GRAPHICAL DETERMINATION OF THE DISSOCIATION CONSTANTS FOR TWO-SUBSTRATE ENZYME SYSTEMS*, **

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The kinetics of single-substrate enzyme systems have received a good deal of attention since the publication of the classical equation of MICHAELIS AND MENTEN[1], and numerous efforts to simplify the calculations involved in these studies have been made. LINEWEAVER AND BURK[2] converted the Michaelis equation into a useful linear form, and since then several other linear modifications of the equation have been pointed out. DIXON[3] suggested further graphical simplifications of the methods of LINEWEAVER AND BURK based on extrapolations to the baseline (ordinate = zero).

The more complicated enzyme systems in which two substrates are involved have only recently begun to receive extensive attention. (See for example SCHWERT AND HAKALA[4].) ALBERTY[5] has suggested a number of mechanisms for such reactions, and has made available criteria for evaluating these possibilities on the basis of kinetic data. Recently FRIEDEN[6] has discussed the calculation of dissociation constants for two-substrate systems and has used a graphical method very similar to that of this report to calculate dissociation constants from data already published for several systems.

ALBERTY's most general mechanism, and the corresponding dissociation constants, are as follows*:

$$E + A \rightleftharpoons EA \qquad K_1 = \frac{[E][A]}{[EA]}$$

$$E + B \rightleftharpoons EB \qquad K_2 = \frac{[E][B]}{[EB]}$$

$$EA + B \rightleftharpoons EAB \qquad K_3 = \frac{[EA][B]}{[EAB]}$$

$$EB + A \rightleftharpoons EAB \qquad K_4 = \frac{[EB][A]}{[EAB]}$$

$$EAB \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ECD$$

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** In this paper the symbols used have their usual significance. Brackets denote molar concentration. The following comment will assist in identifying them: E is enzyme; A, B, C and D are substrates; k_1 and k_2 are rate constants; v is the initial reaction velocity; V_f is the maximum initial reaction velocity for the forward reaction (when both substrates are present in high concentration); $[E]_T$ is total enzyme concentration; S indicates substrate.

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$$ECD \rightleftharpoons EC + D \qquad K_6 = \frac{[EC]\,[D]}{[ECD]}$$

$$ECD \rightleftharpoons ED + C \qquad K_7 = \frac{[ED]\,[C]}{[ECD]}$$

$$EC \rightleftharpoons E + C \qquad K_8 = \frac{[E]\,[C]}{[EC]}$$

$$ED \rightleftharpoons E + C \qquad K_9 = \frac{[E]\,[D]}{[ED]}$$

The initial velocity of the forward reaction by this mechanism is given by equation (1), assuming that the rate-limiting step is the conversion of EAB into ECD, and that all the other equilibria are adjusted rapidly.

$$v = \frac{V_f}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A]\,[B]}} \tag{1}$$

The dissociation constants (K_1—K_9) can be determined from kinetically determined Michaelis constants (K_A, K_B, K_{AB}) according to the following equations:

$$K_1 = \frac{K_{AB}}{K_B} \qquad K_6 = K_D$$

$$K_2 = \frac{K_{AB}}{K_A} \qquad K_7 = K_C$$

$$k_1 = \frac{V_f}{[E]_T}$$

$$K_3 = K_B \qquad K_8 = \frac{K_{CD}}{K_D}$$

$$k_2 = \frac{V_f}{[E]_T}$$

$$K_4 = K_A \qquad K_9 = \frac{K_{CD}}{K_C}$$

The present paper describes a simple graphical method for the calculation of K_A, K_B, and K_{AB} from kinetic data obtained by measuring the effect of variations in [A] and [B] on the initial velocity. These simplifications may be regarded as extensions of the earlier suggestions of DIXON.

Equation (1) may be readily converted to the slope-intercept form by inverting and rearranging terms.

$$\frac{1}{v} = \frac{K_A + \frac{K_{AB}}{[B]}}{V_f} \cdot \frac{1}{[A]} + \frac{1 + \frac{K_B}{[B]}}{V_f} \tag{2}$$

Thus, a series of straight lines (Fig. 1) is obtained when $1/v$ is plotted against $1/[A]$ for a series of constant values of [B]. (Experimentally, [A] and [B] should be of the order of magnitude of their Michaelis constants, K_A and K_B.) Extrapolation of these lines to the ordinate axis gives a series of intercepts each of which equals $1/V_{M_A}$, where V_{M_A} corresponds to the maximum velocity obtainable at infinite [A] and given [B], and is defined by equation (3).

$$V_{M_A} = \frac{V_f}{1 + \frac{K_B}{[B]}} \tag{3}$$

Additional information may be obtained from these plots of reciprocal initial velocity *versus* reciprocal substrate concentration. When two different concentrations of B

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are employed ($[B]_1$ and $[B]_2$), two lines of different slopes and intercepts will result, and the coordinates of their point of intersection may be derived. Equation (2) is written for the two different concentrations of B and the resulting equations solved in turn for $1/v$ and $1/[A]$. The two $1/v$ expressions are set equal and solved for $1/[A]$, which corresponds to the abscissa of the point of intersection. The two $1/[A]$ expressions are then set equal and solved for $1/v$, which yields the ordinate of the point of intersection.

Setting the $1/v$ expressions equal:

$$\frac{1}{v} = \frac{K_A + \frac{K_{AB}}{[B]_1}}{V_f} \cdot \frac{1}{[A]} + \frac{1 + \frac{K_B}{[B]_1}}{V_f} = \frac{K_A + \frac{K_{AB}}{[B]_2}}{V_f} \cdot \frac{1}{[A]} + \frac{1 + \frac{K_B}{[B]_2}}{V_f} \tag{4}$$

and solving for $1/[A]$:

$$\frac{1}{[A]} = \frac{-K_B}{K_{AB}} \tag{5}$$

Similarly setting the $1/[A]$ expressions equal and solving for $1/v$:

$$\frac{1}{v} = \frac{K_{AB} - K_A K_B}{K_{AB} V_f} \tag{6}$$

Thus it is apparent from equations (5) and (6) that the coordinates of the point of intersection of the $1/v$ *versus* $1/[A]$ lines for two values of $[B]$ are independent of $[A]$, $[B]$, and v, and hence the lines for any number of values of $[B]$ can be expected to meet in a single point, as shown in Fig. 1. The behavior predicted here is quite similar to that pointed out by Dixon for cases of enzyme inhibition.

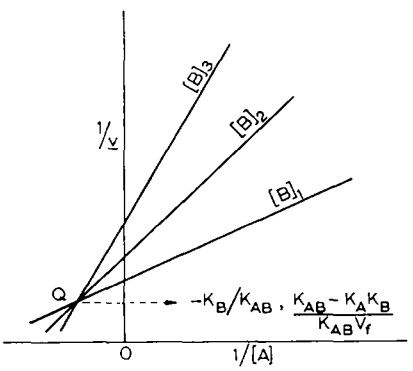

Fig. 1. $1/v$ *vs.* $1/[A]$. Intercepts on $1/v$ axis are $1/V_{M_A}$ values. Coordinates of common intersection point, Q, are $-K_B/K_{AB}$, $K_{AB} - K_A K_B/K_{AB} V_f$.

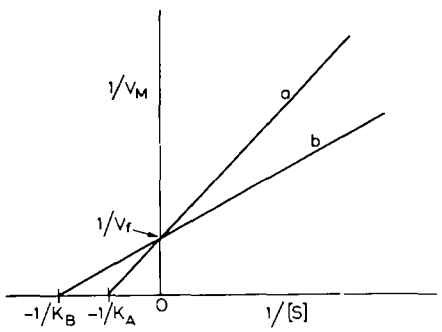

Fig. 2. $1/V_M$ *vs* $1/[S]$; Curve a: $1/V_{M_B}$ *vs* $1/[A]$; Curve b: $1/V_{M_A}$ *vs.* $1/[B]$.

Since equation (1) is symmetrical with respect to $[A]$ and $[B]$, the point of intersection of $1/v$ *vs.* $1/[B]$ lines can readily be demonstrated to have the coordinates, $-K_A/K_{AB}$, $K_{AB} - K_A K_B/K_{AB} V_f$. Similar relationships apply to the reverse reaction.

The Michaelis constants, K_A and K_B, can be readily evaluated by plotting the $1/V_{M_A}$ and $1/V_{M_B}$ values obtained on the previous graphs against the corresponding $1/[B]$ and $1/[A]$ values, respectively, as shown in Fig. 2. Rearrangement of equation

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(3) shows that this will give a straight line of ordinate intercept $1/V_f$ and abscissa intercept $-1/K_B$ when $1/V_{M_A}$ is plotted against $1/[B]$.

$$\frac{1}{V_{M_A}} = \frac{K_B}{V_f} \cdot \frac{1}{[B]} + \frac{1}{V_f} \tag{7}$$

The two lines obtained have the same ordinate intercept, which corresponds to the reciprocal of V_f, the maximum velocity obtainable at infinite $[A]$ and $[B]$, under the conditions of the experiment. Thus from this third graph can be obtained values of K_A, K_B, and V_f. These data, coupled with the K_B/K_{AB} and K_A/K_{AB} values obtained from the previous graphs, allow calculation of all the dissociation constants for the forward reaction based, of course, only on ALBERTY's most general mechanism. The constants for the reverse reaction can be determined in a completely analogous manner.

It should be noted that the ordinates of the points of intersection for the $1/[A]$ and $1/[B]$ plots are equal. Since the calculated value for this term involves all four kinetic quantities, it may be compared to the graphically obtained values as an index of the accuracy of the data and self-consistency of an experiment.

The ordinate of the point of intersection is also an indication of the effect that the presence of one substrate on the active site of the enzyme has upon complexing of the second substrate. If the ordinate is equal to zero, then K_{AB} equals $K_A K_B$, and hence $K_A = K_1 = K_4$ and $K_B = K_2 = K_3$. This situation corresponds to ALBERTY's special case of his Mechanism I, 1, when the presence of one substrate has no effect on the complexing of the second substrate.

In conclusion, it may be pointed out that the above analysis has been applied to the liver lactic dehydrogenase system. The full results will be published elsewhere.

SUMMARY

A simple graphical method for the determination of the dissociation constants for two-substrate enzyme systems has been presented.

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