variation of $V_F/V_M$ or $K_F/K_M$ with $p$H, 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 $p$H is varied from 6.0 to 9.0. Since the equilibrium constant does not vary in this $p$H 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

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[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 $p$H 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 m$\mu$ 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 $p$H dependence of the electromotive force for an oxidation or reduction reaction or in terms of the $p$H 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).

Thus if there is more than one binding site on the enzyme molecule, it is assumed that these sites are independent and have the same intrinsic dissociation constant.[5] If there are $n$ independent sites per enzyme molecule, $[E]_T$ in the following equations is $n$ times the molar concentration of the enzyme. The concentration of bound C may be expressed in terms of $D_{EC}$, $[C]_T$ and $[E]_T$. Two special cases may be considered. If $[E]_T \gg [C]_T$

$$[EC] = \frac{[C]_T}{1 + D_{EC}/[E]_T} \qquad (10)$$

If $[E]_T \ll [C]_T$

$$[EC] = \frac{[E]_T}{1 + D_{EC}/[C]_T} \qquad (11)$$

while in the general case [EC] must be obtained as the solution of a quadratic equation.

The value of the ratio $K_{app}/K_{eq}$ at high concentrations of the enzyme may be obtained by introducing equation (10) into (7). As the enzyme concentration is increased the limiting equation becomes

$$(K_{app}/K_{eq})_{(E)_T \to \infty} = [E_T]/D_{EC} \qquad (12)$$

Thus if a single product is bound the apparent equilibrium constant obtained in the presence of high concentrations of enzyme will be directly proportional to the enzyme concentration. If two product molecules are bound the apparent equilibrium constant will be proportional to the square of the total enzyme concentration at high enzyme concentrations, etc.

The fact that Theorell and Bonnichsen[1] find definite indications that $K_{app}$ reaches a maximum value for reaction (2) indicates that it is necessary to consider, in addition, the binding of a reactant. If B and C are both bound by the enzyme to an appreciable extent, either at the same or different sites, and the enzyme concentration is high

$$\frac{K_{app}}{K_{eq}} = \frac{f_B}{f_C} = \frac{1 - [1 + (D_{EB}/[E]_T)]^{-1}}{1 - [1 + (D_{EC}/[E])_T]^{-1}} \qquad (13)$$

which in the limit of increasing enzyme concentration reduces to

$$(K_{app}/K_{eq})_{(E)_T \to \infty} = D_{EB}/D_{EC} \qquad (14)$$

Thus at sufficiently high enzyme concentrations the apparent equilibrium constant will be independent of the concentration of the enzyme and of the concentrations of the reactants. This is an important relation because it offers a means for calculating the ratio of the dissociation constants for the reactant and product which are bound. Thus the ratio of the dissociation constants for the complex of ADH with DPN and DPNH is 200 at $p$H 7.[1]

**The Binding of DPN and DPNH by Liver Alcohol Dehydrogenase.**—In the case of horse liver alcohol dehydrogenase Theorell and Chance[3] found the dissociation constant ($D_{red}$) for the ADH–DPNH complex at low enzyme concentration to be 0.1 $\mu M$ at $p$H 7 (based on the fact that ADH binds 2 DPNH) and roughly 3 $\mu M$ at $p$H 10 (based on the fact that ADH binds one DPNH). Since the ratio of the dissociation constants is 200 at $p$H 7 and approximately unity at $p$H 10, the dissociation constant for DPN ($D_{ox}$) would be 20 $\mu M$ at $p$H 7 and about 3 $\mu M$ at $p$H 10.

The values of the ratio $K_{app}/K_{eq}$ determined by Theorell and Bonnichsen[1] at $p$H 7 over a range of enzyme concentrations from 0.3 to 79 $\mu M$ are given in Table I along with the total concentrations of DPN and DPNH at equilibrium. The

TABLE I

COMPARISON OF EXPERIMENTAL VALUES FOR $K_{app}/K_{eq}$[a] AT $p$H 7 AND 20° WITH THOSE CALCULATED WITH $D_{red}$ = 0.1 $\mu M$ AND $D_{ox}$ = 20 $\mu M$

| Expt. no. | 2[ADH]T, $\mu M$ | [DPNH]T, $\mu M$ | [DPN]T, $\mu M$ | $K_{app}/K_{eq}$ Exptl. (1) | Compet. calcd. (2) | Non-compet. calcd. (3) | DPNH only (4) |
|---|---|---|---|---|---|---|---|
| 1 | 0.6 | 31.8 | 8.2 | 1.08 | 1.02 | 0.998 | 1.02 |
| 2 | 0.6 | 16.0 | 24.0 | 0.92 | 1.04 | 1.025 | 1.04 |
| 3 | 2.4 | 21.7 | 23.3 | 1.16 | 1.12 | 1.06 | 1.12 |
| 4 | 5.0 | 22.7 | 22.3 | 1.32 | 1.28 | 1.14 | 1.28 |
| 5 | 10.0 | 21.1 | 23.9 | 1.98 | 1.78 | 1.50 | 1.89 |
| 6 | 19.8 | 20.9 | 24.1 | 6.04 | 7.73 | 6.61 | 10.3 |
| 7 | 19.8 | 9.18 | 35.82 | 11.7 | 36.8 | 75.0 | 108 |
| 8 | 29.6 | 23.0 | 22.0 | 20.7 | 32.3 | 36.1 | 70 |
| 9 | 39.4 | 18.0 | 27.0 | 35.1 | 74.1 | 96.9 | 216 |
| 10 | 39.4 | 8.84 | 25.16 | 72.1 | 96.7 | 135 | 306 |
| 11 | 59.2 | 21.7 | 23.3 | 87.3 | 111 | 119 | 377 |
| 12 | 79.0 | 21.1 | 23.9 | 163 | 136 | 143 | 580 |
| 13 | 79.0 | 13.0 | 27.0 | 133 | 141 | 168 | 661 |
| 14 | 158.0 | 12.4 | 22.6 | 173 | 173 | 184 | 1460 |

[a] The value of $K_{eq}$ was taken to be 1.11 $\mu M$, the average of the two experimental values at the lowest enzyme concentration.

values of the ratio $K_{app}/K_{eq}$ at $p$H 7 to be expected from the values of $D_{red}$ and $D_{ox}$ obtained by Theorell and Chance have been calculated for the case that there is no competition between DPN and DPNH for the binding sites (column 3), that there is competition for the same site (column 2) and that only DPNH is bound (column 4). If there is no competition the following equations may be used for the calculation

$$\frac{K_{app}}{K_{eq}} = \frac{1 - ([ADH\text{-}DPN]/[DPN]_T)}{1 - ([ADH\text{-}DPNH]/[DPNH]_T)} \qquad (15)$$

$$D_{red} = \frac{(2[ADH]_T - [ADH\text{-}DPNH])([DPNH]_T - [ADH\text{-}DPNH])}{(ADH\text{-}DPNH)} \qquad (16)$$

$$D_{ox} = \frac{(2[ADH]_T - [ADH\text{-}DPN])([DPN]_T - [ADH\text{-}DPN])}{[ADH\text{-}DPN]} \qquad (17)$$

where $[ADH]_T$ is the molar concentration of the enzyme which is multiplied by 2 since there are two binding sites. In case DPN and DPNH do compete for the same site, a supposition which is supported by certain observations,[3] it is necessary to write the concentration of free enzyme as (2 [ADH] − [ADH–DPN] − [ADH–DPNH]) in equations (16) and (17). Values of $K_{app}/K_{eq}$ calculated for the case that there is competition are also plotted in Fig. 1 for comparison with the experimental values of Theorell and Bonnichsen. The values of $K_{app}/K_{eq}$ are in good agreement with the experimental values and offer an independent test of the dissociation constants obtained by spectrophotometric titration.

(5) The relationship between the intrinsic constant and the usual equilibrium constant has been discussed by H. S. Simms, THIS JOURNAL, **48**, 1239 (1926).

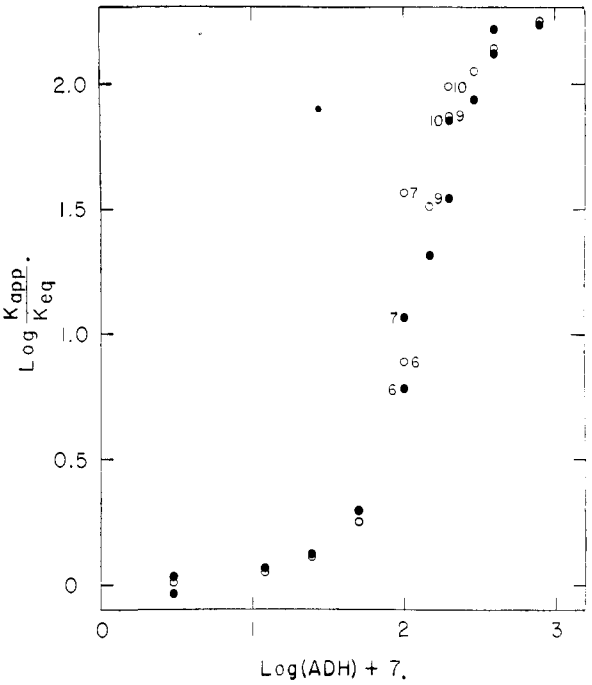


Fig. 1.—Plot of log ($K_{app}/K_{eq}$) *versus* log $[ADH]_T$ at *p*H 7 and 20°: ●, experimental data of Theorell and Bonnichsen[1]; O, values calculated from equations (15)–(17) using $D_{red} = 0.1\ \mu M$ and $D_{ox} = 20\ \mu M$.

The ratio $K_{app}/K_{eq}$ is actually not a continuous function of $[E]_0$ since the concentrations of DPN and DPNH at equilibrium vary from experiment to experiment. There are three pairs of equilibrium experiments in which the total enzyme concentration is constant but the concentrations of DPN and DPNH are varied (experiments 6 and 7, 9 and 10, 12 and 13). In each case there are quite appreciable differences between the experimentally determined apparent equilibrium constants. It is interesting to see the extent to which the theory predicts these differences. In the first pair of experiments (6 and 7) the concentration of DPNH is less in experiment 7 so that it would be expected that a larger fraction would be bound. If a larger fraction of DPNH is bound it would be expected that $K_{app}/K_{eq}$ would be larger than in experiment 6, as is actually observed. In the second pair of experiments (9 and 10) the total concentration of DPNH is lower in experiment 10, so that the larger value of $K_{app}/K_{eq}$ in this experiment is in agreement with the theory. In the third pair of experiments (12 and 13) the theory predicts relatively little effect of the change of the $[DPNH]_T/[DPN]_T$ ratio, while an effect in the opposite direction is observed.

The values of $D_{red}$ at *p*H 8 and 9 have not been determined spectrophotometrically, but since the apparent equilibrium constants have been measured over a range of enzyme concentration under these conditions, the values for $D_{red}$ may be calculated from the equilibrium data. A method of successive approximations has been used to obtain $D_{red}$ for the case that the two forms of the coenzyme compete for the same site. The values obtained in this way are given in the last column of Table II

TABLE II

VALUES OF $D_{red}$ AND $D_{ox}$ FOR ALCOHOL DEHYDROGENASE AT 20°

| *p*H | $D_{ox}/D_{red}$[a] | $D_{red}$ in $\mu M$: From spect. titr.[3] | $D_{red}$ in $\mu M$: Calcd. from equil. data |
|---|---|---|---|
| 7 | 200 | 0.1 | 0.1 |
| 8 | 140 | ... | 0.2 |
| 9 | 30 | ... | 6 |
| 10 | 1 | 3 | ... |

[a] From the value of $K_{app}/K_{eq}$ approached at the highest enzyme concentrations.

and the ratios $K_{app}/K_{eq}$ calculated from these values may be compared with the experimental results in Fig. 2.

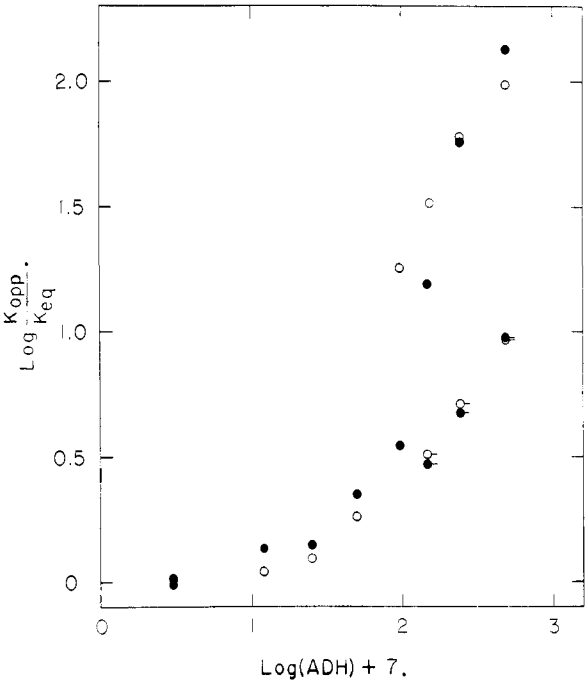


Fig. 2.—Plot of log ($K_{app}/K_{eq}$) *versus* log $[ADH]_T$ at *p*H 8 and 9 and 20°: ●, experimental data of Theorell and Bonnichsen[1]; O, values calculated from equations (15)–(17) using at *p*H 8, $D_{red} = 0.2\ \mu M$, $D_{ox} = 28\ \mu M$ and at *p*H 9, $D_{red} = 6\ \mu M$, $D_{ox} = 180\ \mu M$. The points at *p*H 9 are represented by ●- (exptl.) and O- (theoret.), and only the points at the three highest enzyme concentrations are included since at the lower enzyme concentrations the ratio is unity within the experimental error.

**The Binding of DPN and DPNH by Heart Lactic Dehydrogenase.**—Neilands[6] finds that the apparent equilibrium constant for the reaction

$$\text{Lactate} + \text{DPN} \rightleftharpoons \text{DPNH} + \text{Pyruvate} + H^+ \quad (18)$$

is dependent upon the concentration of heart lactic dehydrogenase and values of $K_{app}/K_{eq}$ of 50 and 36 were reached at *p*H 7 and 8, respectively, at 25°. The values of $K_{app}/K_{eq}$ did not appear to approach a maximum for the enzyme concentrations used as was true in the case of alcohol dehydrogenase. Chance and Neilands[7] have measured the dissociation constant for the complex of DPNH with the enzyme spectrophotometrically at *p*H 7.15 and 5°

(6) J. Neilands, *J. Biol. Chem.*, **199**, 373 (1952).
(7) B. Chance and J. Neilands, *ibid.*, **199**, 383 (1952).

and obtain 7 $\mu M$. Their experiments also indicate that at 25° the value of $D_{red}$ is greater than at 4°. Calculations such as described above show that if DPNH is the only substrate appreciably bound such a value for $D_{red}$ is too large to account for the increase in $K_{app}/K_{eq}$ which is observed. A value of $D_{red}$ of 0.8 $\mu M$ would be required if this were the case. As pointed out by Neilands[6] the low value of the Michaelis constant for pyruvate suggests that this substance is also strongly bound. If it is assumed that the dissociation constant for pyruvate is the same as that for DPNH (that is, 7 $\mu M$) the values of $K_{app}/K_{eq}$ calculated with equations (15)–(17) are in agreement with the experimental values. If the binding of DPN is negligible the value of $K_{app}/K_{eq}$ would be expected to be proportional to the enzyme concentration squared at higher enzyme concentrations.

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MADISON, WISCONSIN

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

# The Relationship between Michaelis Constants, Maximum Velocities and the Equilibrium Constant for an Enzyme-catalyzed Reaction

BY ROBERT A. ALBERTY

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In the case of reversible reactions which are catalyzed by enzymes the Michaelis constants and maximum velocities may be determined for both the forward and reverse reactions. The steady state treatment of a number of commonly used mechanisms has been used to obtain the equations for the initial rates of the forward and reverse reactions. It is possible to show that for these mechanisms there is a relationship between the various kinetic constants and the equilibrium constant for the over-all reaction. These relations offer a means of testing the suitability of a particular mechanism for an enzyme-catalyzed reaction. These equations also call attention to important new kinetic constants.

In the case of enzyme-catalyzed reactions for which the equilibrium is not displaced strongly in favor of reactants or products it is possible to determine the maximum velocities and Michaelis constants for both the forward and reverse reactions. The question then arises as to whether the values of these constants are independent of the equilibrium constant for the over-all reaction under the same conditions. Haldane[1] was the first to show that such relations exist. As shown in the preceding paper[2] the Haldane relationship is satisfied by the fumarase reaction. The purpose of the present article is to discuss such relationships for more complicated reactions and mechanisms.

The objective of completely describing the kinetics of a reaction requires the determination of the mechanism and the individual rate constants. In the absence of means for directly studying the intermediate complexes in an enzymatic reaction this objective is not generally attained and the maximum amount of information must be obtained from a study of the kinetics of the over-all reaction; that is, the rate of disappearance of substrate or appearance of product when the reaction is in a nearly steady state. The problem is then to interpret the constants obtainable for the over-all reaction in terms of the individual rate constants of the appropriate mechanism. The system of differential equations for a particular mechanism may be solved in a perfectly general way,[3] but frequently one of two approximations is satisfactory. The first is that made by Michaelis and Menten[4] who assumed that the enzyme–substrate complex is in rapid equilibrium with free substrate and enzyme. A less restrictive assumption is that introduced to enzyme kinetics by Briggs and Haldane[5] who assumed that the enzyme–substrate complex may be considered to be in a steady state; that is, the rate of formation of the complex is equal to its rate of decomposition. It should be pointed out that the steady state assumption used in enzyme kinetics is somewhat different from the assumption of unstable intermediates frequently used in kinetics[6] since the usual corollary of the latter assumption is that an appreciable fraction of *none* of the reactants is in the form of the intermediate. In the case of enzymatic mechanisms it is generally assumed that an appreciable fraction of the enzyme, but of no other reactant, may be in the form of an intermediate complex. A number of mechanisms for reversible enzymatic reactions may not be treated by the rapid equilibrium method since contradictory assumptions would be involved in treating the forward and reverse reactions. Therefore, in the following discussion only the more general steady state method[7] will be used except in

(1) J. B. S. Haldane, "Enzymes," Longman's, Green and Co., London, 1930, pp. 80–82.

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

(3) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943); B. Chance, D. S. Greenstein, J. Higgins and C. C. Yang, *Arch. Biochem. Biophys.*, **37**, 332 (1952).

(4) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

(5) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(6) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1940, p. 105.

(7) In order to avoid details in subsequent discussions the following description of the steady state method is given: The rate equations for the $n$ enzyme–substrate complexes involved in the mechanism are written and the rates of change for the concentrations of these complexes are set equal to zero. The concentration of free enzyme is eliminated by use of $[E] = [E]_0 - \sum_{i=1}^{i=n} [ES_i]$, where $[E]_0$ is the total concentration of the enzyme. The resulting $n$ equations in $n$ un-