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On the Determination of Rate Constants for Coenzyme Mechanisms[1]

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Investigation of the inhibition of a coenzyme reaction by its products offers the possibility of obtaining a further check on a proposed mechanism and an additional way for determining the rate constants in the mechanism. By means of experiments of this type it is possible to distinguish between the Theorell-Chance mechanism, the ternary complex mechanism, and a general type of mechanism involving four binary complexes and two ternary complexes with the interconversion of the two ternary complexes being rate determining. In addition to these mechanisms the effects of adding one product at a time is discussed for related mechanisms containing substrate or product inhibition steps. It is shown that a ternary complex may be made kinetically important even though it may not be important under the usual steady-state conditions. Since adding a product initially brings new terms into the steady state-rate law for the forward reaction it is possible to obtain the values of rate constants which do not appear in the usual steady-state rate law for the forward reaction.

The evaluation of the rate constants for individual steps in a coenzyme reaction has been beautifully illustrated by Theorell and co-workers[2-5] for the liver alcohol dehydrogenase reaction. From studies of the steady-state kinetics of the forward and reverse reactions it has proved possible to evaluate all six rate constants in the mechanism analyzed by Theorell and Chance.[3] In order to add weight to their conclusions it is desirable to check the values of the rate constants in other ways. The ratios of certain rate constants in the mechanism may be determined by spectrophotometric[1] or ultracentrifugal[6] studies of the binding of substrates by the enzyme. In addition the kinetic constants should satisfy the appropriate relation between the kinetic constants and the equilibrium constant for the over-all reaction.[7] Dalziel[8] has pointed out the relations which must exist between the kinetic constants for the forward and reverse reactions for various mechanisms.

In order to obtain further checks on the mechanism and the values of individual rate constants the effects of adding one of the products of the reaction along with the reactants may be investigated. This has the effect of introducing further terms in the steady-state rate law and may cause an intermediate normally present at a very low concentration in the steady-state to become kinetically important.

For the over-all reaction

$$A + B \rightleftarrows C + D \qquad (1)$$

the effect of D, for example, on the initial steady-state velocity of the forward reaction for an assumed mechanism may be obtained by the usual steady-state calculation, including all terms in the rate equations involving the concentrations of A, B or D and excluding those involving the concentration of C.[9] Since it is assumed that the initial concentrations of A, B and D are large compared with the initial concentration, $(E)_0$, of enzymatic sites, the steady-state velocity calculated in this way corresponds to the velocity obtained by extrapolation of experimental data to $t = 0$. In certain cases it may require very great sensitivity of the analytical method to obtain such *initial* steady-state velocities.[10]

(1) This research was supported by Grant G1585 of the National Science Foundation.

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

(3) H. Theorell and B. Chance, *ibid.*, **5**, 1127 (1951).

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

(5) K. Dalziel and H. Theorell, in preparation.

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

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

(8) K. Dalziel, *Acta Chem. Scand.*, in press.

(9) The effect of D on the steady-state velocity for the Theorell-Chance mechanism has already been given (R. A. Alberty, *Advances in Enzymology*, **17**, 1 (1956)).

(10) R. A. Alberty and B. M. Koerber, THIS JOURNAL, **79**, 6379 (1957).

As shown by Dalziel[11] it is convenient to use equations for the initial steady state velocity v expressed in the form

$$\frac{(E)_0}{v} = \phi_0 + \frac{\phi_1}{a} + \frac{\phi_2}{b} + \frac{\phi_{12}}{ab} \qquad (2)$$

where a and b represent the initial concentrations of these substrates and ϕ_0, ϕ_1, ϕ_2 and ϕ_{12} are parameters which may be obtained readily from the experimental data. For example, if the concentration of A is held constant for a series of experiments at different concentrations of B, a plot of $(E)_0/v$ *vs.* $1/b$ will be linear as shown by

$$\frac{(E)_0}{v} = \left[\phi_0 + \frac{\phi_1}{a}\right] + \frac{1}{b}\left[\phi_2 + \frac{\phi_{12}}{a}\right] \qquad (3)$$

By plotting the intercept and slope of this plot *vs.* $1/a$ all four coefficients may be obtained from intercepts and slopes. Dalziel has pointed out that for the Theorell–Chance mechanism $\phi_1\phi_2/\phi_{12} = \phi_0'$ where ϕ_0' is the constant term for the reverse reaction. Under special conditions this relation may be obeyed by the ternary complex mechanism or by more complicated mechanisms. Thus it is desirable to have further means for distinguishing between various mechanisms which all represent the initial steady-state velocities equally well. For example, the kinetic data for lactic dehydrogenase[12,13] may be represented in terms of mechanisms II and III given below. The kinetic data for yeast alcohol dehydrogenase[14] can at present[8] only be represented by mechanism III, and it is to be hoped that a simpler mechanism can be found.

The Theorell–Chance (T–C) Mechanism.[3]—

$$\begin{array}{ll} (1) & E + A \rightleftarrows EA \\ (2) & EA + B \rightleftarrows EC + D \\ (3) & EC \rightleftarrows E + C \end{array} \qquad (I)$$

The steady-state rate equations for the forward and reverse reactions are well known, and it is convenient to use the following forms where the subscripts on the rate constants refer to the step in the mechanism and a minus sign is used to designate the reverse step.

A + B + E

$$\frac{(E)_0}{v} = \frac{1}{k_3} + \frac{1}{k_1 a} + \frac{1}{k_2 b} + \frac{k_{-1}}{k_1 k_2 ab} \qquad (I, 1)$$

C + D + E

$$\frac{(E)_0}{v} = \frac{1}{k_{-1}} + \frac{1}{k_{-3}c} + \frac{1}{k_{-2}d} + \frac{k_3}{k_{-2}k_{-3}cd} \qquad (I, 2)$$

Theorell, Nygaard and Bonnichsen[4] calculated k_1, k_2 and k_3 from the initial velocities of the forward reaction and k_{-1}, k_{-2} and k_{-3} from the initial velocities of the reverse reaction, thus obtaining all six rate constants in mechanism I. Dalziel[8] has shown that by use of the coefficient of the ab term in equation I, 1 it is possible to get an additional value of k_{-1} and that by use of the coefficient of the cd term in equation I, 2 it is possible to get an additional value of k_3. This provides two checks on whether the experimental data really can be represented in terms of mechanism I. Further checks may be obtained by use of experiments in which one of the products is added along with two reactants.

A + B + C + E

$$\frac{(E)_0}{v} = \frac{1}{k_3} + \frac{1}{k_1 a}\left(1 + \frac{k_{-3}c}{k_3}\right) + \frac{1}{k_2 b} + \frac{k_{-1}}{k_1 k_2 ab}\left(1 + \frac{k_{-3}c}{k_3}\right) \qquad (I, 3)$$

Since C competes with A for free E, the terms in the rate law containing a are competitively inhibited, and the inhibition constant for C is simply the equilibrium constant for reaction 3. Thus by determining the effect of C on the initial steady-state velocity it is possible to obtain k_{-3}/k_3 and consequently a value of k_{-3} from the forward reaction, since k_3 is known.

A + B + D + E

$$\frac{(E)_0}{v} = \frac{1}{k_3} + \frac{1}{k_1 a} + \frac{1}{k_2 b}\left(1 + \frac{k_{-2}d}{k_3}\right) + \frac{k_{-1}}{k_1 k_2 ab}\left(1 + \frac{k_{-2}d}{k_3}\right) \qquad (I, 4)$$

Since the addition of D tends to reverse reaction 2 there is a competitive effect on the terms in the rate law containing b. The fact that D is inhibitory does not prove, of course, that there is an equilibrium E + D $\rightleftarrows$ ED, and the competitive inhibition constant, k_3/k_{-2}, is not an equilibrium constant. Thus by determining the effect of D on the initial velocity it is possible to obtain k_{-2}, since k_3 is known. *Thus by use of experiments of the types A + B + C + E and A + B + D + E it is possible to determine all six rate constants in mechanism I from only steady state velocity data on the forward reaction.* As a check these rate constants must be consistent with the equilibrium constant for the over-all reaction, that is, $K_{eq} = k_1k_2k_3/k_{-1}k_{-2}k_{-3}$.

C + D + A + E and C + D + B + E. The rate equations for such experiments are analogous to I, 3 and I, 4 above and may be obtained by inspection since the mechanism is symmetrical. From studies of the steady-state velocities of the reverse reaction including these two types of experiments all six rate constants in mechanism I may be calculated, thus providing an independent check on the values obtained by studies of the forward reaction.

Mechanism Involving a Single Ternary Complex.—

$$\begin{array}{ll} (1) & E + A \rightleftarrows EA \\ (2) & EA + B \rightleftarrows EXY \\ (3) & EXY \rightleftarrows EC + D \\ (4) & EC \rightleftarrows E + C \end{array} \qquad (II)$$

The steady-state rate equations for the usual types of experiments are[7]

A + B + E

$$\frac{(E)_0}{v} = \left(\frac{1}{k_3} + \frac{1}{k_4}\right) + \frac{1}{k_1 a} + \frac{(1 + k_{-2}/k_3)}{k_2 b} + \frac{k_{-1}(1 + k_{-2}/k_3)}{k_1 k_2 ab} \qquad (II, 1)$$

C + D + E

$$\frac{(E)_0}{v} = \left(\frac{1}{k_{-1}} + \frac{1}{k_{-2}}\right) + \frac{1}{k_{-4}c} + \frac{(1 + k_3/k_{-2})}{k_{-3}d} + \frac{k_4(1 + k_3/k_{-2})}{k_{-3}k_{-4}cd} \qquad (II, 2)$$

(11) K. Dalziel, *Biochem. J.*, **66**, 34P (1957).

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

(13) Y. Takemaka and G. W. Schwert, *ibid.*, **223**, 157 (1956).

(14) A. P. Nygaard and H. Theorell, *Acta Chem Scand.*, **9**, 1551 (1955).

By studying the initial steady-state velocities of the forward reaction, k_1, k_{-1} and the quantities $(1/k_3 + 1/k_4)$ and $(1 + k_{-2}/k_3)/k_2$ may be calculated. Similarly for the reverse reaction, k_4, k_{-4} and the quantities $(1/k_{-1} + 1/k_{-2})$ and $(1 + k_3/k_{-2})/k_{-3}$ may be obtained. By combining these data all eight rate constants in mechanism II may be calculated from steady-state data only.

Since the dissociation constants of EA and EC are obtained from the rate equation in exactly the same way as for the T-C mechanism, agreement between equilibrium measurements and the kinetically determined dissociation constants is to be expected for both mechanisms I and II.

As shown by Dalziel[8] if $k_{-2} >> k_{-1}$ and $k_3 >> k_4$, $\phi_1\phi_2/\phi_{12} = \phi_0'$, and $\phi_1'\phi_2'/\phi_{12}' = \phi_0$, where ϕ_0 is the reciprocal maximum velocity for the forward reaction and ϕ_0' is the reciprocal maximum velocity for the reverse reaction. Since these relations are obeyed by the rate equations for the T–C mechanism, the existence of the ternary complex would not be inferred from steady-state velocities if $k_{-2} >> k_{-1}$ and $k_3 >> k_4$. The steady-state data would obey equations I, 1 and I, 2, but the value of k_2 calculated from equation I, 1 would be equal to $k_2/(1 + k_{-2}/k_3)$ if there was indeed a ternary complex but $k_{-2} >> k_{-1}$ and $k_3 >> k_4$. However, the existence of the ternary complex could be brought out by experiments in which D is added initially.

A + B + C + E

$$\frac{(E)_0}{v} = \left(\frac{1}{k_3} + \frac{1}{k_4}\right) + \frac{1}{k_1 a}\left(1 + \frac{k_{-4}c}{k_4}\right) + \frac{(1 + k_{-2}/k_3)}{k_2 b} + \frac{k_{-1}(1 + k_{-2}/k_3)}{k_1 k_2 a b}\left(1 + \frac{k_{-4}c}{k_4}\right) \quad \text{(II, 3)}$$

Since C competes with A for free enzyme, the a terms are competitively inhibited and the inhibition constant is simply the equilibrium constant for the dissociation of EC. This equation is of the same type as I, 3 and so the addition of C initially is of no value for distinguishing between the T–C and ternary complex mechanisms.

A + B + D + E. The initial rate equation may be written in two ways, the first to show how inhibition by D enters and the second to show what combinations of the kinetic constants can actually be determined.

$$\frac{(E)_0}{v} = \frac{1}{k_3}\left(1 + \frac{k_{-3}d}{k_4}\right) + \frac{1}{k_4} + \frac{1}{k_1 a} + \frac{1}{k_2 b}\left[1 + \frac{k_{-2}}{k_3}\left(1 + \frac{k_{-3}d}{k_4}\right)\right] + \frac{k_{-1}}{k_1 k_2 a b}\left[1 + \frac{k_{-2}}{k_3}\left(1 + \frac{k_{-3}d}{k_4}\right)\right] \quad \text{(II, 4)}$$

$$\frac{(E)_0}{v} = \left(\frac{1}{k_3} + \frac{1}{k_4}\right)\left[1 + \frac{k_{-3}d}{(k_3 + k_4)}\right] + \frac{1}{k_1 a} + \frac{(1 + k_{-2}/k_3)}{k_2 b}\left[1 + \frac{k_{-2}k_{-3}d}{(k_{-2} + k_3)k_4}\right] + \frac{k_{-1}(1 + k_{-2}/k_3)}{k_1 k_2 a b}\left[1 + \frac{k_{-2}k_{-3}d}{(k_{-2} + k_3)k_4}\right] \quad \text{(II, 5)}$$

The first form shows that D exerts a competitive effect on the terms containing k_3. This is a result of the fact that D tends to reverse this reaction, and consequently the inhibition constant is not an equilibrium constant. However, because of the form of equation II, 4, the inhibition constant k_{-3}/k_4 cannot be calculated from the experimental data, but only $k_{-3}/(k_3 + k_4)$ and $k_{-2}k_{-3}/(k_{-2} + k_3)k_4$ as shown by equation II, 5. It is of interest to note that D affects the maximum velocity which is approached as the concentrations of a and b are increased indefinitely.

Since equation II, 5 is of a different form from equation I, 4 it should be possible to differentiate between mechanisms I and II by experiments in which one of the products is added initially. This distinction can be made even when $k_{-2} >> k_{-1}$ and $k_3 >> k_4$ so that the relationships between the coefficients for the forward and reverse reactions are the same for the two mechanisms.

Since as pointed out above, the 8 rate constants in mechanism II can be calculated from A + B + E and C + D + E experiments, the effect of adding either one of the products could be predicted in advance.

C + D + A + E and C + D + B + E. The rate equations for such experiments are analogous to II, 3 and II, 4 above and may be obtained by inspection since mechanism II is symmetrical.

The rate equations for the corresponding mechanism with two ternary intermediates could readily be written down, but there seems to be no profit in this since such a mechanism cannot be distinguished from the present one in the absence of means for actually detecting the two different ternary complexes.

Mechanism Involving Four Binary and Two Ternary Complexes.—

(1)	$E + A \rightleftarrows EA$	(6)	$ECD \rightleftarrows EC + D$	
(2)	$E + B \rightleftarrows EB$	(7)	$ECD \rightleftarrows ED + C$	
(3)	$EA + B \rightleftarrows EAB$	(8)	$EC \rightleftarrows E + C$	(III)
(4)	$EB + A \rightleftarrows EAB$	(9)	$ED \rightleftarrows E + D$	
(5)	$EAB \rightleftarrows ECD$			

The simpler mechanism involving only one ternary complex rather than two is already sufficiently complicated that it does not appear useful to write out the steady-state rate equations. These terms may be written out readily using the schematic method of King and Altman,[15] and their probability considerations show that the denominator of the steady state rate law for mechanism III will be a sum of 336 terms. The steady-state rate equation for the special case of mechanism III that the products dissociate very rapidly has been derived.[16,17] This steady-state rate equation contains 192 terms in the denominator and shows some unusual kinetic consequences.[17]

Mechanism III provides for the possibility that one ternary complex may be converted into the other slowly, and if this process is sufficiently slow the rate equation may be derived on the assumption that steps 1–4 and 6–9 remain in equilibrium. When this is true the rate equation is of the same type as for the preceding mechanism. Dissociation constants are represented by capital K's.

A + B + E

$$\frac{(E)_0}{v} = \frac{1}{k_5} + \frac{K_4}{k_5 a} + \frac{K_3}{k_5 b} + \frac{K_1 K_3}{k_5 a b} \quad \text{(III, 1)}$$

C + D + E

$$\frac{(E)_0}{v} = \frac{1}{k_{-5}} + \frac{K_7}{k_{-5}c} + \frac{K_6}{k_{-5}d} + \frac{K_7 K_9}{k_{-5}d} \quad \text{(III, 2)}$$

(15) E. L. King and C. Altman, *J. Phys. Chem.*, **60**, 1375 (1956).
(16) L. I. Ingraham and B. Makower, *ibid.*, **58**, 266 (1954).
(17) E. L. King, *ibid.*, **60**, 1378 (1956).

From these types of experiments k_5, k_{-5} and the eight equilibrium constants may be calculated since $K_1K_3 = K_2K_4$ and $K_6K_8 = K_7K_9$.

A + B + C + E

$$\frac{(E)_0}{v} = \frac{1}{k_5} + \frac{K_4}{k_5 a} + \frac{K_3}{k_5 b} + \frac{K_1K_3}{k_5 ab}\left(1 + \frac{c}{K_8}\right) \quad \text{(III, 3)}$$

The inhibitory effect of added product is restricted to the ab term. This is the term which predominates at low concentrations of a and b where the enzyme is mainly in the uncombined form and there is competition between A, B and C. Consequently the effect of adding C is different from that for the preceding two mechanisms. Such experiments would provide a check on the value of K_8 obtained from C + D + E experiments.

A + B + D + E

$$\frac{(E)_0}{v} = \frac{1}{k_5} + \frac{K_4}{k_5 a} + \frac{K_3}{k_5 b} + \frac{K_1K_3}{k_5 ab}\left(1 + \frac{d}{K_9}\right) \quad \text{(III, 4)}$$

The effect of D is of the same type as C because the mechanism is symmetrical in C and D. *The effect of D on the initial velocity is therefore distinctly different for mechanisms I, II and III (rapid equilibrium case).*

Inhibition by Product or Substrate

At higher substrate concentrations further terms in the rate law may become significant, and this has actually been observed. Theorell, Nygaard and Bonnichsen[4] observed that ethanol concentrations above 10 mM caused inhibition of liver alcohol dehydrogenase. Hakala, Glaid and Schwert[12] found inhibition of lactic dehydrogenase by pyruvate, but not by lactate. These observations, but not the activation of yeast alcohol dehydrogenase by high concentration of DPN and alcohol,[18] may be explained by the combination of a substrate, say alcohol, with the enzymatic site in such a way as to prevent the binding of the other substrate, say DPN. The mutually exclusive binding of both products by the enzyme is provided for by the following mechanism.

TC Mechanism with Product Inhibition.—

$$\begin{array}{ll} (1) & E + A \rightleftarrows EA \\ (2) & EA + B \rightleftarrows EC + D \\ (3) & EC \rightleftarrows E + C \\ (4) & ED \rightleftarrows E + D \end{array} \quad \text{(IV)}$$

A + B + E and A + B + C + E. The equations for the initial steady-state rates are exactly the same as when the last step is not present (see equations I, 1 and I, 3).

A + B + D + E

$$\frac{(E)_0}{v} = \frac{1}{k_3} + \frac{1}{k_{1a}}\left(1 + \frac{k_{-4}d}{k_4}\right) + \frac{1}{k_2 b}\left(1 + \frac{k_{-2}d}{k_3}\right) + \frac{k_{-1}}{k_1k_2ab}\left(1 + \frac{k_{-4}d}{k_4}\right)\left(1 + \frac{k_2 d}{k_3}\right) \quad \text{(IV, 1)}$$

In addition to the effect of D in mechanism I the terms containing a are now multiplied by a factor $(1 + k_{-4}d/k_4)$ since D competes with A for free enzyme. As a result the ab term will show a more complicated dependence on d. The binding constant of D on the free enzyme will be most easily obtained by determining the effect of d on the intercept of a plot of $(E)_0/v$ *vs.* $1/b$. The existence of this type of mechanism may of course be inferred from studies of the reverse reaction (see mechanism VI), but experiments of the present type may be simpler to interpret.

Ternary Complex Mechanism with Product Inhibition.—

$$\begin{array}{lll} (1) & E + A \rightleftarrows EA & (4)\ EC \rightleftarrows E + C \\ (2) & EA + B \rightleftarrows EXY & (5)\ ED \rightleftarrows E + D \\ (3) & EXY \rightleftarrows EC + D & \end{array} \quad \text{(V)}$$

A + B + E and A + B + C + E. The equations for the initial steady-state rates are exactly the same as when the last step is not present (see equations II, 1 and II, 3).

A + B + D + E. Using the form of equation II, 5 the initial steady state-rate equation is

$$\frac{(E)_0}{v} = \left(\frac{1}{k_3} + \frac{1}{k_4}\right)\left[1 + \frac{k_{-3}d}{(k_3 + k_4)}\right] + \frac{1}{k_1 a}\left(1 + \frac{k_{-5}d}{k_5}\right) + \frac{(1 + k_{-2}/k_3)}{k_2 b}\left[1 + \frac{k_{-2}k_{-3}d}{(k_{-2} + k_3)k_4}\right] + \frac{k_{-1}(1 + k_{-2}/k_3)}{k_1k_2ab}\left[1 + \frac{k_{-2}k_{-3}d}{(k_{-2} + k_3)k_4}\right]\left[1 + \frac{k_{-5}d}{k_5}\right] \quad \text{(V, 1)}$$

Since D competes with A for the free enzyme, as well as displacing equilibrium 3, the terms containing a are inhibited by the factor $(1 + k_{-5}d/k_5)$. This rate equation is readily distinguishable from IV, 1 because of the effect of D on the maximum velocity. Thus the ternary complex mechanism with product inhibition readily may be distinguished from the T–C mechanism with product inhibition.

T–C Mechanism with Substrate Inhibition.—

$$\begin{array}{ll} (1) & E + A \rightleftarrows EA \\ (2) & E + B \rightleftarrows EB \\ (3) & EA + B \rightleftarrows EC + D \\ (4) & EC \rightleftarrows E + C \end{array} \quad \text{(VI)}$$

This mechanism is of course just the reverse of mechanism IV, but it is convenient to rewrite it in this way so that rate equations for A + B reactions may be compared more directly.

A + B + E

$$\frac{(E)_0}{v} = \frac{1}{k_4} + \frac{1}{k_1 a}\left(1 + \frac{k_{-1}k_2}{k_{-2}k_3}\right) + \frac{1}{k_3 b} + \frac{k_{-1}}{k_1k_3ab} + \frac{k_2 b}{k_1k_{-2}a} \quad \text{(VI, 1)}$$

This rate equation, which is given by Dalziel,[8] contains a b/a term in addition to constant $1/a$, $1/b$, and $1/ab$ terms. It may alternatively be written in the form

$$\frac{(E)_0}{v} = \left(\frac{1}{k_4} + \frac{1}{k_3 b}\right) + \frac{1}{k_1 a}\left(1 + \frac{k_2 b}{k_{-2}}\right)\left(1 + \frac{k_{-1}}{k_3 b}\right) \quad \text{(VI, 2)}$$

Thus by plotting $(E)_0/v$ *vs.* $1/a$, the rate constants k_3 and k_4 may be calculated from the effect of b on the intercept. If $k_2b/k_{-2} << 1$, the equation reduces to that for the T–C mechanism. If $k_{-1}/k_3b << 1$, the binding constant for B at the enzymatic site readily can be determined. However, if k_{-2}/k_2 is of the same magnitude as k_{-1}/k_3, there will not be a range of B concentrations where k_{-2}/k_2 or k_{-1}/k_3 may be calculated from such simple plots. The combinations of rate constants which can be determined in this case are indicated by re-

(18) A. P. Nygaard and H. Theorell, *Acta Chem. Scand.*, **9**, 1300 (1955).

writing the rate equation in the form

$$\frac{(E)_0}{v} = \left(\frac{1}{k_4} + \frac{1}{k_3 b}\right) + \frac{(1 + k_{-1}k_2/k_{-2}k_3)}{k_1 a}\left[1 + \frac{k_{-1}}{k_3(1 + k_{-1}k_2/k_{-2}k_3)b} + \frac{k_2 b}{k_{-2}(1 + k_{-1}k_2/k_{-2}k_3)}\right] \quad \text{(VI, 3)}$$

Since the slope of the plot of $(E)_0/v$ *vs.* $1/a$ has the same form as the equation for the effect of hydrogen ion concentration on the reciprocal maximum velocity for a simple mechanism, the same method[19] may be employed for the calculation of the constants. If the reciprocal of the slope is plotted *vs.* $-\log b$, a symmetrical bell-shaped curve should be obtained. The values of $(1 + k_{-1}k_2/k_{-2}k_3)/k_1$, $k_{-1}/k_3(1 + k_{-1}k_2/k_{-2}k_3)$ and $k_2/k_{-2}(1 + k_{-1}k_2/k_{-2}k_3)$ may be obtained and from them k_{-1}/k_1k_3 and k_2/k_1k_{-2} calculated. Since k_3 is known from the intercept and k_{-1} may be obtained from studies of the reverse reaction, and the values of k_1 and k_2/k_{-2} may be calculated. From the steady-state kinetics of the reverse reaction the values of k_{-1}, k_{-3}, k_4, k_{-4} and k_2/k_{-2} may be calculated so that check values are obtained for k_4 and k_2/k_{-2}.

A + B + C + E

$$\frac{(E)_0}{v} = \frac{1}{k_4} + \frac{1}{k_1 a}\left(1 + \frac{k_{-1}k_2}{k_{-2}k_3} + \frac{k_{-4}c}{k_4}\right) + \frac{1}{k_3 b} + \frac{k_{-1}}{k_1k_3ab}\left(1 + \frac{k_{-4}c}{k_4}\right) + \frac{k_2 b}{k_1k_{-2}a} \quad \text{(VI, 4)}$$

Although C competes with both A and B for free enzymatic sites, the $1/b$ term is not inhibited since it arises from the third step in the mechanism which is unaffected by C. The velocity at high A concentrations is unaffected by C, and the $1/ab$ term is affected by a simple competitive factor. There is a competitive effect on the $1/a$ term, but one with a different competitive inhibition constant.

A + B + D + E

$$\frac{(E)_0}{v} = \frac{1}{k_4} + \frac{1}{k_1 a}\left[1 + \frac{k_{-1}k_2}{k_{-2}k_3}\left(1 + \frac{k_{-3}d}{k_4}\right)\right] + \frac{1}{k_3 b}\left(1 + \frac{k_{-3}d}{k_4}\right) + \frac{k_{-1}}{k_1k_3ab}\left(1 + \frac{k_{-3}d}{k_4}\right) + \frac{k_2 b}{k_1k_{-2}a} \quad \text{(VI, 5)}$$

Since D tends to reverse the reaction with rate constant k_3, the terms in the rate law containing k_3 are inhibited by the factor $(1 + k_{-3}d/k_4)$. In contrast with mechanism I, the coefficient of the $1/a$ term is affected. By studying the effect of D on the $1/b$ term, the ratio k_{-3}/k_4 is readily determined and k_{-3} calculated since k_4 is known from the maximum velocity.

Ternary Complex Mechanism with Substrate Inhibition.—

$$\begin{array}{ll} (1) & E + A \rightleftarrows EA \\ (2) & E + B \rightleftarrows EB \\ (3) & EA + B \rightleftarrows EXY \\ (4) & EXY \rightleftarrows EC + D \\ (5) & EC \rightleftarrows E + C \end{array} \quad \text{(VII)}$$

This mechanism is of course just the reverse of mechanism V, but it is convenient to rewrite it in this way so that rate equations for A + B reactions may be more directly compared.

(19) R. A. Alberty and V. Massey, *Biochem. Biophys. Acta*, **13**, 347 (1954).

A + B + E

$$\frac{(E)_0}{v} = \left(\frac{1}{k_4} + \frac{1}{k_5}\right) + \frac{1}{k_1 a}\left[1 + \frac{k_{-1}k_2}{k_{-2}k_3}\left(1 + \frac{k_{-3}}{k_4}\right)\right] + \frac{1}{k_3 b}\left(1 + \frac{k_{-3}}{k_4}\right) + \frac{k_{-1}}{k_1k_3ab}\left(1 + \frac{k_{-3}}{k_4}\right) + \frac{k_2 b}{k_1k_{-2}a} \quad \text{(VII, 1)}$$

The general form of this equation is the same as VI, 1, but it is possible to distinguish between these mechanisms by comparing the quantities determined in A + B + E experiments with those determined in C + D + E experiments. The existence of the b/a term may make the calculation of rate constants rather difficult as discussed in connection with the preceding mechanism. It will be noted that since EXY can break down in two ways there is a $(1 + k_{-3}/k_4)$ factor every place there is a k_3.

A + B + C + E

$$\frac{(E)_0}{v} = \left(\frac{1}{k_4} + \frac{1}{k_5}\right) + \frac{1}{k_1 a}\left[1 + \frac{k_{-1}k_2}{k_{-2}k_3}\left(1 + \frac{k_{-3}}{k_4}\right) + \frac{k_{-5}c}{k_5}\right] + \frac{1}{k_3 b}\left(1 + \frac{k_{-3}}{k_4}\right) + \frac{k_{-1}}{k_1k_3ab}\left(1 + \frac{k_{-3}}{k_4}\right)\left(1 + \frac{k_5 c}{k_{-5}}\right) + \frac{k_2 b}{k_1k_{-2}a} \quad \text{(VII, 2)}$$

Although C competes with both A and B for free enzymatic sites, the $1/b$ term is not inhibited since it arises from the third and fourth steps in the mechanism which are not affected by C. The competitive effects on the $1/a$ and $1/ab$ terms yield different competitive inhibition constants. The effect of added C is of the same type as for mechanism VI.

A + B + D + E

$$\frac{(E)_0}{v} = \left[\frac{1}{k_4}\left(1 + \frac{k_{-4}d}{k_5}\right) + \frac{1}{k_5}\right] + \frac{1}{k_1 a}\left\{1 + \frac{k_{-1}k_2}{k_{-2}k_3}\left[1 + \frac{k_{-3}}{k_4}\left(1 + \frac{k_{-4}d}{k_5}\right)\right]\right\} + \frac{1}{k_3 b}\left[1 + \frac{k_{-3}}{k_4}\left(1 + \frac{k_{-4}d}{k_5}\right)\right] + \frac{k_{-1}}{k_1k_3ab}\left[1 + \frac{k_{-3}}{k_4}\left(1 + \frac{k_{-4}d}{k_5}\right)\right] + \frac{k_2 b}{k_1k_{-2}a} \quad \text{(VII, 3)}$$

The addition of D affects all the terms in k_4 since the net rate of dissociation of EXY to give EC + D is reduced according to the apparent inhibition constant k_5/k_{-4}. Since the term which is independent of a and b is affected by added D, the ternary complex mechanism with substrate inhibition is distinguishable from the T-C mechanism with substrate inhibition.

T-C Mechanism with Substrate and Product Inhibition.—

$$\begin{array}{ll} (1) & E + A \rightleftarrows EA \\ (2) & E + B \rightleftarrows EB \\ (3) & EA + B \rightleftarrows EC + D \\ (4) & EC \rightleftarrows E + C \\ (5) & ED \rightleftarrows E + D \end{array} \quad \text{(VIII)}$$

A + B + E and A + B + C + E. The equations for the initial steady-state rates are exactly the same as when the last step is not present (see equations VI, 1 and VI, 4).

A + B + D + E

$$\frac{(E)_0}{v} = \frac{1}{k_4} + \frac{1}{k_1 a}\left[1 + \frac{k_{-1}k_2}{k_{-2}k_3}\left(1 + \frac{k_{-3}d}{k_4}\right) + \frac{k_{-5}d}{k_5}\right] + \frac{1}{k_3 b}\left(1 + \frac{k_{-3}d}{k_4}\right) + \frac{k_{-1}}{k_1 k_3 a b}\left(1 + \frac{k_{-5}d}{k_5}\right)\left(1 + \frac{k_{-3}d}{k_4}\right) + \frac{k_2 b}{k_1 k_{-2} a} \quad \text{(VIII, 1)}$$

There is much in common between the effect of D here and in mechanism IV. The effect of D on the $1/ab$ term is complicated, but in the limit of high a concentrations a simple competitive effect is obtained on the $1/b$ term. From studies of the forward reaction alone values of k_1, k_{-1}, k_3, k_{-3}, k_4, k_{-4}, k_{-2}/k_2 and k_5/k_{-5} may be obtained. The corresponding equation for the reverse reaction may be written down by analogy since the mechanism is symmetrical, and it is found that the same quantities can be determined independently from the reverse reaction.

Ternary Complex Mechanism with Substrate and Product Inhibition.—

$$\begin{aligned} &(1)\quad E + A \rightleftarrows EA \\ &(2)\quad E + B \rightleftarrows EB \\ &(3)\quad EA + B \rightleftarrows EXY \\ &(4)\quad EXY \rightleftarrows EC + D \\ &(5)\quad EC \rightleftarrows E + C \\ &(6)\quad ED \rightleftarrows E + D \end{aligned} \qquad \text{(IX)}$$

A + B + E and A + B + C + E. The equations for the initial steady-state rates are exactly the same as when the last step is not present (see equations VII, 1 and VII, 2).

A + B + D + E

$$\frac{(E)_0}{v} = \left[\frac{1}{k_4}\left(1 + \frac{k_{-4}d}{k_5}\right) + \frac{1}{k_5}\right] + \frac{1}{k_1 a}\left\{1 + \frac{k_{-1}k_2}{k_{-2}k_3}\left[1 + \frac{k_{-3}}{k_4}\left(1 + \frac{k_{-4}d}{k_5}\right)\right] + \frac{k_{-6}d}{k_6}\right\} + \frac{1}{k_3 b}\left[1 + \frac{k_{-3}}{k_4}\left(1 + \frac{k_{-4}d}{k_4}\right)\right] + \frac{k_{-1}}{k_1 k_3 a b}\left[1 + \frac{k_{-3}}{k_4}\left(1 + \frac{k_{-4}d}{k_4}\right)\right]\left(1 + \frac{k_{-6}d}{k_6}\right) + \frac{k_2 b}{k_1 k_{-2} a} \quad \text{(IX, 1)}$$

The effect of D on the kinetics is rather complicated and corresponds to a combination of the effects shown by equations V, 1 and VII, 3. Since again the maximum velocity is affected by D, this mechanism readily may be distinguished from VIII. The corresponding equation for the reverse reaction may be written down by analogy because of the symmetry of the mechanism.

Discussion

There are four steady state kinetic methods for distinguishing between various mechanisms for coenzyme reactions: (a) the form of the initial rate equation, *i.e.*, the orders of the substrate concentration terms, (b) the relationship between certain kinetic parameters in the rate equations for the forward and reverse reactions,[8] (c) the relationship between the kinetic parameters and the equilibrium constant for the over-all reaction[7] and (d) the effect of added product on the initial steady-state rate. None of these methods alone can distinguish between all of the mechanisms for coenzyme reactions which have been considered of interest in the recent past. However, when these methods are used together it is possible to distinguish between quite a number of possibilities and to verify the values of individual rate constants in a sufficient number of ways that we may have considerable confidence in them.

For enzymatic reactions following mechanism I or II there is the problem of determining which substrate combines first with the enzymatic site since the rate law alone does not tell. For liver alcohol dehydrogenase[3] and lactic dehydrogenase[20] the order has been established by direct spectrophotometric studies of the complexes with the coenzymes, Since for mechanisms I and II the effects of C and D are different, it is possible to determine the roles of these substances in the mechanism. In the case of mechanism I it would be necessary to know which substrate combines first in order to decide which product combines with the enzymatic site first, but in the case of mechanism II the effect of D on the maximum velocity of the forward reaction would show that it is the substrate which combines second in the reverse reaction. It is to be noted that for mechanisms I, II and III, the dissociation constant for the first substrate is always calculated from the kinetic data in the same way. Frieden[21] has described a graphical method for this calculation.

When a product is added initially the amount of reactant converted in reaching equilibrium is reduced and consequently the steady-state velocity must be determined during a smaller extent of reaction. The fact that the fluorimetric method[22] for DPNH is so sensitive permits considerable optimism concerning studies of reactions involving this coenzyme.

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