[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Studies of the Enzyme Fumarase. I. Kinetics and Equilibrium

BY ROBERT M. BOCK AND ROBERT A. ALBERTY

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The equilibrium constant for the reaction catalyzed by the enzyme fumarase has been determined spectrophotometrically over a range of ionic strength, temperature and *p*H. The variation of the apparent equilibrium constant with *p*H is interpreted in terms of the ionization constants of the two substrates. The Michaelis constants for the two substrates and the ratio of maximum velocities have been determined by use of a recording ultraviolet spectrophotometer. Simple mechanisms postulated for the enzymatic reaction indicate that the two Michaelis constants and the ratio of maximum velocities should be related to the equilibrium constant of the over-all reaction. A simple relation first obtained by Haldane is borne out by our data.

Introduction

The enzyme fumarase catalyzes one of the reactions in the tricarboxylic acid (Krebs) cycle, the hydration of fumarate to form *l*-malate.

$$\text{fumarate} + H_2O \rightleftharpoons \text{l-malate} \qquad (1)$$

Ochoa[1] has reviewed the studies of this enzyme. More recently Massey[2] has described a procedure for obtaining the crystalline enzyme from pig heart muscle. Kinetic and equilibrium studies are readily made since the enzyme is rather stable and spectrophotometric measurements in the ultraviolet may be used to determine the extent of reaction.[3] One especially interesting feature of this reaction is that Michaelis constants and maximum velocities may be obtained for both the forward and reverse reactions. If certain mechanisms are followed by the enzymatic reaction, it is to be expected that there will be a simple relationship between the kinetic constants and the equilibrium constant, as pointed out by Haldane.[4] One of the purposes of this research is to test the Haldane relation.

Experimental

The fumarase used in these studies was isolated from pig heart muscle by procedures developed in this Laboratory which will be described together with electrophoretic and ultracentrifugal studies in a future publication. When assayed in 0.05 *M* phosphate buffer of *p*H 7.3 at 25° the activity of the crystalline material was such that one gram of enzyme would cause the dehydration of 0.7 mole of *l*-malate per minute at maximum velocity which is approximately the same as that obtained by Massey[2] when corrected to his experimental conditions.

In the equilibrium and kinetic measurements the concentration of fumarate was determined by its absorption in the ultraviolet.[3] The apparatus employed consists of a Beckman DU spectrophotometer equipped with a special phototube housing and amplifier supplied by Beckman Instruments Co. and a standard Brown recording potentiometer (strip chart; 50 millivolts full scale; pen speed, 2 sec. full scale; chart speed 30 to 120 inches per hour). The phototube housing is equipped with a switch for placing 500, 2000 or 10,000 megohm resistors in the phototube circuit so that the sensitivity may be varied over a wide range. The amplifier is constructed so that either 0–100% transmission or 80–100% transmission may be recorded on the full scale.

The cell compartment is equipped with double thermospacers (Beckman arrangement D) through which water is circulated from two thermostats to control the temperature of the solution to about 0.1° over the range 4 to 50°. The top of the cell compartment is equipped with a removable plug just above the cuvette in the optical path so that substrate or enzyme may be added without moving the cuvette or closing the shutter. The wave length scale of the monochromator was calibrated with a low pressure mercury lamp.

Eastman Kodak Co. fumaric acid was recrystallized from 1 *N* hydrochloric acid according to the procedure of reference 5 and dried at 110°. This material melted at 296–298° in a sealed tube. Beer's law is followed by sodium fumarate at 250 mμ at concentrations below 10 m*M*. At 250 mμ the optical densities were independent of slit width. The molar extinction coefficients of fumarate are given in Table I.

TABLE I

MOLAR EXTINCTION COEFFICIENTS[a] OF FUMARATE AT 25°

*p*H	λ, mμ	ε, l. mole^{-1} cm.$^{-1}$
7.3	230	4610
7.3	240	2400
7.3	250	1450
7.3	260	930
7.3	270	500
7.3	280	260
7.3	290	109
4.5	250	1400
2.5	250	740
1	250	480

[a] Defined by $\epsilon = D/lc$ where $D = \log_{10}(I_0/I)$, l is the length of the optical path through the cell in cm. and c is concentration in moles l.$^{-1}$.

The *l*-malic acid purchased from the Bios and Pfanstiehl companies was dissolved by refluxing in 15 parts anhydrous ether, decanted, concentrated to one-third volume and crystallized at 0°. The acid was recrystallized until it melted at 105–106°. The extinction coefficient of the disodium *l*-malate prepared from this acid was 0.7 to 0.9 l. mole^{-1} cm.$^{-1}$ at *p*H 7 and 250 mμ compared to 3 to 35 for the commercial samples.

Determination of the Equilibrium Constant.—Stock buffer solutions of double the desired final concentration were prepared and 5 ml. of this buffer delivered into each of two 10-ml. volumetric flasks. The solution in the first flask was diluted to the mark and used as a blank. Enough stock solution of *l*-malate or fumarate was added to the second flask to give a concentration of 2 m*M* upon dilution to the mark. Three-ml. aliquots of these solutions were placed in the cuvettes to which was added 0.05 ml. of enzyme solution containing about 0.01 mg. of protein.

Equilibrium was ordinarily reached within 20 minutes. At low temperatures or at *p*H values far removed from neutrality the amount of enzyme added was increased so that equilibrium was attained in a reasonable length of time. After equilibrium had been reached the temperature and *p*H of the solution were determined.

All equilibrium measurements were checked by starting with both *l*-malate and fumarate. The contribution of *l*-malate to the optical density of the equilibrium mixtures was less than 0.05% of the total and was negligible except below *p*H 5 where a small correction is necessary.

(1) S. Ochoa, "The Enzymes," edited by Sumner and Myrback, Vol. I, Part 2, Academic Press, Inc., New York, N. Y., 1951, Chapt. 40.

(2) V. Massey, *Nature*, **167**, 769 (1951); *Biochem. J.*, **51**, 490 (1952).

(3) E. Racker, *Biochem. Biophys. Acta*, **4**, 211 (1950).

(4) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930, p. 81.

(5) A. H. Blatt, editor, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 302.

The apparent equilibrium constant is defined by

$$K_{app} = \frac{[M]_T}{[F]_T} = \frac{[S]_0 - [F]_T}{[F]_T} \qquad (2)$$

where $[M]_T$ and $[F]_T$ are the total concentrations of *l*-malate and fumarate at equilibrium and $[S]_0$ is the initial concentration of *l*-malate or fumarate. In a 1-cm. cuvette the optical density at equilibrium is

$$D_{eq} = [F]_T\epsilon_F + ([S]_0 - [F]_T)\epsilon_M \qquad (3)$$

Thus the apparent equilibrium constant may be calculated from

$$K_{app} = [S]_0\epsilon_F - D_{eq}/(D_{eq} - [S]_0\epsilon_M) \qquad (4)$$

At *p*H 6 to 9 substrate concentrations of 2 m*M* gave equilibrium optical densities in the range in which they may be determined most accurately.[6]

Measurement of Initial Rates.—In the kinetic measurements 1 ml. of buffered enzyme solution was delivered rapidly into 2 ml. of substrate solution in a cuvette in place in the recording spectrophotometer. The solution was mixed vigorously for 3 to 5 seconds with a small glass rod, and the record of percentage transmission *versus* time was obtained from about 10 seconds after the addition of enzyme and continued for at least 60 seconds. The optical densities at various times were read off of these graphs with specially constructed logarithmic rulers, and the initial reaction velocities were evaluated by plotting the rate of change of optical density *versus* time and extrapolating back to start of the reaction. When rates were determined for fumarate concentrations greater than 5 m*M* it was necessary to use a higher wave length, 270 to 290 mμ, to avoid the very low transmission at 250 mμ.

Results

Variation of Equilibrium Constant with Ionic Strength.—Figure 1 shows the nearly linear variation of the apparent equilibrium constant for reaction (1) at *p*H 7.3 with the square root of the concentration of sodium phosphate buffer. If, however, the ionic strength is maintained constant by the addition of sodium chloride, the apparent equilibrium constant does not vary over the same range of phosphate concentrations.

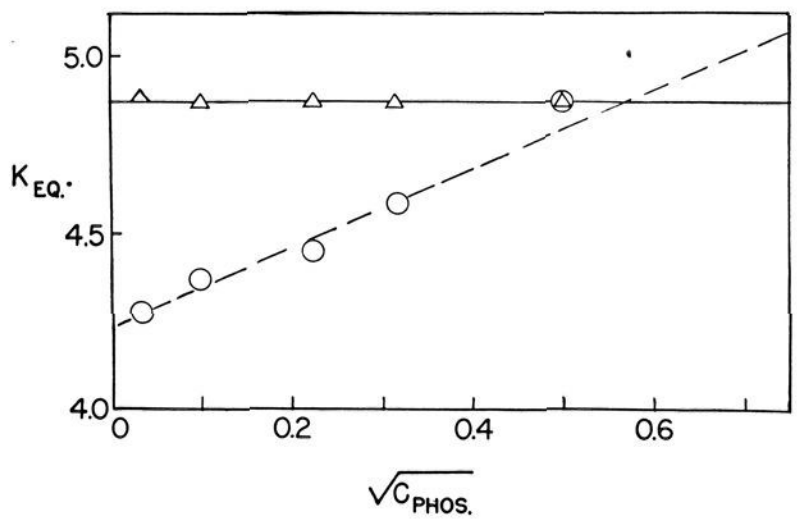

Fig. 1.—Equilibrium constant as a function of ionic strength and phosphate concentration at *p*H 7.3 and 25°: △, ionic strength held at 0.5 by addition of NaCl; ○, ionic strength equal to 0.006 plus 2.2 times the phosphate concentration.

The value of the equilibrium constant at *p*H 7.3 and an ionic strength of 0.50 is 4.8 which may be compared with a value of 3.8 obtained by Scott and Powell[7] under these conditions and 3.1 obtained by Borsook and Schott[8] over a range of *p*H 6.8–7.1 by use of an indirect potentiometric method.

Variation of Apparent Equilibrium Constant with *p*H.—The apparent equilibrium constant which is determined spectrophotometrically for reaction (1) is defined by equation (2) in which $[M]_T$ and $[F]_T$ are the total concentrations of *l*-malate and fumarate, irrespective of the extents to which they exist in different ionic forms. The equilibrium constant defined in this way is dependent upon the *p*H although it is constant over a wide range of *p*H above 7 as shown in Fig. 2.

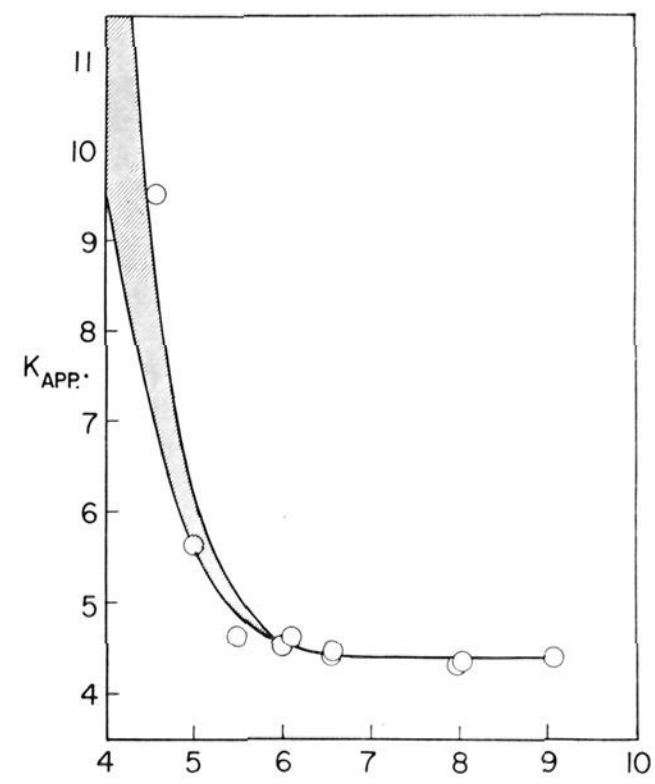

Fig. 2.—Variation of apparent equilibrium constant with *p*H at 0.05 ionic strength and 25°. Experimental values are shown as circles. The shaded area represents the maximum uncertainty introduced in the theoretical plot by an uncertainty of 0.05 in the values of the second pK's of *l*-malate and fumarate.

The variation of K_{app} with *p*H which is to be expected may be calculated by writing equation (2) as[9]

$$K_{app} = [M^=]f_{F^=}/[F^=]f_{M^=} = K_{pH\,7}(f_{F^=}/f_{M^=}) \qquad (5)$$

where $f_{F^=}$ and $f_{M^=}$ represent the fractions of fumarate and *l*-malate in the form of the divalent ions. The value of the apparent equilibrium constant at *p*H 7 and above is represented by the constant K_{pH_7}. Since the experiments are restricted to the range *p*H 4–9 it is necessary to consider only the second ionization constants of fumaric acid (K''_F) and *l*-malic acid (K''_M) in expressing $f_{F^=}$ and $f_{M^=}$. Equation (5) may, therefore, be written

$$K_{app} = K_{pH\,7}\frac{(1 + [H^+]/K''_M)}{(1 + [H^+]/K''_F)} \qquad (6)$$

The values of the ionization constants determined by titrating samples of purified *l*-malic and fumaric acids in 0.10 *M* sodium chloride at 25° are $K_F'' = 10^{-4.18}$ and $K_M'' = 10^{-4.73}$. The values of K_{app} calculated from equation (6) are shown in

(6) W. West, in A. Weissberger, "Physical Methods of Organic Chemistry," Interscience Publishers, Inc., New York, N. Y., 1949, Chapt. XXII, p. 1409.

(7) E. M. Scott and R. Powell, THIS JOURNAL, **70**, 1104 (1948).

(8) H. Borsook and H. F. Schott, *J. Biol. Chem.*, **92**, 559 (1931).

(9) R. A. Alberty, R. M. Smith and R. M. Bock, *ibid.*, **193**, 425 (1951).

Fig. 2 by the line and the shaded area which indicates the uncertainty introduced by a probable error of 0.05 in the pK values for the acids.

Variation of the Apparent Equilibrium Constant with Temperature.—The equilibrium constant has been determined over a range of 5 to 40° at pH 7.3 and 0.10 ionic strength. A plot of log K_{app} *versus* $1/T$ is shown in Fig. 3. The value of ΔH determined from this plot is -3960 ± 100 cal. mole^{-1}.

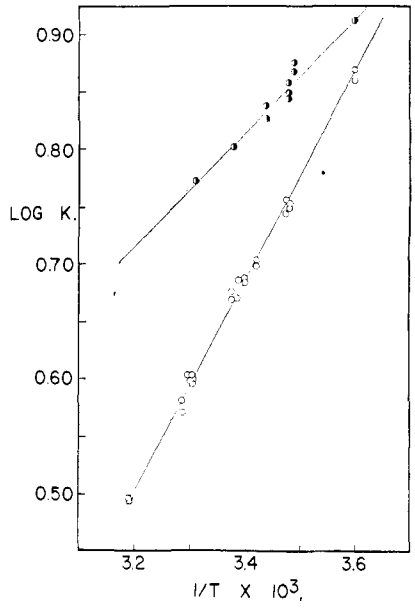

Fig. 3.—Plot of log K_{app} *vs.* $1/T$: O, pH 7.30, 0.05 M phosphate buffer, ◑, pH 4.91, 0.20 M acetate buffer.

Scott and Powell[7] obtained -3560 cal. mole^{-1} for ΔH. A series of experiments in acetate buffer of pH 4.91 and 0.1 ionic strength yielded $\Delta H = -2400 \pm 400$ cal. mole^{-1}. The pH of the buffer was measured only at 25° but the variation of pH of an acetate buffer over the temperature range used (5–35°) is insignificant.[10]

The variation of heat of reaction with pH may be calculated by taking the logarithm of equation (6), differentiating with respect to the absolute temperature and multiplying by RT^2 to obtain

$$\Delta H_{app} = \Delta H_{pH7} + \Delta H_F/(1 + K_F''/[H^+]) - \Delta H_M/(1 + K_M''/[H^+]) \quad (7)$$

where $\Delta H_{app} = RT^2(\partial \ln K_{app}/\partial T)$, $\Delta H_{pH7} = RT^2(\partial \ln K_{pH7}/\partial T)$, and ΔH_F and ΔH_M are the heats of ionization for the second ionizations of fumaric and l-malic acids.

In the absence of information concerning the value of ΔH_M it is not possible to compare the value for ΔH_{app} determined at pH 4.91 with that calculated from equation (7), but this procedure may be reversed and the value of ΔH_M calculated from the value ΔH_{app}, $\Delta H = -3960$ cal. mole^{-1} and $\Delta H_F = -980$ cal. mole^{-1}.[11] The value for ΔH_M obtained in this way is -3000 ± 1500 cal. mole^{-1}, where the large uncertainty arises from an uncertainty of about 0.05 in the second pK for l-malic acid.

Values of Kinetic Constants.—The initial rates have been plotted in Fig. 4 by the method of Lineweaver and Burk[12] to obtain Michaelis constants and maximum velocities. The Michaelis constant for l-malate at pH 7.4 in 0.05 M sodium phosphate buffer at 25° is 4.81 ± 0.28 mM, where the indicated uncertainty is the average deviation from the mean of five determinations. Racker[3] obtained a value of 4.1 in preliminary measurements in 0.05 M potassium phosphate buffer of pH 7.3.

The Michaelis constant for fumarate under the same conditions is 1.37 ± 0.14 mM which is considerably lower than the value of 3 mM reported by Jacobsohn and da Cruz[13] at 37°.

The maximum velocities are proportional to the enzyme concentration, a variable which is not as easily controlled as the others in this system. Therefore, the ratio of the maximum initial velocity with fumarate as the substrate (V_F) to the maximum initial velocity with l-malate as the substrate (V_M) has been determined by adding aliquots of the same enzyme solution to both substrates. The value of V_F/V_M is 1.19 ± 0.03 at 25° which is very considerably lower than the value of about 2 reported by Scott and Powell[7] at pH 7.29 and an ionic strength of 0.5.

Discussion

Mechanism of the Enzymatic Reaction.—Most of the kinetic studies of this enzyme reported in the literature[7,14] have been based upon the rate equation for the reversible first-order reaction

$$F \rightleftarrows M \quad (8)$$

which is

$$k_1 + k_2 = \frac{1}{t} \ln \frac{[F]_0 - [F]_e}{[F] - [F]_e} \quad (9)$$

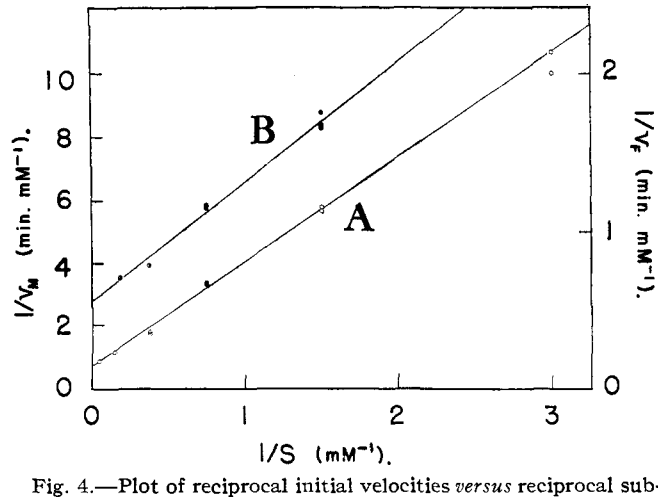

Fig. 4.—Plot of reciprocal initial velocities *versus* reciprocal substrate concentrations at pH 7.4, 0.05 molar phosphate buffer: A, for l-malate; B, for fumarate.

where k_1 and k_2 are the first-order constants for the forward and reverse reactions and [F] is the fumar-

(10) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, N. Y., 1943, p. 580.

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 82.

(12) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(13) K. P. Jacobsohn and A. da Cruz, *IX Congr. intern. quim. pura y aplicada (Madrid)*, **5**, 91 (1934) (published in 1936).

(14) K. P. Jacobsohn, F. B. Pereira and J. Tapadinhas, *Biochem. Z.*, **254**, 112 (1932).

ate concentration if fumarate is the initial substrate and the subscripts o and e indicate initial and equilibrium values. Both Jacobsohn, *et al.*,[14] and Scott and Powell[7] found that different values for $(k_1 + k_2)$ are obtained with fumarate and *l*-malate as the initial substrate and that this equation does not adequately represent the approach to equilibrium. Although equation (8) would probably be followed at very low substrate concentrations, the measurements described above show that substrate concentrations used by these authors were much too high to yield first-order reactions. The simplest mechanism according to which the enzyme-catalyzed reaction might be expected to proceed is

$$F + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} EX \underset{k_4}{\overset{k_3}{\rightleftarrows}} M + E \qquad (10)$$

where EX is an enzyme-substrate complex common to both substrates and E the active site of the enzyme. The concentration of water, which is also a substrate, might be included in k_1 or k_3. The steady state treatment of this mechanism yields the following equation for the reaction rate[4]

$$-\frac{d[F]}{dt} = \frac{d[M]}{dt} = \frac{(V_F/K_F)[F] - (V_M/K_M)[M]}{1 + ([F]/K_F) + ([M]/K_M)} \qquad (11)$$

where maximum initial velocities are represented by V and Michaelis constants by K, and $V_F = k_3[E]_0$, $K_F = (k_2 + k_3)/k_1$, $V_M = k_2[E]_0$ and $K_M = (k_2 + k_3)/k_4$. The substrate concentrations are total concentrations without regard for the degree of ionization. This rate equation is in agreement with the experimental finding that linear plots are obtained when reciprocal initial velocities are plotted *versus* reciprocal initial concentrations of the substrates.

However, it would appear to be more reasonable to expect two complexes so that the combination with water could result from a separate bimolecular reaction.

$$F + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} EX \underset{k_4}{\overset{k_3}{\rightleftarrows}} EY \underset{k_6}{\overset{k_5}{\rightleftarrows}} E + M \qquad (12)$$

where k_3 could include the concentration of water. The complexes have been designated EX and EY to avoid the necessity of specifying the nature of the combination since this is unimportant from a kinetic standpoint. Haldane[4] has also given the steady state treatment for this mechanism for which equation (11) is also obtained with

$$V_F = k_3k_5[E]_0/(k_3 + k_4 + k_5)$$
$$V_M = k_2k_4[E]_0/(k_2 + k_3 + k_4)$$
$$K_F = (k_2k_5 + k_2k_4 + k_3k_5)/(k_3 + k_4 + k_5)k_1$$
$$K_M = (k_2k_5 + k_2k_4 + k_3k_5)/(k_2 + k_3 + k_4)k_6$$

Since both mechanisms yield the same equation it is not possible to distinguish between them or any other mechanism involving additional complexes of the type of EX and EY in the absence of means for detecting these complexes.

Relation between the Kinetic Constants and the Equilibrium Constant.—Haldane[4] has pointed out that the kinetic constants are related to the equilibrium constant for the over-all reaction by

$$K_{app} = V_FK_M/V_MK_F \qquad (13)$$

which can easily be ascertained since $K_{app} = k_1k_3/k_2k_4$ for mechanism (10) or $k_1k_3k_5/k_2k_4k_6$ for mechanism (12). The apparent equilibrium constant K_{app} is used since the maximum velocities and Michaelis constants are expressed in terms of the total concentrations of the substrates rather than in terms of particular ionic forms. All quantities in this equation must be determined under the same conditions (buffer and temperature), and it is to be understood that V_F and V_M are for the same total enzyme concentration, although it is not necessary to know this concentration in molar units. The derivation of similar relations for more complicated enzymatic reactions is given in the following article.[15]

Another way of deriving equation (13) is to point out that according to equation (11) the initial reaction velocities of both the forward and reverse reactions are directly proportional to the substrate concentration at very low substrate concentrations. Under these conditions the first-order constant for the forward reaction is V_F/K_F and for the reverse reaction, V_M/K_M. Since the ratio of the first-order constants should be equal to the equilibrium constant for the reversible first order reaction (8), equation (13) results. It is not practical to make rate measurements at concentrations sufficiently low for the reaction to be first order; therefore, the first-order constants are best calculated from the maximum velocities and Michaelis constants.

Equation (13) has been nearly neglected although Frederich-Freksa[16] recently has derived the same relation for the special case that the enzyme–substrate complexes in mechanism (12) are in equilibrium with free enzyme so that k_3 and k_4 are the rate-determining.

Substitution of the kinetic constants from the preceding section into equation (13) yields 4.2 ± 0.4 for K_{app} in agreement with the directly determined value of 4.45 in 0.05 *M* phosphate buffer of *p*H 7.3 at 25°. The fact that equation (13) holds for the fumarase reaction shows that the over-all kinetics may be represented satisfactorily by either mechanism (10) or (14) although the simpler would be chosen in the absence of additional information.

Scott and Powell[7] attempted to relate the initial reaction rates determined at high concentrations of fumarate and *l*-malate to the equilibrium constant but found that the ratio of the rates was not equal to the equilibrium constant. In terms of mechanism (10) it is easy to see why this ratio is unrelated to the equilibrium constant if the velocities are measured at high concentrations of the substrates. Under these conditions the ratio of the initial rates is simply equal to the ratio of the rates at which the enzyme–substrate complexes dissociate into products. On the other hand, at extremely low substrate concentrations the forward and reverse reactions will be initially first order, and the ratio of these first-order constants would be expected to be equal to the equilibrium constant for the over-all reaction under that particular set of conditions.

Equation (13) makes it possible to predict the

(15) R. A. Alberty, THIS JOURNAL, **75**, 1925 (1953).

(16) H. Frederich-Freksa, *Z. Naturforschung*, **6b**, 398 (1951).

variation of V_F/V_M or K_F/K_M with pH, temperature or other variable provided the equilibrium constant and one of these ratios is known. For example, Scott and Powell[7] have observed that the ratio V_F/V_M varies from 8 to 0.5 as the pH is varied from 6.0 to 9.0. Since the equilibrium constant does not vary in this pH range, K_M/K_F must vary markedly. On the other hand, the variation of V_F/V_M with temperature over the range 8 to 45° nearly parallels the variation of the equilibrium constant so that K_M/K_F must remain nearly constant.

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MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Effect of Enzyme Concentration on the Apparent Equilibrium Constant for an Enzyme-catalyzed Reaction

BY ROBERT A. ALBERTY

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The change in apparent equilibrium constant for an enzyme-catalyzed reaction which is produced by the use of high concentrations of the enzyme depends upon the values of the dissociation constants for complexes of the various reactants and products with the enzyme. A treatment of this problem is given together with applications to the data of Theorell and Bonnichsen on the alcohol dehydrogenase reaction.

Theorell and Bonnichsen[1] found that the value of the equilibrium constant

$$K_{eq} = [DPNH][CH_3CHO][H^+]/[DPN][C_2H_5OH] \quad (1)$$

for the reaction

$$DPN + C_2H_5OH \rightleftharpoons DPNH + CH_3CHO + H^+ \quad (2)^2$$

was increased as much as 200-fold at pH 6.4–7.8 by the addition of large amounts of liver alcohol dehydrogenase (ADH). They concluded that this increase resulted from a stronger binding of DPNH by the enzyme than of any of the other reactants. Since the wave length of maximum absorption of DPNH is shifted from 340 to 328 mμ upon formation of a complex with the enzyme, Theorell and Chance[3] were able to measure the dissociation constant for this complex by a spectrophotometric titration.

Since the magnitude of the shift of the equilibrium constant in such a case depends upon the values of the dissociation constants for complexes of the various reactants and products with the enzyme, the equilibrium data itself offers information concerning such interactions. Such information is of special value in cases in which a spectral shift does not occur upon formation of the complex. Apparent displacements of equilibria such as observed by Theorell and Bonnichsen are more likely to be observed in experiments in which high concentrations of the enzyme are used, a situation more closely resembling that actually encountered in the living organism. Such displacements may be caused not only by interactions of reactants with the enzyme but with other substances which are present. The effect of the hydrogen ion concentration upon equilibria is well known and is frequently expressed in terms of the pH dependence of the electromotive force for an oxidation or reduction reaction or in terms of the pH dependence of the apparent equilibrium constant for a reaction.[4]

Theory.—The term apparent equilibrium constant (K_{app}) will be used to refer to the equilibrium constant calculated without regard for the degree of binding of the reactants by the enzyme. In the case of the reaction

$$A + B \rightleftharpoons C + D \quad (3)$$

$$K_{app} = [C]_T[D]_T/[A]_T[B]_T \quad (4)$$

where the subscript T's represent total concentrations which may be obtained spectrophotometrically, for example. The value of the apparent equilibrium constant obtained in the presence of a vanishingly small concentration of the enzyme so that the amounts bound are negligible will be represented by K_{eq}.

$$K_{eq} = [C][D]/[A][B] \quad (5)$$

These two equilibrium constants are related by

$$K_{app} = K_{eq}(f_A f_B/f_C f_D) \quad (6)$$

where the f's represent fractions of the indicated reactant *not* bound by the enzyme.

If a product of the reaction is bound by the enzyme, K_{app} will be greater than K_{eq}, while if a reactant is bound, K_{app} will be smaller. If, for example, only C is bound, equation (6) becomes

$$f_C = \frac{K_{eq}}{K_{app}} = 1 - \frac{[EC]}{[C]_T} \quad (7)$$

where [EC] is the concentration of C in the form of a complex with the enzyme. The following discussion and calculations will be restricted to the case that the dissociation of this complex may be represented by

$$EC \rightleftharpoons E + C \quad (8)$$

where E represents the site on the enzyme responsible for the binding. The dissociation constant is

$$D_{EC} = [E][C]/[EC] \quad (9)$$

(1) H. Theorell and R. Bonnichsen, *Acta Chem. Scand.*, **5**, 1105 (1951).

(2) DPN, diphosphopyridinenucleotide; DPNH, reduced diphosphopyridinenucleotide.

(3) H. Theorell and B. Chance, *Acta Chem. Scand.*, **5**, 1127 (1951).

(4) R. A. Alberty, R. M. Smith and R. M. Bock, *J. Biol. Chem.*, **193**, 425 (1951).