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The Calculation of an Enzyme–Substrate Dissociation Constant from the Over-all Initial Velocity for Reactions Involving Two Substrates

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Initial velocity measurements at a particular *p*H and buffer concentration for enzymatic reactions involving two substrates are shown to yield an enzyme–substrate dissociation constant (k_2/k_1) for at least one of the substrates. A convenient method is described to determine this dissociation constant from Lineweaver–Burk plots of the data. The values calculated graphically by this method agree closely with dissociation constants determined from equilibrium binding experiments. This is true regardless of whether the dissociation constant is larger, smaller or identical with the apparent Michaelis constants. The advantages of this method are pointed out.

The use of the steady-state assumption in the derivation of the rate equations for enzymatic reactions involving one substrate led to the important concept that the Michaelis constant is not necessarily a dissociation constant for the enzyme–substrate complex.[1] The derived equation shows that certain assumptions must be made as to the values of various rate constants before the experimentally determined Michaelis constant becomes numerically equal to the dissociation constant. This may also be true for more complicated enzymatic reactions since it was proven experimentally for D-glyceraldehyde 3-phosphate dehydrogenase, an enzyme which requires more than one substrate, that the apparent Michaelis and dissociation constants for the coenzyme were not identical.[2]

For enzymatic reactions involving two substrates, it will be shown here that assumptions as to the values of various rate constants are not necessary for the determination of an enzyme–substrate dissociation constant for at least one of the two substrates. Consider the rather general mechanism for the two substrate case in which there is a compulsory binding order, that is, one substrate must be bound to the enzyme before the second may also bind. The even more general case of random addition of substrates will be considered later. Thus for the mechanism

$$\begin{array}{c} \mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \\ \mathrm{EA} + \mathrm{B} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EAB} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{ECD} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{ED} + \mathrm{C} \\ \mathrm{ED} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \mathrm{E} + \mathrm{D} \end{array} \quad (1)$$

the over-all rate equation expressing the initial velocity as a function of the substrate concentration has been shown to be[3]

$$v = \frac{V}{1 + K_A/[A] + K_B/[B] + K_{AB}/[AB]} \quad (2)$$

where[4]

$$\begin{aligned} V &= k_7(E)_0/[(k_6 + k_7)/k_5 + k_7/k_9 + 1] \\ K_A &= k_7/k_1[(k_6 + k_7)/k_5 + k_7/k_9 + 1] \\ K_B &= [k_4k_6 + k_4k_7 + k_5k_7]/k_3k_5[(k_6 + k_7)/k_5 + k_7/k_9 + 1] \\ K_{AB} &= k_2[k_4k_6 + k_4k_7 + k_5k_7]/k_1k_3k_5[(k_6 + k_7)/k_5 + k_7/k_9 + 1] \end{aligned} \quad (3)$$

Plots of the reciprocal initial velocity *vs.* the reciprocal substrate concentration[5] for either substrate will yield a linear plot similar to one of those shown in Fig. 1, 2 or 3. Data from the literature for different enzymes have been found which exemplify each type of plot. Thus data at a particular *p*H and buffer concentration for (a) lactic dehydrogenase,[6] (b) liver alcohol dehydrogenase[7] and (c) yeast alcohol dehydrogenase[8] have been replotted to give Figs. 1, 2 and 3, respectively, where A is considered to be the coenzyme, reduced or oxidized form of diphosphopyridine nucleotide (DPNH or DPN^+). It is these plots of $1/v$ *vs.* $1/[A]$ at varying concentrations of B which are of interest.

It may be shown that the "negative concentration" of A, $-[A']$, at the point of intersection of the lines in Figs. 1–3 (where $1/v_1$ at $[B]_1$ equals $1/v_2$ at $[B]_2$) is numerically equal to K_{AB}/K_B. The reciprocal of this "negative concentration" is shown by the arrows drawn on Figs. 1, 2 and 3.

From the equation given for mechanism (1), it is shown easily that K_{AB}/K_B is k_2/k_1. Therefore since the graphically determined value of A′ is numerically equal to K_{AB}/K_B, A' may be defined as the dissociation constant for the EA complex. This conclusion is quite general. For simpler reaction mechanisms, for example those which involve only one or no ternary complexes, for which the over-all rate equation is represented by equation 2, the expression K_{AB}/K_B always yields the dissociation constant for the EA complex. Presumably the same result would be obtained even if the mechanism involved three or more ternary complexes.

Dissociation constants determined graphically by the method described above compared to the experimentally determined dissociation constants obtained from data in the literature are shown in Table I. In general, the agreement is quite good.

For reaction mechanism (1), it is of course not possible to obtain a dissociation constant for the EB complex since such a complex does not exist. Moreover, the ratio K_{AB}/K_A determined graphically in a manner similar to the determination of K_{AB}/K_B does not reduce to the simple Michaelis or

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

(2) S. F. Velick, J. E. Hayes, Jr., and J. Harting, *J. Biol. Chem.*, **203**, 527 (1953).

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

(4) The expressions for the kinetic parameters given by Alberty[3] have been corrected.

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

(6) M. T. Hakala, A. J. Glaid and G. W. Schwert, *J. Biol. Chem.*, **221**, 191 (1956).

(7) H. Theorell, A. P. Nygaard and R. Bonnichsen, *Acta Chem. Scand.*, **9**, 1148 (1955).

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

TABLE I

COMPARISON OF EXPERIMENTAL AND CALCULATED[a] DISSOCIATION CONSTANTS FOR SEVERAL ENZYMES

Enzyme	Substrate	Dissociation constant: Experimental	Exptl. conditions	Ref.	Dissociation constant: Calculated[a]	Exptl. conditions	Ref.
Lactic dehydrogenase	DPN^+	3×10^{-4} *M*	25°, *p*H 6.8, 0.05 *M* phosphate	9	3.2×10^{-4}	22°, *p*H 6.8, 0.05 *M* phosphate	6
	DPNH	6×10^{-6} *M*	5°, *p*H 7.1, 0.15 *M* phosphate	10	1.7×10^{-6}	12°, *p*H 6.8, 0.05 *M* phosphate	6
Liver alcohol dehydrogenase	DPNH	1×10^{-7} *M*	25°, *p*H 7, 0.01 *M* phosphate	11	4×10^{-7} *M*	23°, *p*H 7.2, 0.05 *M* phosphate	7
		3×10^{-6} *M*	25°, *p*H 10, 0.01 *M* glycine-NaOH	11	4×10^{-6} *M*	23°, *p*H 10, 0.1 *M* glycine	7
Yeast alcohol dehydrogenase	DPNH	1.3×10^{-5} *M*	4°, *p*H 7.8, 0.013 *M* glycine		2.5×10^{-5} *M*	25°, *p*H 7.9, 0.013 *M* glycine	8
	DPN^+	2.6×10^{-4} *M*	0.01 *M* semicarbazide	8	1.7×10^{-4} *M*	0.01 *M* semicarbazide	8
	CH_3CHO	1.1×10^{-4} *M*			1.8×10^{-4} *M*		12

[a] Calculated from the ratio K_{AB}/K_B determined from replotted kinetic data in the literature according to the method described in the text.

dissociation constant for the EAB complex except under very special conditions.

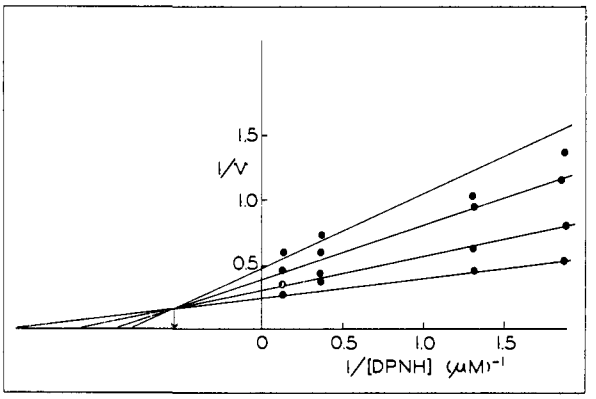

Fig. 1.—Data for lactic dehydrogenase at 11.5° and *p*H 6.8 in 0.05 *M* phosphate buffer (from Hakala, Glaid and Schwert[6]). Arrow shows value of $-1/[A']$ at intersection of the Lineweaver-Burk plots.

Now consider the more general case of random addition of substrates as shown in mechanism (4).

$$\begin{aligned} E + A &\rightleftharpoons EA & E + D &\rightleftharpoons ED \\ E + B &\rightleftharpoons EB & E + C &\rightleftharpoons EC \\ EA + B &\rightleftharpoons EAB & ED + C &\rightleftharpoons ECD \\ EB + A &\rightleftharpoons EAB & EC + D &\rightleftharpoons ECD \\ & EAB \rightleftharpoons ECD \end{aligned} \qquad (4)$$

The rate equations for this mechanism have been derived using the assumptions of either steady state[13] or rapid equilibrium.[3,14,15] When the rapid equilibrium assumption is used, the rate equation becomes identical with that given in equation 2. The apparent Michaelis constants are the dissociation constants for the EAB complex since by definition $K_A = (EB)(A)/(EAB)$ and $K_B = (EA)(B)/(EAB)$. The dissociation constant for the EA complex is obtained graphically in the usual manner from the ratio K_{AB}/K_B.

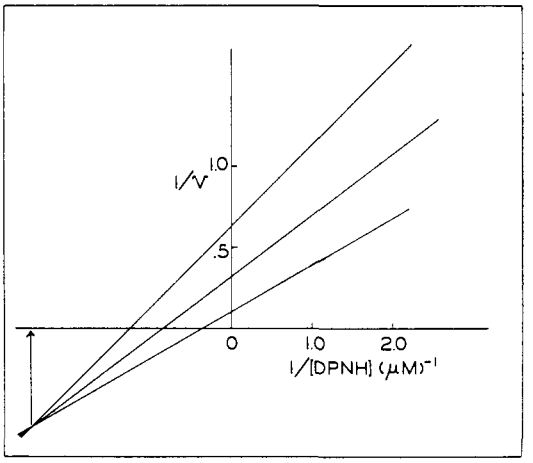

Fig. 2.—Data for liver alcohol dehydrogenase at *p*H 7.2 and 23° in 0.1 ionic strength phosphate buffer. The Lineweaver-Burk plots are calculated from rate constants and Michaelis constants given by Theorell, Nygaard and Bonnichsen.[7]

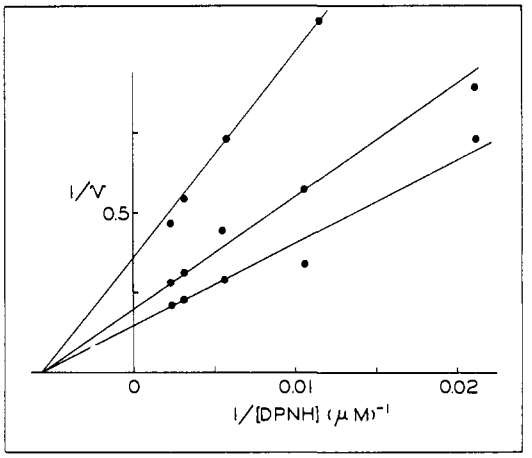

Fig. 3.—Data for yeast alcohol dehydrogenase at *p*H 7.9 and 25° in 0.013 *M* glycine and 0.01 *M* semicarbazide; Lineweaver-Burk plots replotted from data of Hayes and Velick.[8]

(9) G. W. Schwert and Y. Takenaka, *Federation Proc.*, **15**, 351 (1956).
(10) B. Chance and J. Neilands, *J. Biol. Chem.*, **199**, 383 (1952).
(11) H. Theorell and B. Chance, *Acta Chem. Scand.*, **5**, 1127 (1951).
(12) E. Negelein and H. J. Wulff, *Biochem. Z.*, **293**, 351 (1937).
(13) L. L. Ingraham and B. Makower, *J. Phys. Chem.*, **58**, 266 (1954).
(14) H. L. Segal, J. F. Kachmar and P. D. Boyer, *Enzymologia*, **15**, 187 (1952).
(15) K. L. Laidler and I. M. Socquet, *J. Phys. Colloid Chem.*, **54**, 519 (1950).

The steady-state derivation of the rate equations for mechanisms involving random addition is somewhat more complex. The equation has been given by Ingraham and Makower.[13] Theoretically this treatment yields a rate equation very different from equation 2 and non-linear Lineweaver–Burk plots are obtained where the Michaelis constants are rather complicated functions of the substrate concentration. This case will not be discussed since presumably it may be experimentally differentiated from the others.

Discussion

The method outlined in this paper presents a convenient way in which the enzyme–substrate dissociation constant for at least one substrate of a two substrate reaction may be calculated from measurements of the over-all initial reaction velocity. The advantages of this method are quite apparent. First, it is not necessary to perform equilibrium binding experiments using large quantities of enzyme in order to obtain the dissociation constant. In some cases, experimental determination of the dissociation constant is impossible because of enzyme-A interaction or because the dissociation constant is too large to be determined experimentally. Secondly, it is not necessary to go through an extended kinetic analysis to obtain an accurate value for the dissociation constant for the EA complex. The only necessary kinetic data may be obtained from initial velocity experiments at several A concentrations and a minimum of two B concentrations. Thirdly, it is not essential that the enzyme be absolutely pure. Reliable kinetic results may be obtained for enzymes in the presence of other proteins or enzymes provided that such contaminants have no effect upon the velocity of the reaction. It is interesting to note that the data in Fig. 1 show that the dissociation constant for EA is *larger* than any apparent Michaelis constant determined at a particular concentration of B. On the other hand, the dissociation constant in Fig. 2 is *smaller* than any apparent Michaelis constant and in Fig. 3 the apparent Michaelis constant is identical with the dissociation constant at any B concentration. It is even more interesting that examples of each type have been found in the literature.

For most cases, the difference between the experimental and calculated values of the dissociation constant is small, although direct comparison is complicated by the fact that all the experimental conditions were not the same. For the most part, the difference in these two values for the dissociation constant is less than a factor of two. In the two cases where the difference is larger, the *p*H values in the two experiments were not the same and there was some difficulty encountered in the determinations of the experimental values (see the original papers).

Although the ratio K_{AB}/K_B is now defined as the dissociation constant, the ratio K_{AB}/K_A is less clear. While it is true that the dissociation constant may be obtained for one substrate, it is also true that the method will not yield the dissociation constant for the second substrate except under certain assumptions for the values of particular rate constants or for the special case in which all steps in the formation of EAB are in rapid equilibrium. For this reason, it is impossible to describe the meaning of K_{AB}/K_A from kinetic measurements only. For the case of yeast alcohol dehydrogenase, it is apparent that the Michaelis constant and dissociation constant for each of the substrates acetaldehyde, DPNH and DPN^+ are identical. Thus all steps in this reaction appear to be adjusted rapidly except for the conversion of EAB to ECD.

Whereas the over-all rate equation must be given by equation 2, it is presumably not necessary that the reaction be limited to two substrate cases. For example, DPNH–cytochrome c reductase is an enzyme which requires three substrates, yet it is apparent from the over-all reaction velocity that the mechanism is analogous to the two substrate case.[16] For this enzyme, one would predict from the data that the *p*H-independent dissociation constant for DPNH is 4×10^{-5} *M* at 14° in 0.01 *M* acetate.[16,17]

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(16) C. Frieden, *Biochem. Biophys. Acta*, in press.

(17) ADDENDUM.—After this paper was submitted, Y. Takenaka and G. W. Schwert, *J. Biol. Chem.*, **223**, 157 (1956), reached the same conclusion that the ratio K_{AB}/K_A is identical with k_2/k_1. They did not, however, present a graphical method of determining the dissociation constant.