been measured in the crystalline state by using a grating spectrophotometer. Based on the experimental results and on our previous analysis of the normal vibrations of N-methylacetamide, a complete assignment has been made of the fundamental vibrational frequencies of acetylglycine N-methylamide observed in the 1800–400 cm.$^{-1}$ region.

Introduction

Acetylglycine N-methylamide, $CH_3CONHCH_2$-$CONHCH_3$, can be regarded as a unit of the polypeptide chain containing two peptide bonds. The infrared spectra of this molecule were measured in detail in the 3 μ region in our laboratory.[1–6] The measurement of the infrared spectra in the NaCl region also have been made.[7] From the results we could draw some important conclusions on the molecular structure of the polypeptide chain. Since progress has been made recently in the analysis of the vibrational spectra of the molecules containing peptide bonds,[8–10] further researches on this molecule have been made in our laboratory. An exact measurement of infrared spectra of acetylglycine N-methylamide in solid state were made with a grating spectrophotometer. The variation in the intensity of the observed absorption bands with the direction of the incident beam was measured. Furthermore, the measurement on the N-deuterated molecule was made. Based on the result of the measurements and on our analysis of the normal vibrations of simpler molecules, a complete assignment of the observed frequencies was made. The results are reported in the present paper.

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