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Studies of the Enzyme Fumarase. V.[1] Calculation of Minimum and Maximum Values of Constants for the General Fumarase Mechanism[2]

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Generally steady-state studies of enzymatic reactions yield only rate parameters which are combinations of individual rate constants for the catalytic mechanism. However, when the rate parameters are determined for both the forward and reverse reactions, it becomes possible to calculate individual rate constants for certain mechanisms. From studies of the effect of *p*H on the initial steady-state velocities of the forward and reverse reactions catalyzed by fumarase, it is possible to calculate 6 apparent acid dissociation constants and 4 *p*H independent kinetic parameters (2 Michaelis constants and 2 maximum initial velocities). However, the simplest mechanism which will represent the observations involves 12 constants. An investigation has been made of the extent to which the values of the desired constants are determined by the experimental data. The two acid dissociation constants of the enzymatic site are given directly by the experimental data, and it is found that the values of two others are fixed within their experimental uncertainties. The values of 2 of the individual rate constants are established within their experimental uncertainties, and minimum or maximum values are obtained for the remaining constants. It is of special interest that the *minimum* values for the second-order rate constants for the combination of enzyme and substrate are of the order of 10^9 sec.$^{-1}$ M^{-1} which is larger than any directly measured second-order rate constant for a protein–ion reaction.

Introduction

The determination of individual rate constants in enzymatic mechanisms by studies of the transient state of the reaction was pioneered by Chance.[3,4] In such studies determination of the time course of the concentration of substrate, catalytic intermediate, or product make it possible to calculate the values of the individual rate constants. The feasibility of such experiments is limited by (a) the sensitivity of the experimental method for differentiating the intermediate from the free enzyme or detecting low concentrations of the product, (b) the speed of mixing the solutions of enzyme and substrate, and (c) the speed of response of the measuring instrument. Recently, there has been increased interest in the technique[5,6] and theory[6-9] of transient state experiments.

The information about individual rate constants in enzymatic mechanisms which is obtainable from steady-state kinetic studies is usually limited since only certain combinations of the individual rate constants are obtained. However, if the parameters in the rate law for the reverse reaction are also determined, it is possible to calculate the values of the individual rate constants for certain mechanisms. Two examples of such mechanisms are (1)

$$\mathrm{E} + \mathrm{F} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EX} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{E} + \mathrm{M} \qquad (1)^{10}$$

which is discussed below, and mechanism (2), which is illustrated by the liver alcohol dehydrogenase reaction for which Theorell and co-workers[11,12] calculated the six specific reaction rate constants from only rate measurements in the steady state.

$$\begin{aligned} \mathrm{E} + \mathrm{A} &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \\ \mathrm{E} + \mathrm{B} &\underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EC} + \mathrm{D} \\ \mathrm{EC} &\underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{C} \end{aligned} \qquad (2)$$

Calculation of Individual Rate Constants for the Fumarase Mechanism Involving a Single Intermediate Complex

The steady-state treatment of mechanism 1 assuming the substrate and product concentrations are large in comparison with the concentration of enzymatic sites yields[13]

$$-\frac{\mathrm{d(F)}}{\mathrm{d}t} = \frac{\mathrm{d(M)}}{\mathrm{d}t} = \frac{V_\mathrm{F}(\mathrm{F})/K_\mathrm{F} - V_\mathrm{M}(\mathrm{M})/K_\mathrm{M}}{1 + (\mathrm{F})/K_\mathrm{F} + (\mathrm{M})/K_\mathrm{M}} \qquad (3)$$

where V_F and V_M are the maximum steady-state velocities for fumarate and L-malate and K_F and K_M are the Michaelis constants. When fumarate is in the initial substrate the second term in the numerator is negligible so long as the reaction is far from equilibrium, but this is not necessarily true of the term involving (M) in the denominator. However, by means of integration of equation 3 and use of series expansions in time it is possible to show that the steady-state velocities for the forward, v_f, and reverse, v_r, reactions obtained by extrapolation to $t = 0$ are given by[14]

$$v_\mathrm{f} = \frac{V_\mathrm{F}}{1 + K_\mathrm{F}/(\mathrm{F})} \text{ and } v_\mathrm{r} = \frac{V_\mathrm{M}}{1 + K_\mathrm{M}/(\mathrm{M})} \qquad (4)$$

where for mechanism 1

$$V_\mathrm{F} = k_3(\mathrm{E})_0 \qquad V_\mathrm{M} = k_2(\mathrm{E})_0$$
$$K_\mathrm{F} = \frac{k_2 + k_3}{k_1} \qquad K_\mathrm{M} = \frac{k_2 + k_3}{k_4} \qquad (5)$$

(1) The preceding article in this series is C. Frieden, R. G. Wolfe and R. A. Alberty, THIS JOURNAL, **79**, 1523 (1957).

(2) This research was supported by the National Science Foundation and by the Research Committee of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

(3) B. Chance, *Science*, **92**, 455 (1940).

(4) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943).

(5) F. J. W. Roughton, *Faraday Soc. Disc.*, **17**, 116 (1954).

(6) H. Gutfreund, *ibid.*, **20**, 167 (1955).

(7) M. F. Morales and D. E. Goldman, THIS JOURNAL, **77**, 6069 (1955).

(8) K. J. Laidler, *Canadian J. Chem.*, **33**, 1614 (1955).

(9) L. Ouellet and K. J. Laidler, *ibid.*, **34**, 146 (1956).

(10) G. B. Kistiakowsky and P. C. Mangelsdorf, Jr., THIS JOURNAL, **78**, 2964 (1956), have mentioned the possibility of calculating all four rate constants in this mechanism for enzymatic ester hydrolysis.

(11) H. Theorell, A. P. Nygaard and R. Bonnichsen, *Acta Chem. Scand.*, **8**, 1490 (1954).

(12) H. Theorell, A. P. Nygaard and R. Bonnichsen, *ibid.*, **9**, 1148 (1955).

(13) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930.

(14) R. A. Alberty and B. E. Meyers, unpublished.

These four kinetic parameters are not independent but are related through the equilibrium constant for the over-all reaction[13] as has been shown.[15,16] However, for present purposes it is simpler not to eliminate one of these equations by introduction of the equilibrium constant. All the rate constants calculated in this article will be consistent with the equilibrium expression because the experimental data are consistent. Equation 3 is suitable for representing kinetic data for the fumarase reaction only at sufficiently low substrate concentrations that the effect of substrate activation or inhibition is avoided.[16]

Since the two Michaelis constants and two maximum initial velocities have been determined over a range of *p*H values it is of interest to calculate the corresponding values of k_1, k_2, k_3 and k_4. To show how these constants vary with *p*H for 0.01 ionic strength "tris"[17] acetate buffers, the values have been calculated for *p*H 6, 7 and 8 using

$$k_1 = \frac{V_F/(E)_0 + V_M/(E)_0}{K_F} \quad (6)$$

$$k_2 = V_M/(E)_0 \quad (7)$$

$$k_3 = V_F/(E)_0 \quad (8)$$

$$k_4 = \frac{V_F/(E)_0 + V_M(E)_0}{K_M} \quad (9)$$

One of the striking things about the values of the specific rate constants which are summarized in Table I is that k_1 and k_4 are very large, of the order of 10^8 sec.$^{-1}$ M^{-1}. These are very large second-order constants for a reaction of a protein. Unfortunately, mechanism 1 is too simple to represent the effect of *p*H on the kinetics since according to this mechanism it would be expected that k_2 and k_3 would vary in the same way with *p*H. It is evident from Table I that this is not the case.

However, it is of interest to inquire as to the duration of the transient phase of the reaction using the values of the rate constants of Table I. Since the transient phase is short the product concentration term in the equation for d(EX)/dt may be ignored and the initial substrate concentration $(F)_0$ may be considered constant during the transient phase. It is shown[8] readily that the concentration of EX increases in a first-order manner with a half-life of $t_{1/2} = 0.693/[k_1(F)_0 + (k_2 + k_3)]$. At low substrate concentrations the half-lives are independent of substrate concentration and would be 4.0×10^{-4}, 3.3×10^{-4} and 5.4×10^{-4} sec. at *p*H 6, 7 and 8, respectively. Thus it does not appear that the transient state of this enzymatic reaction could be studied by direct methods using presently available techniques.

It is of interest to note that in the case of mechanism 1 the concentration of EX may either increase or decrease during the steady-state phase of the reaction. Provided the substrate and product concentrations during the steady state are large in comparison with the concentration of enzymatic sites

$$\frac{(EX)}{(E)_0} = \frac{k_4(F)_0 + (k_1 - k_4)(F)}{(k_2 + k_3) + k_4(F)_0 + (k_1 - k_4)(F)} \quad (10)$$

According to the values of the rate constants in Table I when fumarate is the initial reactant, the concentration of EX increases in the steady state so that d(EX)/dt does not actually equal zero until equilibrium is reached. If L-malate is the initial reactant, the concentration of EX goes through a maximum very early in the reaction.

TABLE I

KINETIC PARAMETERS FOR THE FUMARASE REACTION FOR 25° AND 0.01 IONIC STRENGTH "TRIS" ACETATE BUFFER

*p*H	6	7	8
Experimental parameters			
$[V_F/(E)_0] \times 10^{-3}$ (sec.$^{-1}$)	1.45	1.20	0.30
$[V_M/(E)_0] \times 10^{-3}$ (sec.$^{-1}$)	0.26	0.93	0.98
$K_F \times 10^6$ (M)	5.7	4.7	7.3
$K_M \times 10^6$ (M)	4.6	15.9	103
Derived rate constants			
$k_1 \times 10^{-9}$ (sec.$^{-1}M^{-1}$)	0.30	0.45	0.18
$k_2 \times 10^{-3}$ (sec.$^{-1}$)	0.26	0.93	.98
$k_3 \times 10^{-3}$ (sec.$^{-1}$)	1.45	1.20	.30
$k_4 \times 10^{-9}$ (sec.$^{-1}M^{-1}$)	0.38	0.13	.012

Calculation of Individual Rate Constants for the General Fumarase Mechanism

In order to provide a basis for the interpretation of the effect of *p*H on the fumarase reaction it is necessary to extend mechanism 1 to include two intermediate enzyme–substrate complexes and to provide for two proton dissociations of each complex and of the free enzymatic site.[18] The following mechanism is believed to be the simplest one which may be used to represent the effect of hydrogen ion concentration on the reaction.

$$\begin{array}{ccccccc} E & & EF & & EM & & E \\ K_{aE}\uparrow\downarrow & & K_{aEF}\uparrow\downarrow & & K_{aEM}\uparrow\downarrow & & K_{aE}\uparrow\downarrow \\ F + EH & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & EHF & \underset{k_4}{\overset{k_3}{\rightleftharpoons}} & EHM & \underset{k_6}{\overset{k_5}{\rightleftharpoons}} & EH + M \\ K_{bE}\uparrow\downarrow & & K_{bEF}\uparrow\downarrow & & K_{bEM}\uparrow\downarrow & & K_{bE}\uparrow\downarrow \\ EH_2 & & EH_2F & & EH_2M & & EH_2 \end{array} \quad (11)$$

In this mechanism k_1 to k_6 are the individual rate constants for the interconversions of EH, EHF and EHM; while K_{aE}, K_{bE}, K_{aEF}, K_{bEF}, K_{aEM} and K_{bEM} are the first and second acid dissociation constants for the ionization of the enzymatic site. As expected for the steady-state treatment of a mechanism of this type it is found that only the *equilibrium* constants for the acid dissociations are involved and not the individual rate constants for the proton dissociations and associations. Furthermore the steady-state rate equation may be arranged in the form of equation 3. The Michaelis constants and maximum velocities are given by

$$V_F = \frac{V'_F(E)_0}{1 + (H^+)/K'_{aEF} + K'_{bEF}/(H^+)} \quad (12)$$

$$K_F = K'_F \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K'_{aEF} + K'_{bEF}/(H^+)} \quad (13)$$

$$V_M = \frac{V'_M(E)_0}{1 + (H^+)/K'_{aEM} + K'_{bEM}/(H^+)} \quad (14)$$

$$K_M = K'_M \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K'_{aEM} + K'_{bEM}/(H^+)} \quad (15)$$

(15) R. M. Bock and R. A. Alberty, THIS JOURNAL, **75**, 1921 (1953).

(16) R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, *ibid.*, **76**, 2485 (1954).

(17) The abbreviation "tris" will be used for tris-(hydroxymethyl)-aminomethane cation.

(18) C. Frieden and R. A. Alberty, *J. Biol. Chem.*, **212**, 859 (1955).

The evaluation of the parameters in the right-hand side members of these equations has been discussed in the preceding article.[1] It is of interest to note that two kinetic constants (K_{aE} and K_{bE}) for mechanism 11 are obtained directly from the experimental data. The *p*H-independent maximum initial velocities and Michaelis constants are related to the rate constants of mechanism 11 by

$$V_F' = \frac{k_2k_5}{k_3 + k_4 + k_5} \quad (16)$$

$$K'_F = \frac{k_2k_5 + k_2k_4 + k_3k_5}{k_1(k_3 + k_4 + k_5)} \quad (17)$$

$$V'_M = \frac{k_2k_4}{k_2 + k_3 + k_4} \quad (18)$$

$$K'_M = \frac{k_2k_5 + k_2k_4 + k_3k_5}{k_6(k_2 + k_3 + k_4)} \quad (19)$$

The *apparent* acid dissociation constants (primed) are related to those in mechanism 11 by[19]

$$K'_{aEF} = \frac{k_3 + k_4 + k_5}{(k_4 + k_5)/K_{aEF} + k_3/K_{aEM}} \quad (20)$$

$$K'_{bEF} = \frac{(k_4 + k_5)K_{bEF} + k_3K_{bEM}}{k_3 + k_4 + k_5} \quad (21)$$

$$K'_{aEM} = \frac{k_2 + k_3 + k_4}{(k_2 + k_3)/K_{aEM} + k_4/K_{aEF}} \quad (22)$$

$$K'_{bEM} = \frac{(k_2 + k_3)K_{bEM} + k_4K_{bEF}}{k_2 + k_3 + k_4} \quad (23)$$

The values of the ten experimental parameters for the fumarase reaction in "tris" acetate buffers at 25° are given in Table I of the preceding article.

Although the 8 non-linear relationships (equations 16–23) between the ten unknown quantities are insufficient to determine the values of these constants, they do place certain restrictions on the values of these constants. The 8 non-linear relationships are such that it is possible to write the 10 unknown constants in terms of the 8 measurable quantities and two combinations of specific rate constants given by

$$a = \frac{k_4 + k_5}{k_3 + k_4 + k_5} \quad (24)$$

$$b = \frac{k_2 + k_3}{k_2 + k_3 + k_4} \quad (25)$$

which are the only combinations of constants appearing in equations 20–23. The first equation given below is obtained by eliminating K_{aEM} between equations 20 and 22 after introducing 24 and 25, and the subsequent equations are obtained in a similar fashion.

$$K_{aEF} = \frac{a + b - 1}{\frac{b}{K'_{aEF}} - \frac{(1 - a)}{K'_{aEM}}} \quad (26)$$

$$K_{aEM} = \frac{a + b - 1}{\frac{a}{K'_{aEM}} - \frac{(1 - b)}{K'_{aEF}}} \quad (27)$$

$$K_{bEF} = \frac{bK'_{bEF} - (1 - a)K'_{bEM}}{a + b - 1} \quad (28)$$

$$K_{bEM} = \frac{aK'_{bEM} - (1 - b)K'_{bEF}}{a + b - 1} \quad (29)$$

The individual reaction rate constants $k_1 - k_6$ are expressed in terms of a and b and the experimental parameters by

$$k_1 = \frac{aV'_M + (1 - b)V'_F}{K'_F(1 - b)} \quad (30)$$

$$k_2 = \frac{V'_M}{1 - b} \quad (31)$$

$$k_3 = \frac{bV'_F + (1 - a)V'_M}{a + b - 1} \quad (32)$$

$$k_4 = \frac{aV'_M + (1 - b)V'_F}{a + b - 1} \quad (33)$$

$$k_5 = \frac{V'_F}{1 - a} \quad (34)$$

$$k_6 = \frac{bV'_F + (1 - a)V'_M}{K'_M(1 - a)} \quad (35)$$

These equations are obtained by first eliminating k_5 between equations 16 and 24 and k_2 between equations 18 and 25. The two simultaneous equations in k_3, and k_4 are then solved to obtain equations 32 and 33.

It remains now to determine how a and b must be restricted so that the 10 unknown constants will all be positive. From the definition of a and b we immediately have that $0 < a < 1$ and $0 < b < 1$, and from equations 32 and 33 we have that $(a + b - 1) > 0$. With these restrictions on a and b it follows that k_1, k_2, k_3, k_4, k_5 and k_6 will all be positive. It is apparent from equations 26–29 that the acid dissociation constants will be positive if

$$bK'_{aEM} - (1 - a)K'_{aEF} > 0 \quad (36)$$

$$aK'_{aEF} - (1 - b)K'_{aEM} > 0 \quad (37)$$

$$bK'_{bEF} - (1 - a)K'_{bEM} > 0 \quad (38)$$

$$aK'_{bEM} - (1 - b)K'_{bEF} > 0 \quad (39)$$

If the inequality signs are replaced with equal signs the plots in $a - b$ plane of these four equations are shown in Fig. 1. This figure is for the special case, always observed for fumarase, that $K'_{aEF} > K_{aEM}$ and $K'_{bEF} > K'_{bEM}$. Since the inequality signs require that we use values of a and b to the right and above the corresponding straight lines, the shaded area in this figure gives the range of permitted values of a and b.

In considering the ranges of values for the ten unknown constants we can interpret these ten unknowns geometrically as surfaces above the $a - b$ plane, and we can consider the heights of the surfaces over the shaded area in the $a - b$ plane. Except for k_2 and k_5, which can be handled quite simply, it turns out that the surfaces for the remaining eight unknowns have a constant height above either the boundary imposed by equation 36 or the boundary imposed by equation 39. It turns out further that for each of the remaining eight unknowns, the partial derivatives with respect to a and b are both positive, or both negative. If they are positive the constant height above the boundary due to equation 36 or 39 will be the minimum value of the unknown, and the maximum value is obtained at $a = b = 1$. If the partial derivatives are both negative, the constant height above the boundary due to equation 36 or 39 will be the maximum value of the unknown, and the minimum value is obtained at $a = b = 1$. For example, k_1 has a constant height above the boundary due to equation 39 and substituting $a/(1 - b) = K'_{bEF}/K'_{bEM}$ in equation 30 yields $k_1 = [(K'_{bEF}/K'_{bEM})\ V'_M + V'_F]/K_F$. Since $\partial k_1/\partial a > 0$ and $\partial k_1/\partial b > 0$ this is the mini-

(19) R. A. Alberty, *J. Cell. Comp. Physiol.*, **47**, 245 (1956).

TABLE II

SPECIFIC REACTION RATE CONSTANTS AND pK VALUES FOR MECHANISM 11 AT 25°

	μ = 0.001		μ = 0.005		μ = 0.010		μ = 0.020		μ = 0.100	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
$k_1 \times 10^{-9}$ (sec.$^{-1}$ M^{-1})	17	∞	25	∞	11 (5)	∞	11	∞	0.8	∞
$k_2 \times 10^{-3}$ (sec.$^{-1}$)	30	∞	45	∞	27 (13)	∞	45	∞	28	∞
$k_3 \times 10^{-3}$ (sec.$^{-1}$)	1.2	2.0	2.0	2.2	2.3 (2.1)	2.5 (2.4)	3.2	3.5	2.2	3.0
$k_4 \times 10^{-3}$ (sec.$^{-1}$)	1.2	1.3	1.8	2.0	1.7 (1.6)	2.0 (2.1)	1.8	2.0	1.8	2.0
$k_5 \times 10^{-3}$ (sec.$^{-1}$)	5	∞	40	∞	46 (29)	∞	51	∞	14	∞
$k_6 \times 10^{-9}$ (sec.$^{-1}$ M^{-1})	0.8	∞	5	∞	5 (3)	∞	5	∞	0.1	∞
pK_{aE}	6.5		6.3		6.2		6.3		7.4	
pK_{aEF}	$-\infty$	6.5	$-\infty$	5.8	$-\infty$	5.3	$-\infty$	5.6	$-\infty$	6.9
pK_{aEM}	7.1	7.1	7.1	7.1	6.6	6.6	6.8	6.8	7.7	7.7
pK_{bE}	6.9		6.8		6.8		6.9		7.4	
pK_{bEF}	7.0	7.1	6.9	6.9	7.3	7.3	7.3	7.3	7.7	7.8
pK_{bEM}	8.5	∞	8.3	∞	8.5	∞	8.7	∞	9.0	∞

mum value k_1 can assume. The maximum value is obtained at $b = 1$ where $k_1 = \infty$. The limits obtained in this way are summarized by

$$\frac{(K'_{bEF}/K'_{bEM})V'_M + V'_F}{K'_F} < k_1 < \infty \quad (40)$$

$$(K'_{bEF}/K'_{bEM})V'_M < k_2 < \infty \quad (41)$$

$$V'_F < k_3 < \frac{(K'_{aEF}/K'_{aEM})V'_F + V'_M}{(K'_{aEF}/K'_{aEM}) - 1} \quad (42)$$

$$V'_M < k_4 < \frac{(K'_{bEF}/K'_{bEM})V'_M + V'_F}{(K'_{aEF}/K'_{bEM}) - 1} \quad (43)$$

$$(K'_{aEF}/K'_{aEM})V'_F < k_5 < \infty \quad (44)$$

$$\frac{(K'_{aEF}/K'_{aEM})V'_F + V'_M}{K'_M} < k_6 < \infty \quad (45)$$

$$K'_{aEF} < K_{aEF} < \infty \quad (46)$$

$$\frac{(K'_{bEF}/K'_{bEM}) - 1}{(K'_{bEF}/K'_{bEM})(1/K'_{aEM}) - (1/K'_{aEF})} < K_{aEM} < K'_{aEM} \quad (47)$$

$$K'_{bEF} < K_{bEF} < \frac{(K'_{aEF}/K'_{aEM})K'_{bEF} - K'_{bEM}}{(K'_{aEF}/K'_{aEM}) - 1} \quad (48)$$

$$0 < K_{bEM} < K'_{bEM} \quad (49)$$

The limits would be different if $K'_{aEF} < K'_{bEF}$ and $K'_{aEM} < K'_{bEM}$, but could be worked out readily in the same way.

The minimum and maximum allowed values for these constants at three ionic strength values and 25° are summarized in Table II. It is of interest to note the symmetry in the values of the specific reaction rate constants. Because of the similar nature of the two substrates it is not surprising that $k_1 \approx k_6$ (minimum values) and $k_2 \approx k_5$ (minimum values), but the fact that $k_3 \approx k_4$ must have a deeper significance. The most noteworthy effect of increasing ionic strength is to decrease k_1 and k_6 without as pronounced an effect on the other constants. The minimum values of the six specific reaction rate constants are related by[13]

$$K_{eq} = (M)_{eq}/(F)_{eq} = k_1k_3k_5/k_2k_4k_6 \quad (50)$$

The values for 0.01 ionic strength given in parentheses were calculated to indicate the magnitude of the uncertainty resulting from the experimental errors. The maximum uncertainty in the experimental rate parameters is about ± 10%, and the maximum uncertainty in the apparent acid dissociation constants is about ± 20%. The values given in parentheses for μ = 0.01 are obtained when these maximum errors combine in such a way as to give the *lowest possible* values of k_1 and k_6.

It is of considerable interest that the values of the constants are fixed to the extent they are for this underdetermined set. Actually, of course, it is physically impossible that pK_{bEM}, k_1, k_2, k_5 and k_6 could have values of infinity, or that pK_{aEF} could have a value of $-\infty$. Originally it was hoped that by placing reasonable limits on certain of these constants, the ranges for others would be more narrowly restricted. However, the restrictions which it was felt could safely be applied did not alter the ranges of the other constants sufficiently to make a detailed discussion worthwhile.

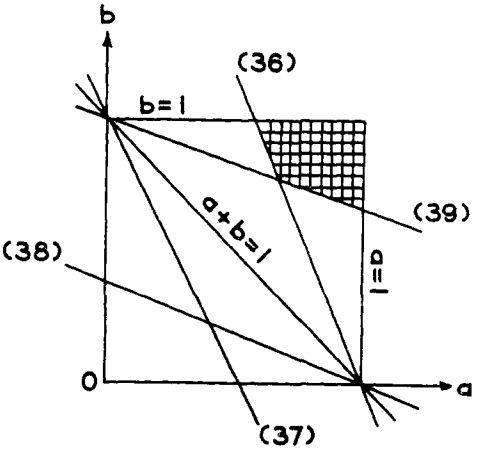

Fig. 1.—The shaded area in the diagram shows the range of permitted values of a and b. The numbers on the lines are the respective equation numbers, the inequality signs being replaced with equal signs. The lines in this diagram are only for the case that $K'_{aEF} > K'_{aEM}$ and $K'_{bEF} > K'_{bEM}$.

The calculation of the half time for the transient phase for mechanism 11 would be rather complicated. While the rates of proton associations and dissociations do not come into the steady-state treatment, information about these rates would be required for the transient state treatment. However, the rates of proton reactions are presumably

very fast, and an idea as to the duration of the transient state may be obtained by noting that the half times for the interconversions of the two enzyme substrate complexes is less than a millisecond. The rapid approach to the steady state which is indicated by these calculations justifies the use of the steady-state approximation in deriving equations for the representation of experimental data and shows that the error made in using total elapsed time in integrated equations rather than time after establishment of the steady state is negligible.

Discussion

The minimum values of the second-order rate constants for the reactions of fumarase with its substrates are greater than any which have been measured for reactions of enzymes. Chance[20] obtained 1.2×10^8 sec.$^{-1}$ M^{-1} for the reaction of yeast cytochrome-*c* peroxidase with cytochrome-*c* by the flow method. The second order rate constants[11,12] for the combination of alcohol dehydrogenase with DPN and DPNH are of the order of 10^6 sec.$^{-1}$ M^{-1}. The high minimum values of the second-order rate constants for the fumarase reaction raise the question, which is to be discussed in a future article, as to how this rate compares with what would be expected from collision theories and diffusion theories of reactions in solution.

The values of the equilibrium constants for the first and last steps of mechanism 11 cannot be calculated from the steady-state kinetic data, but the maximum and minimum values consistent with the experimental data may be calculated using equations 30, 31, 34 and 35 and inequalities 36 and 39. The maximum and minimum factors by which k_2/k_1 differ from K'_F at 0.001, 0.01 and 0.10 ionic strengths are 1.3–0.96, 1.05–0.90, 1.2–0.95, respectively. Thus although the exact values of the equilibrium constants cannot be calculated, it can be stated that they are equal to the Michaelis constants within approximately the experimental error. The same conclusion has been reached for chymotrypsin[21] and urease[22] by other methods.

The calculations of specific reaction rate constants in this article are all based upon the assumption of one enzymatic site per fumarase molecule. If there are n sites the calculated rate constants will all be decreased by this factor. Since the titration curve for fumarase has been determined[23] in 0.1 M NaCl at 25° it is possible to estimate the maximum number of enzymatic sites per molecule. The kinetic studies with 0.1 ionic strength buffer (0.09 M NaCl plus 0.01 M "tris" acetate) show that there are two groups per enzymatic site with pK = 7.4. The slope of the titration curve at pH 7.4 indicates a maximum of 12 groups with this pK value or a maximum of 6 catalytic sites per molecule

Although the present calculations have been made using data from kinetic studies of both the forward and reverse reactions, similar calculations could be made for reactions which go essentially to completion, provided the inhibition constant of the product has been measured. Since this constant is identical with the Michaelis constant for the product, the maximum initial velocity for the reverse reaction could be calculated using $K_{eq} = V_F K_M / V_M K_F$ provided the free energy change or equilibrium constant for the over-all reaction are known. For reactions involving two reactants or two products the mechanism would have to be known so that the correct form of the relation between kinetic parameters and the equilibrium constant for the over-all reaction could be chosen.[24]

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(20) B. Chance, in J. T. Edsall, "Enzymes and Enzyme Systems," Harvard University Press, Cambridge, Mass., 1951.

(21) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(22) G. B. Kistiakowsky and W. E. Thompson, *ibid.*, **78**, 4821 (1956).

(23) N. Shavit, R. G. Wolfe, Jr., and R. A. Alberty, unpublished.

(24) R. A. Alberty, THIS JOURNAL, **75**, 1928 (1953).

MADISON, WISCONSIN

[CONTRIBUTION NO. 1410 FROM THE STERLING CHEMISTRY LABORATORY OF YALE UNIVERSITY]

Bolaform Electrolytes. VI. Conductance of Bis-(trimethylammonium)-polymethylene Iodides and Related Compounds in Methanol and in Ethanol

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The following conductance data are presented: bis-(trimethylammonium)-trimethylene diiodide, bis-(trimethylammonium)-tetramethylene diiodide, bis-(trimethylammonium)-pentamethylene diiodide, tetramethylammonium iodide, methylpyridinium iodide and dimethylpiperidinium iodide in ethanol at 25°; tetramethylammonium iodide and bromide, methylpyridinium iodide, dimethylpiperidinium iodide and 1,1-methane-N,N'-bispyridinium diiodide in methanol at 25°. In ethanol, electrostatic attraction is strong enough to stabilize structures in which two anions associate with the bolaform cations. The association decreases with increasing distance between the cationic sites of the latter.

It has been shown that association of an anion to one end of a bisquaternary salt of the structure

(+).....(+)

is sensitive to the distance between the cationic sites. Most of our previous work has been in methanol, where the dielectric constant (33.62) is sufficiently high to permit neglect of configurations in which anions associate with both cationic sites, at

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