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Multiple Intermediates in Steady-state Enzyme Kinetics. III. Analysis of the Kinetics of Some Reactions Catalyzed by Dehydrogenases

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Steady-state kinetic data for several dehydrogenase systems are analyzed in the fashion described in reference 2. Various kinetic parameters and lower limits for the rate constants are calculated. Evidence requiring the consideration of isomers of the binary complexes in some of these systems is cited. A consideration of the dual role of hydrogen ion in affecting the experimentally determined kinetic parameters and calculated lower limits is presented. It is suggested that diffusion-controlled reactions in the combination of substrates with the enzyme may be indicated. Finally, an examination is made of the feasibility of determining certain kinetic parameters by direct experimental measurement.

Introduction

In the preceding paper[2] (designated as II), we have obtained some formal results for the steady state kinetics of enzyme-catalyzed reactions involving two reactants and two products. In particular the form of the rate law, the expressions for the kinetic parameters and their interrelationships, and the lower limits for the various rate constants presented there are valid irrespective of the number of intermediates in the reaction. We have, however, assumed an ordered, or more precisely a preferred, path of reaction.

In the present paper we apply these results to the data for a number of reactions catalyzed by the dehydrogenases, which comprise a relatively well studied class of such systems.

It was found profitable in paper II, in the interests of writing the over-all rate law in a succinct and symmetrical form, to depart somewhat from the customary nomenclature used by other workers in this field. To facilitate transposition of results by the reader, we present in Table I a summary of the two other widely used schemes and the equivalents in our symbolism. It is to be noted that consideration of the *complete* rate law in paper II for such reactions has necessitated the introduction of further parameters not considered by these authors.

TABLE I

EQUIVALENT EXPRESSIONS FOR SOME OF THE KINETIC PARAMETERS IN THE RATE LAW FOR THE FORWARD REACTION

I[a]	II[b]	III[c]
V_{AB}	V_{AB}^*	$(E)_0/\phi$
K_A	K_{AB}^*/K_B^*	ϕ_{12}/ϕ_2
K_B	K_{AB}^*/K_A^*	ϕ_{12}/ϕ_1
K_{AB}	K_{AB}^*	ϕ_0/ϕ_2

[a] Reference 2, paper II. [b] See for example, ref. 15. [c] K. Dalziel, *Acta Chem. Scand.*, 11, 1706 (1957). The equivalent quantities for the reverse reaction are designated by superscript primes in this reference.

In the succeeding analysis we shall calculate values of the parameters on the reasonable assumption that account must be taken of *ternary* complexes in the mechanism. As may be seen from paper II, the expressions for the general lower limits on the rate constants are identical whether ternary or only binary complexes are considered.

The only difference between the rate laws for the two mechanisms is in the presence of terms involving (A)(B)(Q) and (B)(Q)(R) in the denominator of the former. It may be shown that the expressions 9a and 9b of reference 2, which are used to calculate K_{ABQ} and K_{BQR} in terms of kinetic parameters for the forward and reverse reactions alone, will give rise to infinite values for these quantities if ternary complexes are non-existent or kinetically insignificant. Such a situation indeed appears to be the case for the reactions catalyzed by liver alcohol dehydrogenase and malic dehydrogenase considered below.

Lactate Dehydrogenase

Schwert and co-workers[3a,b] have extensively studied this system and have provided sufficient data for determination of the relevant kinetic parameters at four *p*H values.[3b]

In determining the entries in Table II we have assumed an order of combination with the enzyme in the forward reaction of DPN^+ before lactate, and in the reverse direction of DPNH before pyruvate. In the absence of kinetic studies of the type described in the previous paper[2] and earlier[4] to elucidate the order of reaction, we may refer to binding studies of the type cited below. The redundancy relations 8a–9b of reference 2 have been used to calculate K_{AQ}, K_{BR}, K_{ABQ} and K_{BQR}.

Isomers of the binary complexes derived from the combination of the enzyme with DPN^+ are strongly suggested by the fact that the values of K_{AB}-$V_{QR}/V_{AB}K_AK_B$ are greater than unity at *p*H 6.15 and 6.98. This is a sufficient condition for requiring the consideration of binary isomers.[2] At *p*H 6.15 a bimolecular rate constant for the combination of DPNH with the enzyme of at least the order of $10^8\ M^{-1}$ sec.$^{-1}$ is apparent.

Somewhat disconcertingly, the values of K_{ABQ} and K_{BQR} at *p*H 6.98 and 8.02 are negative. The calculation of these parameters involves a subtraction of quantities of considerable numerical uncertainty. The negative values may simply arise from the accumulation of experimental errors.

The value of the dissociation constant of the lactate dehydrogenase–DPN^+ complex of $3.9 \pm 0.7 \times 10^{-4}\ M$ from an ultracentrifugal determination[2] in 0.1 M phosphate buffer at *p*H 6.80 at 25° agrees only moderately well with K_A at *p*H 6.98 in Table II. The difference in experimental

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(2) V. Bloomfield, L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **84**, 4367 (1962).

(3) (a) Y. Takenaga and G. W. Schwert, *J. Biol. Chem.*, **223**, 157 (1956); (b) A. D. Winer and G. W. Schwert, *ibid.*, **231**, 1065 (1958).

(4) R. A. Alberty, *J. Am. Chem. Soc.*, **80**, 1777 (1958).

TABLE II

KINETIC PARAMETERS FOR DEHYDROGENASES AND CALCULATED LOWER LIMITS OF RATE CONSTANTS
(A = $ADPN^+$, R = DPNH)

Enzyme	Lactate dehydrogenase[3b] B = lactate, Q = pyruvate				Yeast alcohol dehydrogenase[5] B = C_2H_5OH, Q = CH_3CHO		Ribitol dehydrogenase[8] B = D-ribitol, Q = D-ribulose	Liver alcohol dehydrogenase[15] B = C_2H_5OH, Q = CH_3CHO		Malate dehydrogenase B = L-malate, Q = oxalacetate
pH	6.15	6.98	8.02	9.01	7.15	6.0	8.0	7.0	9.0	8.0
$[V_{AB}/(E)_0] \times 10^{-2}$, sec.$^{-1}$	0.75	1.25	2.9	4.7	0.62	0.38		0.031	0.049	1.67
$K_A \times 10^4$, M	2.6	2.5	1.8	1.3	2.6	1.7	6.38	1.3	.16	7.0
$K_B \times 10^2$, M	3.7	3.3	1.7	1.1	8.4	12.3	1.41	0.55	.092	0.28
$K_{AB} \times 10^6$, M^2	6.6	3.3	1.1	1.0	4.2	16	3.19	0.033	.0089	0.56
$[V_{QR}/(E)_0] \times 10^{-2}$ sec.$^{-1}$	10.3	9.2	7.7	7.0	5.0	3.8		0.74	.082	5.7
$K_R \times 10^6$, M	3.9	3.8	2.6	11.3	7.1	4.2	71.5	0.28	.86	5.0
$K_Q \times 10^5$, M	2.8	2.2	6.0	100	13	5.15	133	1.0	7.9	1.1
$K_{QR} \times 10^9$, M^2	0.37	0.53	1.2	1.3	5.5	1.3	34.7	0.067	0.113	0.19
$K_{AQ} \times 10^9$, M^2	7.3	5.5	10.8	130	33	8.8	850	1.3	1.3	7.7
$K_{BR} \times 10^7$, M^2	1.4	1.2	0.44	1.2	6.0	5.1	10.1	0.015	0.0079	0.14
$K_{ABQ} \times 10^9$, M^3	1.3	−0.57	−0.035	5.0	3.0	−3.0	4.0	"∞"	"∞"	"∞"
$K_{BQR} \times 10^{11}$, M^3	7.2	−1.9	−0.41	48	31	−5.8	27.8	"∞"	"∞"	"∞"
$k_1 \times 10^{-6}$, M^{-1} sec.$^{-1}$	0.42	1.25	4.5	5.1	1.2	0.24		0.53	0.51	0.83
$k_f \times 10^{-6}$, M^{-1} sec.$^{-1}$	0.30	0.95	4.8	6.1	0.38	0.041		.012	.0089	0.21
$k_{(\alpha+1)}$, $k_{(\beta+1)}$, $k_{(\gamma+1)} \times 10^{-2}$, sec.$^{-1}$	0.75	1.25	2.9	4.7	0.62	0.38		.031	.049	1.67
$k_{-(n+1)} \times 10^{-7}$, M^{-1} sec.$^{-1}$	7.8	3.8	3.8	5.4	1.2	1.5		1.1	.57	3.3
$k_{-g} \times 10^{-6}$, M^{-1} sec.$^{-1}$	10.9	6.6	1.7	0.61	0.65	1.2		0.37	.062	16
$k_{-\alpha}$, $k_{-\beta}$, $k_{-\gamma} \times 10^{-2}$, sec.$^{-1}$	10.3	9.2	7.7	7.0	5.0	3.8		0.74	.082	5.9
$K_{QR}V_{AB}/V_{QR}K_QK_R$	0.25	0.86	2.9	0.77	0.67	0.61	0.241	1.0	1.0	1.0
$K_{AB}V_{QR}/V_{AB}K_AK_B$	9.4	2.9	0.94	1.05	1.6	7.6	0.538	1.1	1.0	1.0

conditions for these two measurements may be sufficient to account for this. Agreement between these differently determined dissociation constants can be construed as support for the presumed order of combination of substrates with the enzyme.

Yeast Alcohol Dehydrogenase

The alcohol dehydrogenase from yeast has been subjected to fairly detailed kinetic study.[5] In Table II are found values at two pH's for the kinetic parameters appearing in eq. 3 of reference 2. It should be emphasized that as in the previously discussed case of lactate dehydrogenase a mechanism invoking ternary complexes has been presumed coupled with the assumption that the coenzyme combines with the enzyme before the other substrate. Accordingly, use has been made of the redundancy relations, eq. 8a–9b of reference 2, to calculate several of the parameters in Table II from those supplied by direct experimental measurement.[5]

We note the existence of lower limits for the bimolecular rate constants of 10^6–10^7 M^{-1}sec.$^{-1}$. The negative values of K_{ABQ} and K_{BQR} at pH 6.0 are again probably the result of experimental uncertainties. However, the fact that $K_{AB}V_{QR}/V_{AB}K_AK_B$ is greater than unity at both pH values at which data are available argues for isomers of the binary complexes arising from association of DPN^+ with the enzyme. A comparison of K_A and K_R in Table II at pH 7.15 with the values of the dissociation constants, 2.6×10^{-4} M and $1.3 \times 10^{-5}M$, respectively, determined by an ultracentrifugal technique at pH 7.8 (at 0–5°) is reasonably good.[6,7]

Ribitol Dehydrogenase

Recently, Nordlie and Fromm have presented the results of a steady state study of the kinetics of the reaction of D-ribitol with DPN^+ to produce D-ribulose and DPNH.[8] The molecular weight of the enzyme, ribitol dehydrogenase, is unknown, so calculations of $V_{AB}/(E)_0$ and $V_{QR}/(E)_0$ cannot be made.

From the reported kinetic parameters,[8] it is, however, possible to determine certain others by use of relations previously given.[2] The values of these constants appear in Table II. In determining the additional parameters use has been made of the value $V_{AB}/V_{QR} = 0.66$ from measurements of the maximum velocities in the forward and reverse directions at the same concentration of

(5) A. P. Nygaard and H. Theorell, *Acta Chem. Scand.*, **9**, 1300 (1955).

(6) J. E. Hayes, Jr., and S. F. Velick, *J. Biol. Chem.*, **207**, 225 (1954).

(7) A. P. Nygaard and H. Theorell, *Acta Chem. Scand.*, **9**, 1551 (1955).

(8) R. C. Nordlie and H. J. Fromm, *J. Biol. Chem.*, **234**, 2523 (1959).

enzyme.[8] It is assumed that the coenzyme combines with the enzyme before the other substrate.

It is to be noted that there is no kinetic evidence of the type cited above requiring consideration of isomers of the binary complexes. The nature of this criterion[2] is such that when both $V_{AB}K_{QR}/V_{QR}K_QK_R$ and $V_{QR}K_{AB}/V_{AB}K_AK_B$ are less than unity no conclusions can be drawn concerning the relative importance of binary isomers.

Owing to the lack of information of the concentration of enzymatic sites, calculations of the minimal values of the various rate constants cannot be made. However, from the fact that $K_B > K_A$, it follows that the lower limit for k_1 must be greater than for k_f (*cf.* relations 12a and 13a of reference 2). Similarly as $K_Q > K_R$, the lower limit for $k_{-(n+1)}$ must be greater than that for k_{-g}(*cf.* relations 12b and 13b of reference 2).

Liver Alcohol Dehydrogenase

This enzyme obtained from horse liver was chronologically the first dehydrogenase to be studied extensively.[9-15] We can now consider the results of 10 years of study of the kinetics of this reaction carried out by Theorell and coworkers. The most recent data, obtained by Theorell and McKee[15] and presented in Table II, show some interesting features not encountered with lactate dehydrogenase, yeast alcohol dehydrogenase and ribitol dehydrogenase.

In terms of the Dalziel notation, Theorell and McKee[15] found that $\phi_1\phi_2/\phi_{12} = \phi_0'$ and $\phi_1'\phi_2'/\phi_{12}' = \phi_0$ at both *p*H 7.0 and 9.0. In our symbolism these relations are equivalent to the kinetic criteria for isomers of binary complexes being equal to unity. As a further consequence, the calculated values of K_{ABQ} and K_{BQR} are effectively infinite. More precisely, these latter parameters are so large that the steady state concentration of the ternary complexes is essentially negligible compared to that of the binary complexes.[2] The mechanism without kinetically significant ternary complexes was originally proposed for this reaction by Theorell and Chance.[10]

We see from Table II that the lower limits for the bimolecular rate constants k_1 and $k_{-(n+1)}$ for the combination of DPN and DPNH, respectively, with the enzyme are of the usual order of magnitude, 10^6 to 10^7 M^{-1} sec.$^{-1}$. However, k_f and k_{-g} (the latter would be k_{-f} if there were in fact *no* ternary complexes) are significantly smaller than the corresponding rate constants for the other systems considered here. While part of this effect may be due to the influence of *p*H (see below), this result finds rationalization in the idea that there must be rather stringent orientational requirements, and hence a large negative entropy of activation,[17] in the combination of ethanol and acetaldehyde with the enzyme–coenzyme complex if ternary complexes are kinetically insignificant.

The values of K_A at *p*H 7 and 9 compare reasonably well with the values of the equilibrium constant for the dissociation of the enzyme–DPN^+ complex determined by a spectrophotofluorimetric technique[15]: $1.6 \times 10^{-4}M$ and $0.12 \times 10^{-4}M$, respectively. Similarly, K_R at the two *p*H values is in essential agreement with the equilibrium values of 0.31×10^{-6} M (*p*H 7) and 0.65×10^{-6} M (*p*H 9).

It should be remarked that K_A and K_R as derived from kinetic studies represent specifically defined dissociation constants. For them to be comparable to the quantities determined by spectrofluorimetry, the latter technique must specifically distinguish free enzyme from the bound coenzyme, however many isomers of the latter may be present. Departures from agreement may be attributed in part to the failure of the physical measurement used to make this distinction.

Malate Dehydrogenase

Quite recently Raval and Wolfe[18] have published data on the steady state kinetics of malate dehydrogenase isolated from pig hearts.[19] Their results for the four kinetic parameters at *p*H 8.0 for the forward reaction (reduction of DPN^+) and the symmetrically related parameters for the reverse reaction (oxidation of DPNH) were reported in Dalziel's symbolism described in column III of Table I.

We have expressed their results in our notation and calculated the four additional kinetic parameters from equations 8a–9b of reference 2 and the lower limits for the various rate constants.

This enzyme system exhibits the same adherence to the Dalziel criteria for the kinetic insignificance of ternary complexes as liver alcohol dehydrogenase, discussed above. However, the lower limits for the bimolecular rate constants k_f and k_{-g} are not so low as in that case.

Glutamic Dehydrogenase

The reaction catalyzed by this enzyme involves two reactants and three products, *viz.* $TPN^+ +$ glutamate $\rightleftarrows$ TPNH + glutarate + NH_4^+. The stoichiometry is like that of all the dehydrogenase-catalyzed reactions, but NH_4^+ is produced rather than H^+. This latter distinction nullifies the simplification employed above of regarding one of the products as held at a fixed concentration, *i.e.*, (H^+) being determined by the added buffer. The steady state kinetics is more complicated than for the previously discussed cases. We summarize here some of the results which are of principal utility in an analysis of kinetic data for such a system. Reference 20 contains a fuller account of the kinetic formulation.

For an enzyme-catalyzed reaction A + B = P + Q + R with a mechanism of the type

(9) H. Theorell and R. Bonnichsen, *Acta Chem. Scand.*, **5**, 1105 (1951).
(10) H. Theorell and B. Chance, *ibid.*, **5**, 1127 (1951).
(11) H. Theorell, A. P. Nygaard and R. Bonnichsen, *ibid.*, **9**, 1148 (1955).
(12) H. Theorell and A. D. Winer, *Arch. Biochem. Biophys.*, **83**, 291 (1959).
(13) H. Theorell, *Federation Proc.*, **20**, 967 (1961).
(14) H. Theorell and J. McKee, *Nature*, **192**, 47 (1961).
(15) H. Theorell and J. McKee, *Acta Chem. Scand.*, **15**, 1797 (1961).
(16) H. Theorell and J. McKee, **15**, 1811, 1834 (1961).
(17) See, *e.g.*, S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw–Hill Book Co., Inc., New York, N. Y., 1941.
(18) D. N. Raval and R. G. Wolfe, *Biochem.*, **1**, 263 (1962).
(19) R. G. Wolfe and J. B. Neilands, *J. Biol. Chem.*, **221**, 61 (1956).
(20) V. Bloomfield, Doctoral Dissertation, Dept. of Chemistry, University of Wisconsin, 1962.

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \rightleftharpoons \cdots \rightleftharpoons X_\alpha \underset{k_{-(\alpha+1)}}{\overset{k_{(\alpha+1)}}{\rightleftharpoons}} \cdots \rightleftharpoons X_{b-1}$$

$$B + X_{b-1} \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} X_b \rightleftharpoons \cdots \rightleftharpoons X_\beta \underset{k_{-(\beta+1)}}{\overset{k_{(\beta+1)}}{\rightleftharpoons}}$$

$$\cdots \underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}} X_p + P \quad (1)$$

$$X_p \rightleftharpoons \cdots \rightleftharpoons X_\gamma \underset{k_{(\gamma+1)}}{\overset{k_{(\gamma+1)}}{\rightleftharpoons}} \cdots \underset{k_{-q}}{\overset{k_q}{\rightleftharpoons}} X_q + Q$$

$$X_q \rightleftharpoons \cdots \rightleftharpoons X_\delta \underset{k_{-(\delta+1)}}{\overset{k_{(\delta+1)}}{\rightleftharpoons}} \cdots X_n \underset{k_{-(n+1)}}{\overset{k_{(n+1)}}{\rightleftharpoons}} R + E$$

the steady-state rate equation is of the form

$$v = \frac{(V_{AB}/K_{AB})(A)(B) - (V_{PQR}/K_{PQR})(P)(Q)(R)}{1 + \frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(P)}{K_P} + \frac{(R)}{K_R} + \frac{(A)(B)}{K_{AB}} + \frac{(P)(Q)}{K_{PQ}} + \frac{(P)(R)}{K_{PR}} + \frac{(Q)(R)}{K_{QR}} + \frac{(P)(Q)(R)}{K_{PQR}} + \frac{(A)(P)}{K_{AP}} + \frac{(A)(P)(Q)}{K_{APQ}} + \frac{(A)(B)(P)}{K_{ABP}} + \frac{(R)(B)}{K_{RB}} + \frac{(A)(B)(Q)}{K_{ABQ}} + \frac{(Q)(R)(B)}{K_{QRB}} + \frac{(A)(B)(P)(Q)}{K_{ABPQ}} + \frac{(P)(Q)(R)(B)}{K_{PQRB}}} \quad (2)$$

It may be shown that lower limits on the rate constants are given by the expressions

$$k_1 \geq V_{AB}K_B/K_{AB}\,(E)_0 \quad (3a)$$

$$k_{-(n+1)} \geq V_{PQR}K_{PQ}/K_{PQR}\,(E)_0 \quad (3b)$$

$$k_{-q} \geq V_{PQR}K_PK_R/K_{PQR}\,(E)_0 \quad (3c)$$

$$k_b > V_{AB}K_A/K_{AB}\,(E)_0 \quad (3d)$$

$$k_{-p} > V_{PQR}K_{QR}/K_{PQR}\,(E)_0 \quad (3e)$$

$$k_{(\alpha+1)}, k_{(\beta+1)}, k_{(\gamma+1)}, k_{(\delta+1)} > V_{AB}/(E_0) \quad (4a)$$

$$k_{-\alpha}, k_{-\beta}, k_{-\gamma}, k_{-\delta} > V_{PQR}/(E)_0 \quad (4b)$$

If

$$V_{PQR}K_{AB}/V_{AB}K_AK_B > 1 \quad (5a)$$

more than one binary intermediate of type X_α must be considered while if

$$V_{AB}K_{PQR}(K_PK_{QR} + K_{PQ}K_Q)/V_{PQR}K_PK_{QR}K_{PQ}K_R > 1 \quad (5b)$$

there is more than one complex X_δ and/or more than one X_γ.

Frieden has made a fairly detailed study of this enzyme system.[21-23] His analysis of data for the oxidation of TPNH was based on a rate law derived for a scheme with a specific number of intermediates whose *form*[23] is confirmed by eq. 2 on setting (A) = (B) = 0. Frieden's results were presented using the symbolism of column II of Table I and with the *experimentally justified* simplifications[24] in terms of our nomenclature (column I) that (1) $K_PK_R = K_{PR}$, (2) $K_{PQR} = K_PK_{QR}$ and (3) $K_{PQR} = K_RK_{PQ}$. The above simplifications are, however, not of general validity.[20]

From the steady state kinetics of the oxidation of TPNH, Frieden inferred that NH_4^+ combined with the enzyme before glutarate.[24] With the customary assumption that TPNH reacts first with the enzyme, the order is completely specified.

In Table III we present Frieden's data[23] for the reaction in the symbolism of column I of Table I. The minimum values for the various rate constants according to relations 3a–4b have also been calculated. It is to be noted that inasmuch as $V_{PQR}K_{AB}/V_{AB}K_AK_B$ equals 6.2 under the condition of study more than one binary complex must be considered in the combination of TPN^+ with the enzyme.

TABLE III

KINETIC DATA FOR GLUTAMIC DEHYDROGENASE, AS STUDIED BY FRIEDEN,[23] AND LOWER LIMITS OF VARIOUS RATE CONSTANTS. 0.01 *M* TRIS ACETATE BUFFER. *p*H 8.0, 25°
A, TPN; B, glutamate; P, glutarate; Q, NH_4^+; R, TPNH

$V_{AB}/(E)_0$, sec.$^{-1}$	33
K_A, *M*	2.3×10^{-4}
K_B, *M*	8.9×10^{-3}
K_{AB}, M^2	4.2×10^{-7}
$V_{PQR}/(E)_0$, sec.$^{-1}$	1.0×10^3
K_P, *M*	7×10^{-4}
K_R, *M*	2.6×10^{-5}
K_{QR}, M^2	8.3×10^{-8}
K_{PR}, M^2	7.8×10^{-8}
K_{PQ}, M^2	2.2×10^{-6}
K_{PQR}, M^3	5.8×10^{-11}
k_1, M^{-1} sec.$^{-1}$	7.0×10^5
k_b, M^{-1} sec.$^{-1}$	1.8×10^4
$k_{(\alpha+1)}$, $k_{(\beta+1)}$, $k_{(\gamma+1)}$, $k_{(\gamma+1)}$, sec.$^{-1}$	33
$k_{-(n+1)}$, M^{-1} sec.$^{-1}$	3.8×10^7
k_{-q}, M^{-1} sec.$^{-1}$	3.1×10^5
k_{-p}, M^{-1} sec.$^{-1}$	1.4×10^6
$k_{-\alpha}$, $k_{-\beta}$, $k_{-\gamma}$, $k_{-\delta}$, sec.$^{-1}$	1.0×10^3
$V_{PQR}K_{AB}/V_{AB}K_AK_B$	6.2
$\frac{V_{AB}K_{PQR}(K_PK_{QR} + K_{PQ}K_R)}{V_{PQR}K_PK_{QR}K_{PQ}K_R}$	0.066

The Effect of *p*H

It is well known that the experimentally determined kinetic parameters of enzyme reactions, and the rate constants derived from them, are functions of hydrogen ion concentration. In the case of many of the dehydrogenases, this *p*H-dependence is a superposition of two separate effects: the effects of hydrogen ion being in the stoichiometric equation and therefore playing a role as a reactant or product, and ionization of the enzyme and the enzyme–substrate complexes. The first effect may be taken into account by treating such reactions formally as two-substrate, three-product reactions. If hydrogen ion is a product, certain additional terms will be present in the rate law since hydrogen ions are always present.

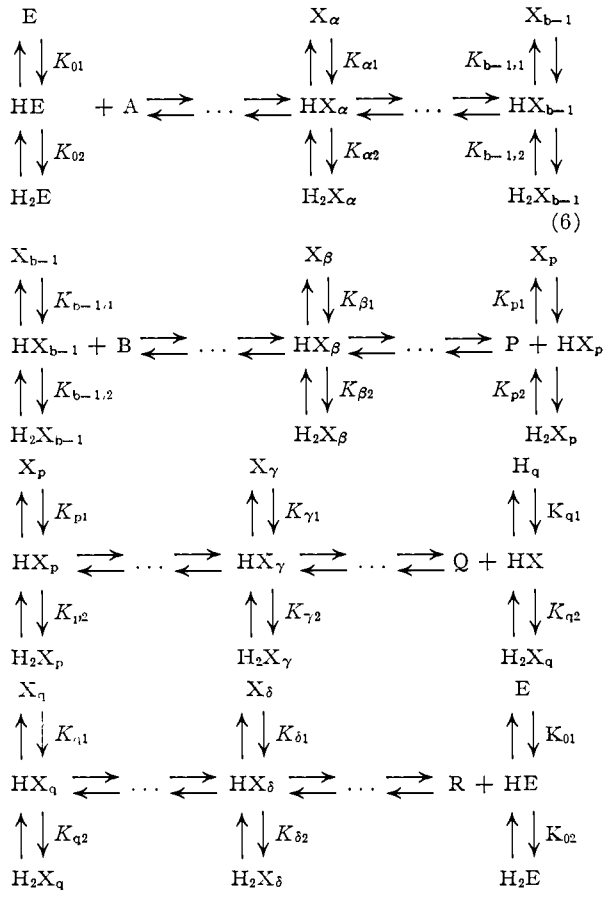
Let us consider a reaction of the type given in eq. 1, in which now one of the products is hydrogen ion. If (H^+) is substituted in eq. 2 for (P), (Q) or (R), and the equation rearranged into the form appropriate to a two-reactant, two-product reaction (eq. 3 of reference 2), we find that the apparent kinetic parameters (designated by primes)

(21) C. Frieden, *J. Biol. Chem.*, **234**, 809 (1959).
(22) C. Frieden, *ibid.*, **234**, 815 (1959).
(23) C. Frieden, *ibid.*, **234**, 2891 (1959).
(24) Such an inference can be conceivably made for this system by examining the contributory terms to the reciprocal rate law when (A) = (B) = 0. This possibility arises because of the presence of terms in (R)(Q) and (P)(Q) but *not* (P)(R) in the expression.[23]

for this reaction are *p*H-dependent. The lower limits on the rate constants, derived from these *p*H-dependent parameters according to the appropriate relations, are of course also *p*H-dependent. The true lower limits will be *greater than or equal to* those computed without taking explicit cognizance of the H^+. The *p*H-dependence of these quantities is given in Table IV.

The modification of eq. 2 to include the effect of different possible states of ionization of the enzyme and the various enzyme–substrate complexes on the steady-state kinetic behavior is quite straightforward. We follow a procedure published earlier.[25] It is assumed that the enzyme and each of the enzyme–substrate complexes can have three states of ionization: doubly protonated, singly protonated and unprotonated. The inclusion of more states of ionization would simply lead to higher terms in (H^+) or its inverse. We further assume that only the singly protonated form of each of these species is converted to the next intermediate.

These assumptions lead to the schematic depiction of the reaction mechanism by eq. 6.



(6)

The steady state rate equations for the species above and below the main line of reaction reduce to equilibrium relations so that acid dissociation constants K_{s1} and K_{s2}, rather than rate constants for these steps, appear in the rate equations.

$$K_{s1} = (X_s)(H^+)/(HX_s) \quad (7a)$$

and

$$K_{s2} = (HX_s)(H^+)/(H_2X_s) \quad (7b)$$

(25) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **81**, 5907 (1959).

We can now write for the *total* concentration of each intermediate

$$(X_s)_t = (HX_s)\left[1 + \frac{K_{s1}}{(H^+)} + \frac{(H^+)}{K_{s2}}\right] = (HX_s)f_s \quad (8)$$

It is this total concentration of each intermediate which is to be used in the conservation equations, while only the concentration of the monoprotonated form appears in the steady state rate equation as it alone is assumed to be capable of reaction.

This treatment leads to a rate equation of the same form as eq. 2. The observable kinetic parameters are simple functions of *p*H and this makes it possible to introduce newly defined parameters indicated by primes, which are independent of *p*H; for example

$$V_{AB} = V_{AB}'\Big/\left[1 + \frac{K_{AB1}}{(H^+)} + \frac{(H^+)}{K_{AB2}}\right] \quad (9a)$$

$$V_{PQR} = V_{PQR}'\Big/\left[1 + \frac{K_{PQR1}}{(H^+)} + \frac{(H^+)}{K_{PQR2}}\right] \quad (9b)$$

$$K_A = K_A'\left[1 + \frac{K_{01}}{(H^+)} + \frac{(H^+)}{K_{02}}\right]\Big/\left[1 + \frac{K_{A1}}{(H^+)} + \frac{(H^+)}{K_{A2}}\right] \quad (10a)$$

and in general

$$K_{\lambda\mu\nu} = K_{\lambda\mu\nu}'\left[1 + \frac{K_{01}}{(H^+)} + \frac{(H^+)}{K_{02}}\right]\Big/\left[1 + \frac{K_{\lambda\mu\nu1}}{(H^+)} + \frac{(H^+)}{K_{\lambda\mu\nu2}}\right] \quad (10b)$$

In each case the primed quantities are given by the expressions for the corresponding unprimed quantities defined by eq. 2. The Greek letter subscripts on the kinetic parameters simply refer to the particular constant under consideration: K_B, K_{PQR}, etc.

In particular, we see from eq. 9a, 10b and 9b and 10b, that

$$\frac{V_{AB}}{K_{AB}} = \frac{V_{AB}'}{K_{AB}'}\Big/\left[1 + \frac{K_{01}}{(H^+)} + \frac{(H^+)}{K_{02}}\right] \quad (11a)$$

and

$$\frac{V_{PQR}}{K_{PQR}} = \frac{V_{PQR}'}{K_{PQR}'}\Big/\left[1 + \frac{K_{01}}{(H^+)} + \frac{(H^+)}{K_{02}}\right] \quad (11b)$$

Thus a plot of, *e.g.*, V_{AB}/K_{AB} *vs.* *p*H, will lead to bell-shaped curves from which the acid dissociation constants of the free enzyme may be determined, just as in the familiar case of a one-reactant, one-product system.[25]

It is evident that a complete analysis of the *p*H dependence of a reaction corresponding to mechanism 6 would be extremely complicated, since a large number of acid dissociation constants and *p*H-independent parameters have been defined by eq. 9 and 10, even though not all of these constants will be independent. The further complexity introduced by the presence of hydrogen ion as a product, as discussed above, makes it appear rather hopeless to attempt to unscramble the *p*H behavior in order to obtain, for example, V_{AB}', which is one of the parameters needed to establish true, *p*H-independent, lower limits on the rate constants.

Discussion

Some general remarks are in order concerning the foregoing analysis of the kinetics of these very similar enzyme systems. We have previously

TABLE IV

*p*H-DEPENDENCE OF APPARENT KINETIC PARAMETERS AND LOWER LIMITS ON RATE CONSTANTS FOR PSEUDO TWO-SUBSTRATE, TWO-PRODUCT REACTIONS WITH H^+ AS A THIRD PRODUCT

The unstarred kinetic parameters are those defined by eq. 2; X and Y are the first and second products to dissociate from the enzyme respectively; *e.g.*, if H^+ is P, X is Q and Y is R

	$H^+ = P$	$H^+ = Q$	$H^+ = R$
V_{AB}'	$\frac{V_{AB}}{1 + (K_{AB}(H^+)/K_{ABH})}$	$\frac{V_{AB}}{1 + (K_{AB}(H^+)/K_{ABH})}$	V_{AB}
V_{XY}'	$\frac{V_{HQR}}{1 + (K_{HQR}/(K_{QR}(H^+))}$	$\frac{V_{PRH}}{1 + (K_{PRH}/K_{PR}(H^+))}$	$\frac{V_{PQH}}{1 + (K_{PQH}/K_{PQ}(H^+))}$
K_{AB}'	$K_{AB}\left[\frac{1 + ((H^+)/K_H)}{1 + (K_{AB}(H^+)/K_{ABH})}\right]$	$\frac{K_{AB}}{1 + (K_{AB}(H^+)/K_{ABH})}$	$K_{AB}\left[1 + \frac{(H^+)}{K_H}\right]$
K_A'	$K_A\left[\frac{1 + ((H^+)/K_H)}{1 + (K_A(H^+)/K_{AH})}\right]$	K_A	$K_A\left[1 + \frac{(H^+)}{K_H}\right]$
K_B'	$K_B\left[1 + \frac{(H^+)}{K_H}\right]$	K_B	$K_B\left[1 + \frac{((H^+)/K_H)}{1+(K_B(H^+)/K_{HB})}\right]$
K_{XY}'	$\frac{K_{HQR}}{K_H}\left[\frac{1 + (K_H/(H^+))}{1 + K_{HQR}/K_{QR}(H^+))}\right]$	$\frac{K_{PRH}}{(H^+) + (K_{PRH}/K_{PR})}$	$\frac{K_{PQH}}{K_H}\left[\frac{1 + (K_H/(H^+))}{1 + (K_{PQH}/K_{PQ}(H^+))}\right]$
K_X'	$\frac{K_{QH}}{K_H}\left[1 + \frac{K_H}{(H^+)}\right]$	$\frac{K_{PH}}{(H^+) + (K_{PH}/K_P)}$	$\frac{K_{PH}}{K_H}\left[\frac{1 + (K_H/(H^+))}{1 + (K_{PH}/K_P(H^+))}\right]$
K_Y'	$\frac{K_{HR}}{K_H}\left[\frac{1 + (K_H/(H^+))}{1 + (K_{HR}/K_R(H^+))}\right]$	$\frac{K_{HR}}{(H^+) + (K_{HR}/K_R)}$	$\frac{K_{QH}}{K_H}\left[1 + \frac{K_H}{(H^+)}\right]$
k_1	$\frac{V_{AB}K_B}{K_{AB}(E)_0}$	$\frac{V_{AB}K_B}{K_{AB}(E)_0}$	$\frac{V_{AB}K_B}{K_{AB}(E)_0[1 + (K_B(H^+)/K_{HB})]}$
k_f'	$\frac{V_{AB}K_A}{K_{AB}(E)_0[1 + (K_A(H^+)/K_{AH})]}$	$\frac{V_{AB}K_A}{K_{AB}(E)_0}$	$\frac{V_{AB}K_A}{K_{AB}(E)_0}$
$k'_{(\alpha+1)}$, $k'_{(\beta+1)}$, $k'_{(\gamma+1)}$	$\frac{V_{AB}}{(E)_0[1 + (K_{AB}(H^+)/K_{ABH})]}$	$\frac{V_{AB}}{(E)_0[1 + (K_{AB}(H^+)/K_{ABH})]}$	$\frac{V_{AB}}{(E)_0}$
$k'_{-(h+1)}$	$\frac{V_{HQR}K_{QH}}{K_{HQR}(E)_0}$	$\frac{V_{PRH}K_{PH}}{V_{PRH}(E)_0[1 + (K_{PH}/K_P(H^+))]}$	$\frac{V_{PQH}K_{PH}}{K_{PQH}(E)_0[1 + (K_{PH}/K_P(H^+))]}$
k'_{-g}	$\frac{K_{HQR}K_{HR}}{K_{HQR}(E)_0[1 + (K_{HR}/K_R(H^+))]}$	$\frac{V_{PRH}K_{HR}}{K_{PRH}(E)_0[1 + (K_{HR}/K_R(H^+))]}$	$\frac{V_{PQH}K_{QH}}{K_{PQH}(E)_0}$
$k'_{-\alpha}$, $k'_{-\beta}$, $k'_{-\gamma}$,	$\frac{V_{HQR}}{(E)_0[1 + (K_{HQR}/K_{QR}(H^+))]}$	$\frac{V_{PRH}}{(E)_0[1 + (K_{PRH}/K_{PR}(H^+))]}$	$\frac{V_{PQH}}{(E)_0[1 + (K_{PQH}/K_{PQ}(H^+))]}$

noted that the bimolecular rate constants for the combination of coenzyme with enzyme are *at least* of the order of 10^6 M^{-1}sec.$^{-1}$ but in some instances may be greater than 10^8 M^{-1}sec.$^{-1}$. From the theory of diffusion-controlled reactions, the Smoluchowski expression as applied to enzyme systems[26] would yield a theoretical upper limit of the order of 10^9 M^{-1}sec.$^{-1}$. This upper limit makes no allowance for any "geometric" requirements for the association reaction other than the fact that the enzymatic site may be approached only from one side of a plane. Considerations of further steric factors would have the effect of diminishing this upper limit. In the light of the experimental data presented earlier[25] as well as those accumulated here, it seems reasonable to regard the first binary complex as being established by a rate process with an activation energy about that of diffusion in water in accordance with the Smoluchowski equation. The orientational requirements of the association reaction might then largely manifest themselves in a negative entropy of activation in the sense of absolute rate theory. In this same vein it should be remarked that in most, though by no means all, instances the lower limit for the bimolecular rate constant for association of the second species is smaller than for the combination of the first species, *i.e.*, the coenzyme. This may be evidence for a further orientational requirement in the second association step.

From the previous discussion concerning the dual effect of *p*H on the kinetics, it may be said that the lower limits for both the unimolecular and bimolecular rate constants would be estimated to be higher if *p*H-independent kinetic parameters were available. With the fumarase-catalyzed interconversion of fumarate and L-malate, the lower limits for the bimolecular association reactions are a factor of 10 to 100 higher when derived from these *p*H-independent parameters.[25,27] We might anticipate an even greater enhancement for the similar quantities in the dehydrogenase reactions as a consequence of the additional role of H^+ in the kinetics. Lastly, it should be observed that the nature of the buffer species will probably affect the magnitude of these limits. For the fumarase system, lower limits on the bimolecular rate constants are a factor of 10 higher in tris-(hydroxymethyl)-aminomethane acetate than in a phosphate buffer at the *same* *p*H.[28]

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In the preceding paper[2] we alluded to the possibility of obtaining kinetic evidence for the order of combination of species by examination of the steady state rate law under certain conditions, *e.g.*, both reactants but only *one* product present initially. Underlying the foregoing analyses has been the assumption that this sequence is known, *i.e.*, appropriate species could be identified as A, B, etc. A variety of indirect studies has suggested that the pyridine nucleotide coenzyme combines first with the enzyme. We have made this assumption in calculating the quantities in Table II.

However, we are now in a position to examine the feasibility of the procedure outlined in paper II for determining the order of addition of substrates. We can refer to eq. 21b of that manuscript[2] for the steady state velocity for the reduction of DPN^+ by alcohol with the addition of the first product, Q, to dissociate from the enzyme. By choosing (A) $= K_{AB}/K_B$, (B) $= K_{AB}/K_A$ and (Q) $= K_Q$ and selecting representative values of these kinetic parameters from Table II, it can be seen that all terms in the expression for V_{AB}/v_f are of the order of unity with the exception of the last term. This last term, which equals $K_{AB}K_Q/K_{ABQ}$, is of the order of 10^{-2} to 10^{-1}. The above method of assessing the importance of terms in the steady state rate law is an obvious extension of the "rule of thumb" statement that for simple enzymatic reactions one must measure steady state velocities at concentrations somewhat higher than the Michaelis constant to evaluate this parameter. The estimate presented above does not encourage too sanguine a view concerning the possibility of a direct experimental determination of the parameter K_{ABQ}.

A substantially similar situation confronts us if we consider the corresponding term for the velocity of the reverse reaction, *i.e.*, the oxidation of DPNH by an aldehyde with B added initially. By an identical argument, it is the magnitude of the term $K_{QR}K_B/K_{BQR}$ compared to unity which is significant. Again values in the range 10^{-2}–10^{-1} are encountered.

The data are of course quite sparse, and any generalization is perhaps hasty. In passing, it should be noted that for ribitol dehydrogenase values of the order of unity are found for $K_{AB}K_Q/K_{ABQ}$ and $K_{QR}K_B/K_{BQR}$. However, even here a precision of better than 15% in the steady state velocity data would be necessary. It remains possible that particularly propitious experimental conditions for a given dehydrogenase system can be found where such studies will be reasonably practicable. More experimental data from conventional steady state studies must be available before any really unequivocal judgments can be formed.

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Multiple Intermediates in Steady-state Enzyme Kinetics. IV. The Steady State Kinetics of Isotopic Exchange in Enzyme-catalyzed Reactions

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Steady state isotopic exchange kinetics is examined for three different types of enzymatic reactions. The concentration dependence of the rate of interchange of label between various species is derived for mechanisms with an arbitrary number of intermediates. In some instances kinetic parameters not present in the steady state rate law for the reaction appear in these expressions. The relative magnitudes of the exchange rates provides a means of establishing the sequence of combination of substrates with the enzyme.

Introduction

Isotopic exchange experiments have been utilized to elucidate a number of interesting features of enzyme catalyzed reactions. The location of the position of bond scission in substrates for hydrolytic and transferase enzymes[2] provides an illustration of one type of study where isotopic labeling has been a useful tool. A somewhat different sort of study is the demonstration of exchange processes without any over-all enzymatic reaction as exemplified by the exchange between orthophosphate and glucose-1-phosphate catalyzed by sucrose phosphorylase in the absence of fructose.[3] Results of this nature on many systems have been widely interpreted as providing evidence for covalently bonded enzyme substrate complexes.[4]

In this paper we will be concerned with the kinetics of the exchange processes themselves. Recently Boyer[5] has analyzed the kinetics of exchange of label between reactant and product under the conditions that: (1) the enzyme reaction is readily reversible, (2) the unlabeled substrates are present at their equilibrium concentrations, (3) exchange takes place under steady state conditions for the labeled species, (4) exchange occurs *via* the *same* path as the over-all enzymatic reaction, and (5) the effect of isotopic substitution on the kinetic parameters is negligible. Boyer has evaluated the exchange rates for several special mechanisms with a specified number of intermediates. Application of these results has been made

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