

## Initial Steady State Velocities in the Evaluation of Enzyme-Coenzyme-Substrate Reaction Mechanisms

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The initial rate equations for a number of possible reaction sequences for enzyme-coenzyme-substrate reactions deduced by the steady state treatment are compared and contrasted.

For certain mechanisms the initial rate equations may be considered to be derived from a common equation which is most conveniently and simply expressed in terms of four kinetic coefficients. In this equation the reciprocal rate is linearly related to the reciprocal of each substrate concentration. A method for determining the kinetic coefficients from initial rate measurements, as slopes and intercepts of double plots, is described.

The distinctive Haldane relations between kinetic and equilibrium constants which exist for certain mechanisms<sup>5</sup> are a consequence of differences between the initial rate equations: either there are distinctive relations between the kinetic coefficients or certain of the coefficients are equal to zero. These mechanisms can sometimes be rejected on the basis of initial rate data alone, with greater certainty than by the Haldane relations.

Certain more complex mechanisms are considered which make provision for deviations from the linear reciprocal form of initial rate equation at high concentrations of one substrate. Substrate inhibition of various types may be distinguished experimentally, and a possible explanation of substrate activation is also considered.

From initial overall velocity measurements in the steady state, Theorell, Nygaard and Bonnichsen<sup>1</sup> were able to calculate all six rate constants in the mechanism proposed by Theorell and Chance<sup>2</sup> for the liver alcohol dehydrogenase reaction. There was reasonably good agreement between two of the rate constants so obtained and values determined in single reaction steps<sup>2</sup>, and also between the equilibrium constant for the overall reaction calculated from the six rate constants and the value obtained from equilibrium measurements<sup>3</sup>.

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The importance of such comparison of kinetic and equilibrium data as a test of the suitability of a postulated reaction sequence<sup>4</sup> was emphasized by Alberty<sup>5</sup>, who discussed a number of possible mechanisms for enzyme-coenzyme-substrate reactions, and showed that for some the Haldane relation between the overall equilibrium constant and initial rate parameters (maximum rate and Michaelis constants) assumes a distinctive form. Theorell *et al.*<sup>3</sup> and Nygaard and Theorell<sup>6</sup> tested their data for liver and yeast alcohol dehydrogenases by means of these relations, and found the Theorell-Chance mechanism to be satisfactory for the liver enzyme but not for the yeast enzyme, and that certain other reaction sequences could be rejected in both cases.

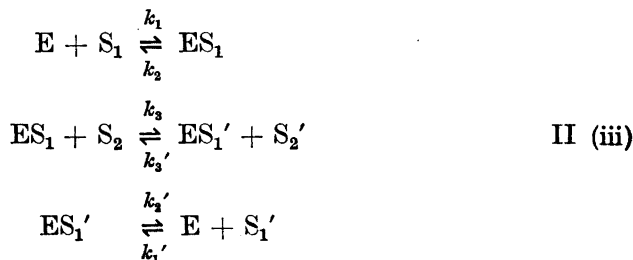
Further kinetic studies of liver alcohol dehydrogenase<sup>7,8</sup> have revealed deviations from the Theorell-Chance mechanism under certain conditions. It is the purpose of the present paper to describe in general terms the theoretical basis and design of those experiments, to extend the discussion by Alberty<sup>5</sup> of possible mechanisms for enzyme-coenzyme-substrate reactions and of the manner in which initial rate data may be used to distinguish them, and to consider more complex reaction sequences which might account for substrate inhibition. The distinctive Haldane relations deduced by Alberty result from differences between the initial rate equations for certain mechanisms, and initial rate data alone can be used, with advantage, to distinguish these alternatives. It is of course essential that the appropriate Haldane relation also be fulfilled by the data if a particular reaction sequence is to be accepted as a satisfactory hypothesis. The relations are simplified by an alternative formulation of the initial rate equations, analogous to that of Lineweaver and Burk<sup>9</sup> for single-substrate reactions, but not employing Michaelis constants.

#### THE STEADY STATE METHOD

The steady state method<sup>10</sup> of deriving the relation between the initial rate, the total enzyme concentration and the initial substrate concentrations is preferable to the more restrictive equilibrium method<sup>11</sup> and except for one mechanism has been used throughout this paper. The assumptions involved are (i) that the enzyme concentration is small compared with the substrate concentrations (ii) that the rate of change of concentration of all enzyme-substrate complexes is zero. For initial rates the product concentrations are set equal to zero, a proceeding which has been widely adopted<sup>4,5</sup> if not theoretically justified<sup>12</sup>. To the succinct description of the steady state method given by Alberty<sup>5</sup> it needs only be added that in deducing the initial rate equation it is simpler to disregard all reactions involving products in setting up the rate equations for the enzyme-substrate complexes than to obtain the general rate equation and then set the product concentrations equal to zero. For single-substrate reactions, the deduction of the initial rate equation is equivalent to using the Michaelis-Menten formulation,  $E + S \rightleftharpoons EX \rightarrow E + S'$ , ignoring the reverse of the second step. For reactions involving a coenzyme and a substrate, however, it is not desirable to group together all steps after the formation of the ternary complex as a single step<sup>13</sup> as the rates of breakdown of binary enzyme-product complexes enter into the steady state expression for the initial rate, and in some cases can be evaluated from initial rate data.

## THE THEORELL-CHANCE MECHANISM

Theorell and Chance<sup>2</sup> proposed that in the liver alcohol dehydrogenase reaction, binary compounds of enzyme and coenzyme (diphosphopyridine nucleotide) react with the substrate (alcohol or acetaldehyde) without the intervention of a rate-limiting ternary complex. If the overall reaction of coenzyme and substrate is represented in general by  $S_1 + S_2 \rightleftharpoons S_1' + S_2'$ , the Theorell-Chance mechanism may be written:



Both the coenzyme,  $S_1$ , and the substrate,  $S_2$ , will be referred to as substrates. Steady state treatment<sup>2</sup> yields the following relations, for the forward and reverse reactions respectively, between the total enzyme concentration  $e$ , the initial rate  $v_0$ , and the initial substrate concentrations:

$$\frac{e}{v_0} = \frac{1}{k_2'} + \frac{1}{k_1[S_1]} + \frac{1}{k_3[S_2]} + \frac{k_2}{k_1 k_3 [S_1][S_2]} \quad \text{1(a)}$$

$$\frac{e}{v_0'} = \frac{1}{k_2} + \frac{1}{k_1'[S_1']} + \frac{1}{k_3'[S_2']} + \frac{k_2'}{k_1' k_3' [S_1'][S_2']} \quad \text{1(b)}$$

Because of the symmetry of the mechanism and of the numbering of the rate constants, the forward and reverse initial rate equations 1(a) and 1(b) are interchangeable by the insertion or deletion of primes on the rate constants, and this will be true throughout. The reciprocal initial rate is linearly related to the reciprocal concentration of each substrate, so that if either substrate concentration is varied whilst the other is kept constant, plots according to Lineweaver and Burk<sup>9</sup> should be linear. If each substrate concentration is varied in turn whilst the other is kept at a sufficiently high level, the last term and either the second or third terms of the above equations will be negligible, and therefore all 6 rate constants could be determined from the slopes and intercepts of four Lineweaver-Burk plots for the forward and reverse reactions. This was in principle the method used by Theorell *et al.*<sup>1</sup>, but because a sufficiently great concentration of the constant partner could not be used in every case, some corrections were necessary. Moreover, elimination of the last terms of 1(a) and 1(b) neglects the important relation between the last three terms of each equation and the first term of the other.

The initial rate eqns. (1) may be written in a general form:

$$\frac{e}{v_0} = \Phi_0 + \frac{\Phi_1}{[S_1]} + \frac{\Phi_2}{[S_2]} + \frac{\Phi_{12}}{[S_1][S_2]} \quad (2)$$

for the forward reaction; for the reverse reaction, the coefficients will be distinguished by primes. Comparison of eqns. (1) and (2) shows that there are complementary relationships between the kinetic coefficients of the forward and reverse reactions:

$$\Phi_0' = \Phi_1\Phi_2/\Phi_{12} \quad 3(a)$$

$$\Phi_0 = \Phi_1'\Phi_2'/\Phi_{12}' \quad 3(b)$$

These relations distinguish the Theorell-Chance mechanism from others which conform to (2). To test them, all 4 coefficients for the forward and reverse reaction must be determined; the determination of  $\Phi_{12}$  and  $\Phi_{12}'$  requires initial rate measurements with low concentrations of both substrates.

It can be shown that the overall equilibrium constant is equal to two functions of the kinetic coefficients (Haldane relations):

$$K_{\text{Eq}} = \Phi_{12}'/\Phi_{12} \quad (4)$$

$$K_{\text{Eq}} = \Phi_0'\Phi_1'\Phi_2'/\Phi_0\Phi_1\Phi_2 \quad (5)$$

Other mechanisms which conform to (2) also conform to (4), but (5) is distinctive for the Theorell-Chance mechanism (*cf.* Alberty<sup>5</sup>, eqn. 9) and is a consequence of relations 3(a) and 3(b) between the coefficients. If experimental data conform to (4) but not to (5), either 3(a) or 3(b), or both, will not be satisfied. These latter relations are a more sensitive test than (5) because they involve fewer experimental quantities, do not involve separate equilibrium measurements, and are independent of one another, whereas (5) is a resultant of both of them.

#### GENERAL FORM OF THE INITIAL RATE EQUATION

It will be seen that the initial rate equations for a number of other mechanisms may also be considered as special cases of eqn. (2), and that certain of these mechanisms may be distinguished by the fact that one or more of the kinetic coefficients is equal to zero, or, as in the case of the Theorell-Chance mechanism, by a relation between the coefficients.

Initial rate equations are usually, but not always<sup>13</sup>, formulated in terms of Michaelis constants, defined as the concentration of the substrate which gives half the maximum rate approached asymptotically with increase of substrate concentration<sup>4</sup>. With this definition, the steady state treatment<sup>10,4</sup> of single-substrate reaction mechanisms in which one or two intermediate enzyme-substrate compounds are postulated gives the same initial rate equation as the equilibrium treatment, *viz.* the Michaelis-Menten equation. For two-substrate reactions, the Michaelis constant for each substrate varies with the concentration of the other<sup>4,2</sup>. Alberty<sup>5</sup> defines the Michaelis constant in this case as the concentration which gives half the maximum rate approached with increase of *both* substrate concentrations, and obtains by steady state treatment of several possible mechanisms an initial rate equation analogous to that of Michaelis and Menten<sup>11</sup>:

$$v_0 = \frac{V}{1 + \frac{K_1}{[S_1]} + \frac{K_2}{[S_2]} + \frac{K_{12}}{[S_1][S_2]}} \quad (6)$$

$V$  is the maximum rate and  $K_1$  and  $K_2$  are the Michaelis constants for  $S_1$  and  $S_2$ .  $K_{12}$  is a new kinetic constant<sup>5</sup>. This equation is equivalent to eqn. (2).

The Michaelis constant has its original theoretical significance as the dissociation constant of a compound of the substrate with the enzyme only when the relative reaction rates are such that the compound can be assumed to be in equilibrium throughout with the free substrate and enzyme. This is true for two-substrate reactions also, the dissociation in this case being from the ternary complex<sup>13</sup> (Mechanism I below). With the less restrictive steady state assumption, the Michaelis constant has no obvious physical significance. For a single-substrate reaction in which one intermediate enzyme complex is postulated, it is still a simple function of rate constants relating to the substrate and its enzyme compound<sup>10</sup>. When more than one intermediate enzyme compound is postulated<sup>4</sup> and for two-substrate reaction mechanisms in general<sup>5</sup>, the Michaelis constant of a substrate is a more or less complex function of several rate constants, some of which relate to reaction steps not involving either the free or bound substrate<sup>2,14</sup>. In the initial rate equation for one mechanism discussed by Alberty, and considered to be a special case of (6),  $V$ ,  $K_1$ , and  $K_2$  do not conform to the definitions given above, and this seems to have led to an erroneous formulation of the kinetic expression for the equilibrium constant (Alberty<sup>5</sup>: mechanism I (6), eqns. (11) and (12)).

Equation (2) is analogous to that of Lineweaver and Burk<sup>9</sup> for single-substrate reactions, which could be written  $e/v_0 = \Phi_0 + (\Phi_1/[S_1])$ . The coefficients are simpler functions of the rate constants than are Michaelis constants, and can be determined as slopes and intercepts by an extension of the Lineweaver-Burk plot (*vide infra*).  $\Phi_0$  has the dimension of a reciprocal first order rate constant, and is equal to the reciprocal of the maximum rate with unit enzyme concentration, or the reciprocal of the maximum turnover number.  $\Phi_1$  and  $\Phi_2$  are equivalent to reciprocal second-order rate constants, and are related to the Michaelis constants, as defined by Alberty<sup>5</sup>, by  $\Phi_1/\Phi_0 = K_1$  and  $\Phi_2/\Phi_0 = K_2$ .  $\Phi_{12}$  has the dimension of a third order rate constant, and  $\Phi_{12}/\Phi_0 = K_{12}$  in Alberty's equation.

#### EXPERIMENTAL DETERMINATION OF THE KINETIC COEFFICIENTS

Rearrangement of eqn. (2) gives:

$$\frac{e}{v_0} = \Phi_0 + \frac{\Phi_2}{[S_2]} + \left( \Phi_1 + \frac{\Phi_{12}}{[S_2]} \right) \frac{1}{[S_1]}$$

If a series of initial rate measurements is made with a constant initial concentration of  $S_2$  (not necessarily large) and different concentrations of  $S_1$ , a plot<sup>9</sup> of  $e/v_0$  against  $1/[S_1]$  should give a straight line, with both slope and intercept linear functions of  $1/[S_2]$ . If several such series of initial rate measurements are made, each with a different constant value of  $[S_2]$ , the slopes and intercepts of the primary plots may be plotted against  $1/[S_2]$ , to give the 4 coefficients directly as slopes and intercepts, thus:

Secondary plots of intercepts: slope =  $\Phi_2$ , intercept =  $\Phi_0$ .

Secondary plots of slopes: slope =  $\Phi_{12}$ , intercept =  $\Phi_1$ .

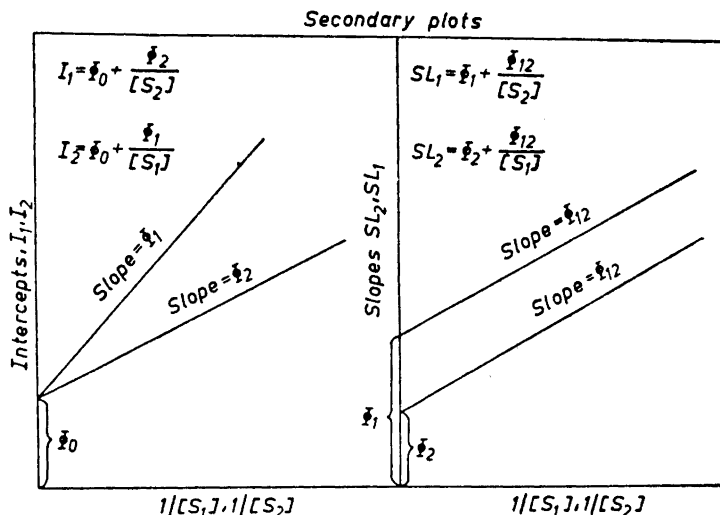


Fig. 1. Illustration of the determination of the kinetic coefficients of eqn. (2) by secondary plots of slopes and intercepts of primary Lineweaver-Burk plots.

The initial rate eqn. (2) is symmetrical in  $[S_1]$  and  $[S_2]$ , and  $[S_2]$  could equally well be chosen as the variable in the primary plots, and  $[S_1]$  as the variable in the secondary plots. If say 5 different concentrations of both substrates are used, the 25 initial rate measurements may be plotted in both ways, thereby grouping them in different sets of 5 for the primary plots. Two sets of values for the 4 coefficients will be obtained from the secondary plots, as illustrated in Fig. 1.

This procedure seems to make the best use of the data, and is a generalisation of those described by Schwert and Hakala<sup>15</sup>, Hakala, Glaid and Schwert<sup>16</sup> and Alberty<sup>5</sup>. It has the advantage of giving all the coefficients in the initial rate equation directly, but it depends upon initial rate measurements with low concentrations of both substrates. Accurate data can only be obtained under these conditions if a sensitive method of analysis is available, so that small enzyme concentrations can be used and measurements made over a small fraction of the total reaction to equilibrium.

#### DISCUSSION OF MECHANISMS CONFORMING TO EQUATION (2)

The most obvious general mechanism for an enzyme-catalysed reaction  $S_1 + S_2 \rightleftharpoons S_1' + S_2'$  is one in which both substrates form binary compounds with the enzyme, providing two alternative pathways for the formation of a ternary complex, which undergoes intramolecular transformation (see I, below). The steady state treatment of this mechanism does not give an initial rate equation of the simple form of (2)<sup>13</sup>. Less complex mechanisms which do<sup>5</sup> may be divided into four types according to the simplifying assumptions. The coefficients in the initial rate eqn. (2) in terms of the rate constants or equilibrium constants for the cases to be discussed are given in Table 1,

Table 1. Initial rate equations (steady state) and Haldane relations for possible mechanisms for an enzyme-catalysed reaction  $S_1 + S_2 \rightleftharpoons S_1' + S_2'$ .

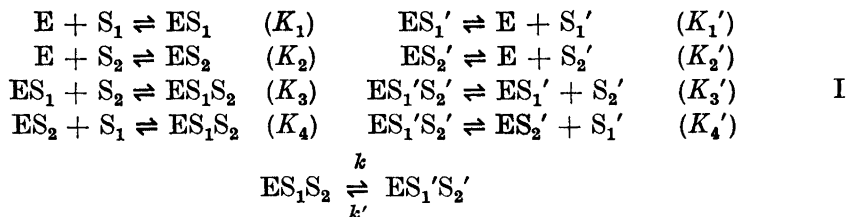
$$\text{General initial rate equation: } \frac{e}{v_0} = \Phi_0 + \frac{\Phi_1}{[S_1]} + \frac{\Phi_2}{[S_2]} + \frac{\Phi_{12}}{[S_1][S_2]}$$

(For description of mechanisms, see text. Only the kinetic coefficients for the forward reactions are given; those for the reverse reaction, denoted by primes, are obtained by inserting or deleting primes on the rate constants.)

Mechanism	Kinetic coefficients				Relations between coefficients	Haldane relation
	$\Phi_0$	$\Phi_1$	$\Phi_2$	$\Phi_{12}$		
I(a)	$\frac{1}{k}$	$\frac{K_4}{k}$	$\frac{K_3}{k}$	$\frac{K_1 K_3}{k}$	$\Phi_1 \Phi_2 = \Phi_0$	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0 \Phi_1' \Phi_2'$
I(b)	$\frac{1}{k}$	$\frac{K_4}{k}$	$\frac{K_3}{k}$	$\frac{K_3 K_4}{k}$		$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$
II(i)	$\frac{1}{k_2'} + \frac{1}{k_4'} + \frac{1}{k} + \frac{k'}{kk_4'}$	$\frac{1}{k_1}$	$\frac{k_4 k_4' + k_4 k' + k_4' k}{k_3 k_4' k}$	$\frac{k_2(k_4 k_4' + k_4 k' + k_4' k)}{k_1 k_3 k_4' k}$	$\Phi_1 \Phi_2 < \Phi_0'$	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$
II(ii,a)	$\frac{1}{k_2'} + \frac{1}{k_4'}$	$\frac{1}{k_1}$	$\frac{k_4 + k_4'}{k_3 k_4'}$	$\frac{k_2(k_4 + k_4')}{k_1 k_3 k_4'}$	$\Phi_1 \Phi_2 < \Phi_0'$	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$
II(ii,b)	$\frac{1}{k_2'}$	$\frac{1}{k_1}$	$\frac{k_4 + k_4'}{k_3 k_4'}$	$\frac{k_2(k_4 + k_4')}{k_1 k_3 k_4'}$	$\Phi_1 \Phi_2 = \Phi_0'$	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$
II(iii)	$\frac{1}{k_2'}$	$\frac{1}{k_1}$	$\frac{1}{k_3}$	$\frac{k_2}{k_1 k_3}$	$\Phi_1 \Phi_2 = \Phi_0'$	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$
III	$\frac{1}{k_4'}$	0	0	$\frac{k_4 + k_4'}{k_3 k_4'}$	—	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$
IV(i)	$\frac{1}{k_2'} + \frac{1}{k_4'}$	$\frac{k_2 + k_3}{k_1 k_2'}$	$\frac{k_4 + k_4'}{k_3 k_4'}$	0	—	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$
IV(ii)	0	$\frac{1}{k_1}$	$\frac{1}{k_2}$	0	—	$\frac{\Phi_{12}'}{\Phi_{12}} = \Phi_0' \Phi_1' \Phi_2'$

together with the Haldane relations between the kinetic coefficients and the overall equilibrium constant, and in some instances relations between the kinetic coefficients themselves. Only the forward initial rate coefficients are given; the expressions for the reverse coefficients are obtained by inserting or deleting primes on the rate constants.

Type I. Four binary and two ternary enzyme complexes are in rapid equilibrium with the free substrates, so that the intramolecular reaction of the ternary complex is the rate-limiting step:



The equilibrium treatment of Michaelis and Menten<sup>11</sup> will be valid and gives the initial rate in terms of the dissociation constants of the enzyme complexes and the rate constants of the intramolecular reaction (Table 1).

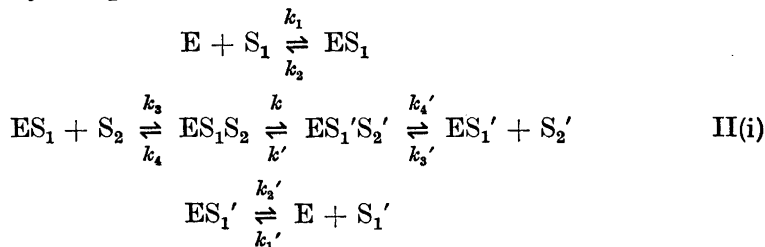
(a) In the general case, there are 6 independent dissociation constants, since  $K_1K_3 = K_2K_4$ , and  $K_1'K_3' = K_2'K_4'$ . All 8 dissociation constants and the two rate constants may be calculated if the 8 kinetic coefficients for the forward and reverse reactions are determined. The Haldane relation  $K_{Eq} = \Phi_{12}'/\Phi_{12}$  is common to this and several other mechanisms, but for this mechanism there is no relation between the coefficients themselves. Since this is the most complicated mechanism considered here, it would only be adopted if all the others had to be rejected. Mechanisms II (i) and II (ii, a) differ from it only in that they require the limiting relations  $\Phi_0' > \Phi_1\Phi_2/\Phi_{12}$  and  $\Phi_0 > \Phi_1'\Phi_2'/\Phi_{12}'$ . The conclusion of Alberty<sup>5</sup> that these three mechanisms cannot be distinguished by initial rate data alone is valid only if these limiting relations are fulfilled.

The maximum rates are  $1/\Phi_0 = k$  and  $1/\Phi_0' = k'$ , and the condition that this mechanism give linear reciprocal plots is that these rates be small compared with those of the other reaction steps. If one of the latter is accessible to direct measurement, it may be possible to test this condition; for example, the spectral shift, which accompanies the combination of reduced diphosphopyridine nucleotide with liver alcohol dehydrogenase<sup>3</sup> made it possible to study this step in isolation<sup>2</sup>. Also, the results of ultracentrifuge studies of enzyme-substrate compounds<sup>17</sup> may be compared with the apparent dissociation constants calculated from initial rate measurements<sup>18</sup>.

(b) In the special case that the combination of the enzyme with one substrate does not influence its affinity for the other<sup>19</sup>, then  $K_1 = K_4$ ,  $K_2 = K_3$ , etc., and there are only 4 independent dissociation constants. There is then a relation between the 4 kinetic coefficients for the reaction in each direction,  $\Phi_0 = \Phi_1\Phi_2/\Phi_{12}$ , which distinguishes the mechanism from all the others considered, and can be used to test the forward and reverse reactions separately. In consequence also, there is an additional distinctive Haldane relation,  $K_{Eq} = \Phi_0\Phi_1'\Phi_1'/\Phi_0'\Phi_1\Phi_2$ .

*Type II.* Only one of the substrates, in each direction, forms a binary enzyme compound, so that there is a compulsory order of reaction.

(i) Two ternary complexes:

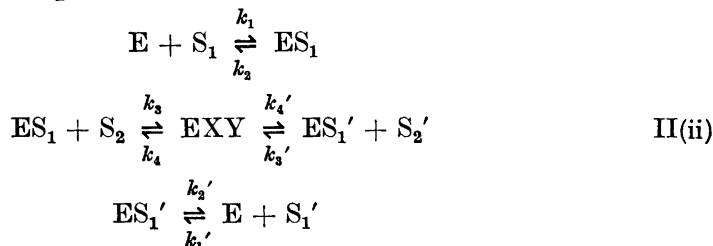


This and the following mechanisms give an initial rate equation of the form of (2) by steady state treatment. The expressions given by Alberty<sup>5</sup> for Michaelis constants and maximum rates in terms of the rate constants in the



mechanism are incorrect. The 10 rate constants cannot all be calculated from the 8 kinetic coefficients for the forward and reverse reactions.

(ii) One ternary complex:



(a) In the general case the 8 rate constants could all be calculated from the kinetic coefficients. This mechanism was the basic one assumed by LuValle and Goddard<sup>20</sup> in their discussion of enzyme oxidation-reduction mechanisms.

(b) In the special case that  $k_4 \gg k_2$  and  $k_4' \gg k_2'$ , the maximum rate becomes equal to the rate of the last step *i.e.*  $\Phi_0 = 1/k_2'$ ,  $\Phi_0' = 1/k_2$ , and there are relations between the kinetic coefficients identical with those for the Theorell-Chance mechanism. But  $\Phi_2$  remains a function of  $k_3$ ,  $k_4$  and  $k_4'$ , although these rate constants cannot now be separately evaluated from the coefficients.

(iii) No ternary complex. This is the Theorell-Chance mechanism described previously.

These mechanisms of Type II have the following features in common.

1. The Haldane relationship,  $K_{E_1} = \Phi_{12}'/\Phi_{12}$ .
2. The rate constants for the formation and dissociation of the binary complex are given by  $k_1 = 1/\Phi_1$  and  $k_2 = \Phi_{12}/\Phi_1\Phi_2$ , and its dissociation constant by  $\Phi_{12}/\Phi_2$ .
3.  $\Phi_0'$  cannot be less than  $\Phi_1\Phi_2/\Phi_{12}$ , since the maximum rate cannot exceed the rate of the last step.

These three mechanisms are not in fact alternatives in a practical sense. Cases (i) and (ii, a) cannot be distinguished, and the second with 8 calculable rate constants would be preferred. Case (iii), in which no ternary complex is included, cannot be distinguished by initial rate measurements of the type considered here from (ii, b), in which the dissociation rate of the ternary complex is fast enough not to limit the maximum rate. The postulate of Theorell and Chance<sup>2</sup> was not intended to explicitly exclude a ternary complex. There is no direct evidence for the formation of a ternary enzyme complex, but it has generally been thought likely<sup>20</sup>, and the recent work of Vennesland and Westheimer<sup>21</sup> makes it probable in the dehydrogenase reactions involving diphosphopyridine nucleotide. Case (ii, b) would therefore be a preferable hypothesis to (iii), although it is not possible to calculate  $k_3$  and  $k_3'$  individually, but only as functions of  $k_4$  and  $k_4'$  (Table 1). Recently Alberty<sup>22</sup> has shown that it may be possible to distinguish mechanisms (ii, b) and (iii) by initial rate measurements with product  $S_2'$  present at the start.

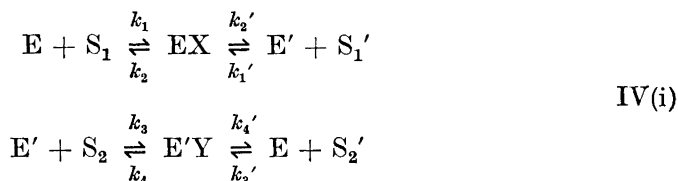
*Type III.* There are no binary complexes; a ternary complex is formed by a termolecular reaction:



Two of the terms in the initial rate eqn. (2) are zero. The intercepts of Lineweaver-Burk plots are independent of the concentration of the constant partner. The mechanism can therefore be distinguished from the others considered, although there is no distinctive Haldane relation. All the rate constants can be calculated from initial rate measurements in both directions.

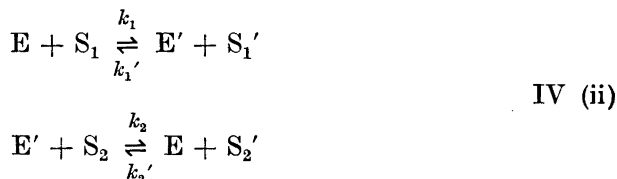
*Type IV.* The enzyme reacts with each substrate in turn to form a product, itself undergoing intermediate chemical change, *e.g.* oxidation and reduction. Such a mechanism is in principle open to direct test with stoichiometric proportions of enzyme and one substrate.

(i) Binary enzyme-substrate compounds are formed:



In recent practical considerations of this mechanism<sup>5,1,6</sup> the fact that it is distinguished from the preceding ones by the absence of the last term of equation (2) from the initial rate equation, as well as by a distinctive Haldane relationship, seems to have been overlooked. The Haldane relationship differs from those for Mechanisms I(b), II(ii, b) and II(iii) only by the ratio  $\Phi_0'/\Phi_0$  or its reciprocal, so that these mechanisms cannot be distinguished by the Haldane relationship when the forward and reverse maximum rates are about the same.

(ii) No enzyme-substrate compounds:



Since there are no enzyme-substrate compounds, the initial rate increases indefinitely with increase of substrate concentration. The slopes of Lineweaver-Burk plots will be independent of the concentration of the constant partner. The intercepts will be inversely proportional to the concentration of the constant partner, and the secondary plots of the intercepts will therefore pass through the origin. The Haldane relation given by Alberty<sup>5</sup>, which apparently distinguishes this case from the preceding one, is indeterminate. The correct Haldane relation is the same as that for the preceding case.

## APPLICATION

Sufficient experimental data for the application of the principles discussed appears to be available for only three enzymes. Two of these will be considered briefly; the third, liver alcohol dehydrogenase, will be discussed in detail elsewhere<sup>8</sup>.

*Yeast alcohol dehydrogenase.* Application of the Haldane relations of Alberty<sup>5</sup> to initial rate and equilibrium data at pH 6.0 and pH 7.15 enabled Nygaard and Theorell<sup>6</sup> to show that the rapid equilibrium mechanism I(a) was a satisfactory hypothesis for the yeast alcohol dehydrogenase reaction, and that the special case of this mechanism, I(b) and the Theorell-Chance mechanism, could be rejected. As regards the "enzyme oxidation" mechanism IV(i), it was considered that the discrepancy of 60% between the value of the equilibrium constant calculated from the appropriate Haldane relationship, and the direct value, was not outside the possible combined experimental error in the kinetic constants involved, but this mechanism was rejected on other grounds. The preceding discussion permits extension of these arguments.

Values for the kinetic coefficients and a distinctive function of the coefficients (Table 1) are shown in Table 2. Mechanism IV(i) requires  $\Phi_{12} = \Phi_{12}' = 0$ , so that this possibility can be rejected from the kinetic data alone. The Theorell-Chance mechanism can be rejected by discrepancies from the required relations between the kinetic coefficients  $\Phi_0 = \Phi_1' \Phi_2' / \Phi_{12}'$  and  $\Phi_0' = \Phi_1 \Phi_2 / \Phi_{12}$  (primes denote coefficients for the  $\text{DPN} + \text{C}_2\text{H}_5\text{OH}$  reaction).

Mechanism I(a) was satisfactory as regards the values of  $K_{\text{Eq}} = 0.81$  and  $1.1 \times 10^{-11}$  M, calculated from the appropriate Haldane relationship, which agree well with the directly determined value of  $0.9 \times 10^{-11}$  M, but the same Haldane relation is also fulfilled by the simpler mechanisms II(i) and II(ii) (*cf.* Table 1) which were not considered by Nygaard and Theorell<sup>6</sup>. The data of Table 2 show, however, that  $\Phi_0 < \Phi_1' \Phi_2' / \Phi_{12}'$  at both the pH values, which is contrary to the requirements of Mechanisms II(i) and II(ii). This same discrepancy from the requirements of all Type II mechanisms has recently been observed with liver alcohol dehydrogenase at pH 7.15<sup>7, 8</sup>.

The special case of the rapid equilibrium mechanism, I(b), was rejected because the appropriate Haldane relation gave values of  $K_{\text{Eq}} = 4.5$  and  $18 \times 10^{-11}$ . This mechanism also requires that  $\Phi_0' = \Phi_1' \Phi_2' / \Phi_{12}'$  and  $\Phi_0 =$

Table 2. Kinetic coefficients for yeast alcohol dehydrogenase, 23°C, calculated from the data of Theorell and Nygaard<sup>6</sup>

Substrates	$\Phi_0$ sec.	$\Phi_1$ $\mu\text{M sec.}$	$\Phi_2$ $\mu\text{M sec.}$	$\Phi_{12}$ $\mu\text{M}^2 \text{sec.}$	$\Phi_1 \Phi_2 / \Phi_{12}$ sec.
pH 6.0					
DPNH, $\text{CH}_3\text{CHO}$ ( $\Phi$ )	0.0027	0.063	0.79	3.4	0.014
DPN, $\text{C}_2\text{H}_5\text{OH}$ ( $\Phi'$ )	0.026	4.2	$2.6 \times 10^3$	$4.2 \times 10^5$	0.026
pH 7.15					
DPNH, $\text{CH}_3\text{CHO}$ ( $\Phi$ )	0.002	0.077	1.12	11.2	0.008
DPN, $\text{C}_2\text{H}_5\text{OH}$ ( $\Phi'$ )	0.016	0.94	$2.95 \times 10^3$	$6.9 \times 10^4$	0.004

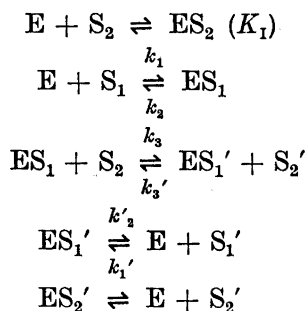
$\Phi_1\Phi_2/\Phi_{12}$ . At pH 6.0 the first of these relations is satisfied, but the second is not and accounts for the whole of the discrepancy of a factor of 5 in the Haldane relation. Thus, Mechanism I(b) is satisfactory for the alcohol-DPN reaction, but not for the aldehyde-DPNH reaction, *i.e.* if mechanism I(a) is accepted, it can also be assumed that at pH 6 the combination of the enzyme with either DPN or alcohol does not affect its affinity for the other. At pH 7.15 this additional assumption is not valid.

*Lactic dehydrogenase.* From tests of initial rate data with the Haldane relations of Alberty, Hakala *et al.*<sup>16</sup> concluded that the Theorell-Chance mechanism and the enzyme oxidation mechanism could be rejected, and that any of the ternary complex mechanisms I, II(i) and II(ii, a) were satisfactory. Examination of their data shows that the limiting relations between the kinetic coefficients required by mechanisms II(i) and II(ii, a) are also satisfied, so that no further conclusions can be drawn, except that the special case I(b) is not satisfactory for either the forward or reverse reactions.

#### SUBSTRATE INHIBITION

Deviations from the linear reciprocal form of initial rate equation at high substrate concentrations are common. Liver alcohol dehydrogenase<sup>1,2</sup> and lactic dehydrogenase<sup>16</sup> show substrate inhibition with high concentrations of alcohol and pyruvate, respectively. With yeast alcohol dehydrogenase on the other hand, deviation towards higher activity occur at high alcohol concentrations<sup>6</sup>. In cases such as lactic dehydrogenase where the reaction conforms, at low substrate concentrations, to the requirements of a Type II mechanism, in which there is a compulsory order of reaction of the two substrates with the enzyme, inhibition at high concentrations of the second substrate might be accounted for by combination of the latter with the enzyme and/or with the active ternary complex to form inactive compounds. It will be seen that the effects of such substrate inhibition on Lineweaver-Burk plots with respect to the first substrate are to a large extent analogous to the effects of inhibition in single-substrate reactions, and competitive, uncompetitive and non-competitive types may be distinguished. At present there does not appear to be sufficiently detailed data available to test these mechanisms, but the treatment may serve as a guide in the planning of further experiments.

*Competitive substrate inhibition.* The second substrate forms an inactive binary complex with the enzyme:



The last step will not influence the initial rate of the forward reaction, nor the first step that of the reverse reaction.

Steady state treatment gives for the forward reaction:

$$\frac{e}{v_0} = \frac{1}{k_2'} + \left(1 + \frac{[S_2]}{K_I}\right) \frac{1}{k_1[S_1]} + \frac{1}{k_3[S_2]} + \left(1 + \frac{[S_2]}{K_I}\right) \frac{k_2}{k_1 k_3 [S_1][S_2]} \quad (7)$$

$$= \Phi_0 + \left(1 + \frac{[S_2]}{K_I}\right) \frac{\Phi_1}{[S_1]} + \frac{\Phi_2}{[S_2]} + \left(1 + \frac{[S_2]}{K_I}\right) \frac{\Phi_{12}}{[S_1][S_2]} \quad (8)$$

$$= \left(\Phi_0 + \frac{\Phi_2}{[S_2]}\right) + \left(1 + \frac{[S_2]}{K_I}\right) \left(\Phi_1 + \frac{\Phi_{12}}{[S_2]}\right) \frac{1}{[S_1]} \quad (8a)$$

Intercept

Slope

The  $\Phi$  coefficients have the same significance in terms of rate constants as in the simple Theorell-Chance mechanism (Table 1, II (iii)), and  $K_I$  is the dissociation constant of the inactive complex. If one or two ternary complexes are postulated, eqn. (8) is also valid, and the  $\Phi$  coefficients are identical with those for mechanisms II(i) and II(ii), respectively. If the inhibition factor is replaced by  $\left(1 + \frac{[I]}{K_I}\right)$ , eqn. (8) is quite general<sup>13</sup> for an inhibitor which competes with  $S_1$  and includes the case when product  $S_1'$  (but not  $S_2'$ ) is present at the start<sup>22</sup>.

With low concentrations of  $S_2$  such that (i)  $[S_2]/K_I \ll 1$ , eqn. (8) reduces to eqn. (2), and initial rate data will conform to the requirements of the corresponding Type II mechanism. Plots of  $e/v_0$  against  $1/[S_2]$  will be linear only when (i) holds, and at some higher value of  $[S_2]$  which will depend upon  $[S_1]$ ,  $e/v_0$  will pass through a minimum. Plots of  $e/v_0$  against  $1/[S_1]$  will be linear for all values of  $[S_2]$  (eqn. 8a), and as in Lineweaver-Burk plots for a single-substrate reaction with competitive inhibition, the slope is increased by the inhibition factor but the intercept is unaffected.  $\Phi_0$  and  $\Phi_2$  can therefore be determined by a secondary plot of the intercepts against  $1/[S_2]$ . The slope is not a linear function of  $1/[S_2]$  except when (i) holds, and will have a minimum value when (ii)  $[S_2]^2 = \Phi_{12}K_I/\Phi_1$ , as shown by differentiation. Theoretically  $\Phi_1$  and  $\Phi_{12}$  can be obtained in the usual way by a secondary plot of the slope against  $1/[S_2]$  for low values of  $[S_2]$  such that (i) holds, and  $K_I$  can then be calculated from the value of  $[S_2]$  at which the slope is minimum by (ii). However, the upper limit imposed on  $[S_2]$  by (i) will make it difficult to get accurate values of  $\Phi_1$  in this way unless  $\Phi_1 > \Phi_{12}/K_I$  (i.e.  $K_I > k_2/k_3$ ), since otherwise  $\Phi_1$  will be negligible compared with  $\Phi_{12}/[S_2]$  over the whole linear range. If the expression for the slope of equation (8a) is rearranged thus,

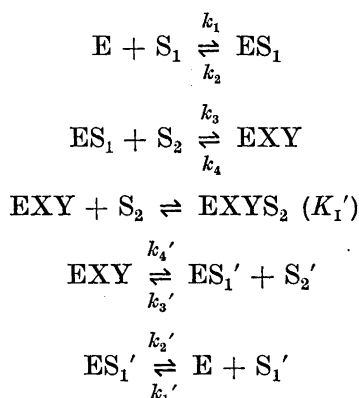
$$\text{Slope} = \Phi_1 + \frac{\Phi_{12}^2}{K_I} + \frac{\Phi_1[S_2]}{K_I} + \frac{\Phi_{12}}{[S_2]}$$

it is seen that a plot of the reciprocal of the slope against  $-\log [S_2]$  over a wide range of the latter should give a bell-shaped curve, from which  $\Phi_1$ ,  $\Phi_{12}$  and  $K_I$  could be determined by the method employed by Alberty and Massey<sup>23</sup>

to calculate the acid dissociation constants of an enzyme-substrate complex and the pH-independent maximum velocity<sup>24</sup>.

When the inhibitor is not a substrate, the determination of the kinetic coefficients and the inhibition constant is obvious. The case in which  $S_1'$  is present at the start, when  $K_I = k_2'/k_1'$ , is discussed by Alberty<sup>22</sup>.

*Uncompetitive substrate inhibition.* In single-substrate reactions, the effect of an inhibitor which combines with the enzyme-substrate complex but not with the free enzyme is to increase the intercept of the Lineweaver-Burk plot without affecting the slope, and this type of inhibition has been termed uncompetitive<sup>25</sup>. Haldane<sup>4</sup> discussed substrate inhibition of this kind, in which a second molecule of the substrate combines with the enzyme-substrate complex to form an inactive compound. An analogous situation in two-substrate systems would be the combination of a second molecule of one substrate with the ternary complex in mechanism II(ii) to give an inactive compound:



Steady state treatment of this mechanism gives, for the forward reaction

$$\frac{e}{v_0} = \frac{1}{k_2'} + \left(1 + \frac{[S_2]}{K_I'}\right) \frac{1}{k_4'} + \frac{1}{k_1[S_1]} + \frac{k_4 + k_4'}{k_3 k_4' [S_2]} + \frac{k_2(k_4 + k_4')}{k_1 k_3 k_4' [S_1][S_2]} \quad (9)$$

$$= \Phi_0 + \frac{[S_2]}{K_I' k_4'} + \frac{\Phi_1}{[S_1]} + \frac{\Phi_2}{[S_2]} + \frac{\Phi_{12}}{[S_1][S_2]} \quad (10)$$

$$= \left(\Phi_0 + \frac{[S_2]}{K_I' k_4'} + \frac{\Phi_2}{[S_2]}\right) + \left(\Phi_1 + \frac{\Phi_{12}}{[S_2]}\right) \frac{1}{[S_1]} \quad (11)$$

The  $\Phi$  coefficients are identical with those for mechanism II (ii,a), (Table 1) and  $K_I'$  is the dissociation constant of the inactive complex. With low concentrations of  $S_2$ , such that  $[S_2]/K_I' k_4' \ll \Phi_0$ , the initial rate data will conform to the requirements of mechanism II(ii). With higher concentrations of  $S_2$ , plots of  $e/v_0$  against  $1/[S_2]$  will pass through a minimum. As for competitive substrate inhibition, plots of  $e/v_0$  against  $1/[S_1]$  will be linear for all values of  $[S_2]$ . In contrast to the preceding case, however, the slope is not affected by the inhibition term, and will yield values of  $\Phi_1$  and  $\Phi_{12}$  by a secondary plot against  $1/[S_2]$ , whilst the intercept is no longer linear in  $1/[S_2]$ . A secondary

plot of the intercept against  $1/[S_2]$  for low values of  $[S_2]$ , such that  $[S_2]/K_1'k_4' \ll \Phi_0$ , would give  $\Phi_0$  as intercept and  $\Phi_2$  as slope and at higher values of  $[S_2]$  there will be a minimum value of the intercept when  $[S_2]^2 = \Phi_2 K_1' k_4'$ , from which  $K_1' k_4'$  could be calculated. Alternatively, a plot of the reciprocal of the intercept against  $-\log[S_2]$  should give a bell-shaped curve from which  $\Phi_0$ ,  $\Phi_2$  and  $K_1' k_4'$  could be determined<sup>4,23</sup>. Since  $k_4'$  can be calculated from the coefficients for the forward and reverse reactions taken together ( $\Phi_0 = (k_2' + k_4'/k_2'k_4')$ ;  $k_2' = \Phi_{12}'/\Phi_1'\Phi_2'$ ),  $K_1'$  can be evaluated.

In contrast to the analogous single-substrate case, the whole maximum rate term in eqn. (11) is not multiplied by an inhibition factor. Only one of the two terms comprising  $\Phi_0$  is affected (eqn. 9), namely  $1/k_4'$ . If  $k_4' \gg k_2'$ , i.e. if the data at low  $S_2$  concentrations conform to the special case II(ii, b),  $k_4'$  and  $K_1'$  cannot be separately evaluated, and also substrate inhibition will be deferred to much higher values of  $[S_2]$ .

*Non-competitive substrate inhibition.* If the second substrate can combine with both the free enzyme and the ternary complex to give inactive compounds, it is obvious from the preceding discussion that the initial rate equation will be

$$\frac{e}{v_0} = \left( \Phi_0 + \frac{[S_2]}{k_4'K_1'} + \frac{\Phi_2}{[S_2]} \right) + \left( 1 + \frac{[S_2]}{K_1'} \right) \left( \Phi_1 + \frac{\Phi_{12}}{[S_2]} \right) \frac{1}{[S_1]} \quad (12)$$

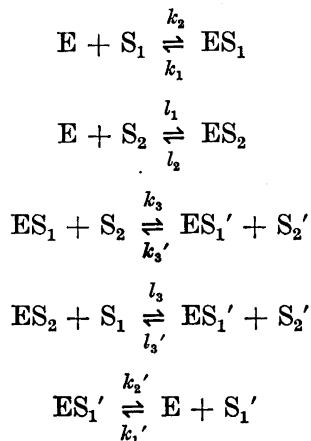
Again the plot of  $e/v_0$  against  $1/[S_1]$  should be linear for all values of  $[S_2]$  but neither the slope nor the intercept will be linear in  $1/[S_2]$ . The kinetic coefficients and the inhibition constants may be determined as in the two previous cases.

The term non-competitive inhibition has usually been applied to single-substrate reactions in which the dissociation constants of the inactive compounds of the inhibitor with the free enzyme and the enzyme-substrate complex are equal, in which case the slope and the intercept of Lineweaver-Burk plots are multiplied by the same inhibition factor. It would seem to be logical to extend the use of the term to the more general case in which the dissociation constants are unequal, and the slope and intercept are affected to different extents. This is true of eqn. (12) even when  $K_1 = K_1'$ .

#### SUBSTRATE ACTIVATION

Steady state treatment of Type I mechanisms in which there are alternative pathways for the overall reaction yields complex initial rate equations<sup>26</sup> which would be difficult to deal with experimentally. Such mechanisms yield the linear reciprocal rate eqn. (2) only if it is assumed that all steps except one are in equilibrium. It is evident, however, that if the enzyme has a much greater affinity for one substrate than for the other, the reaction may be effectively confined to one pathway over a certain range of substrate concentrations, and initial rate data would conform to the requirements of a Type II mechanism. It is of interest to consider under what conditions the initial rate equation obtained by steady state treatment of a mechanism of Type I will reduce to eqn. (2) over a reasonably wide range of substrate con-

centrations, and what kind of deviations might be observed outside this range. A simple case is an extension of the Theorell-Chance mechanism in one direction:



The same rate constants as before are used for the preferred pathway, and  $l_1$  etc. are the rate constants for the alternative pathway which will be assumed to be important only when  $[S_2]/[S_1]$  is large. There is a compulsory relation between the equilibrium constants for the alternative reactions

(i)  $l_1 l_3 / l_2 l_3' = k_1 k_3 / k_2 k_3'$ .

(a) For the forward reaction, steady state treatment gives:

$$\frac{e}{v_0} = \frac{1}{k_2'} + \frac{k_1[S_1](l_3[S_1] + l_2) + (l_3[S_1] + l_2)(k_3[S_2] + k_2) + l_1[S_2](k_3[S_2] + k_2)}{[S_1][S_2]\{k_1 k_3(l_3[S_1] + l_2) + l_1 l_3(k_3[S_2] + k_2)\}} \quad (13)$$

If  $l_1 = 0$ , this mechanism reduces to the Theorell-Chance mechanism, and if  $l_1 \neq 0$  but  $l_3 = 0$  it reduces to the competitive substrate inhibition type. Accordingly, substitution of  $l_1 = 0$  and  $l_3 = 0$ , respectively, in (9) gives eqns. (1a) and (7). If (ii)  $k_1 k_3 l_2 \gg l_1 l_3 k_2$ , eqn. (13) approximates to

$$\frac{e}{v_0} = \frac{1}{k_2'} + \left( \frac{A}{A + l_3 l_1 [S_2] / k_1} \right) \frac{\Phi_2}{[S_2]} + \left( \frac{A + l_1 [S_2]}{A + l_3 l_1 [S_2] / k_1} \right) \left( \frac{\Phi_1}{[S_1]} + \frac{\Phi_{12}}{[S_1][S_2]} \right) \quad (14)$$

where the  $\Phi$  coefficients have the same significance as in the Theorell-Chance mechanism, and  $A = l_3[S_1] + l_2$ . If the first factors of the second and third terms of eqn. (14) are equal to unity, this equation reduces to the Theorell-Chance equation. This will be true if  $l_1[S_2] \ll A$  and  $l_1[S_2] \ll k_1 A / l_3$ . Conditions favouring these requirements are high values of  $k_1/l_1$ ,  $l_2/l_1$  and  $l_3/l_1$ , which are compatible with (ii). Thus, this mechanism may conform to the requirements of the Theorell-Chance mechanism over a wide range of substrate concentrations, even when  $k_3$  and  $l_3$  are of the same order of magnitude, if the complex  $ES_2$  has a large dissociation constant and is formed much more slowly than  $ES_1$ . With sufficiently high values of  $[S_2]/[S_1]$ , Lineweaver-Burk plots against both  $1/[S_1]$  and  $1/[S_2]$  will be non-linear. Consider the



plot of  $e/v_0$  against  $1/[S_2]$  with a fixed concentration of  $S_1$ . The second term of eqn. (14) does not contain  $[S_2]$  in the numerator, and with high concentrations of  $S_2$  will produce deviations in the linear reciprocal plot towards higher activity. This will be true also of the third term if  $l_3 > k_1$ , and with this condition, therefore, substrate activation should be observed at high concentrations of  $S_2$ , regardless of the value of the fixed concentration of  $S_1$ . If  $l_3 < k_1$ , the third term of eqn. (14) will produce deviations towards lower activity, and the effects of the second and third terms will be opposed. Whether activation or inhibition occurs at high values of  $S_2$  will depend upon the relative values of these two terms. High values of the fixed concentration of  $S_1$  will make the third term smaller compared to the second, and therefore favour substrate activation. This situation provides a possible explanation of substrate activation by alcohol in the yeast dehydrogenase reaction, which occurs only with high concentrations of the coenzyme<sup>6</sup>.

(b) For the reverse reaction, steady state treatment gives:

$$\frac{e}{v_0} = \frac{B}{k_2} + \frac{1}{k_1'[S_1']} + \frac{1}{(k_3' + l_3')[S_2']} + \frac{k_2'}{k_1'(k_3' + l_3')[S_1'] [S_2']} \quad (15)$$

$$= B\Phi_0' + \frac{\Phi_1'}{[S_1']} + \frac{\Phi_2'}{[S_2']} + \frac{\Phi_{12}'}{[S_1'] [S_2']} \quad (16)$$

The  $\Phi$  coefficients have the same significance as in the Theorell-Chance mechanism except that, in  $\Phi_2'$  and  $\Phi_{12}'$ ,  $k_3'$  is replaced by  $k_3' + l_3'$ ; and  $B = \left(1 + \frac{l_3'k_2}{k_3'l_2}\right) / \left(1 + \frac{l_3'}{k_3'}\right)$ . Corollaries of assumption (ii) in the preceding section, together with the compulsory relation (i), however, are that  $l_3' \ll k_3'$  and  $B \doteq 1$ . Therefore under conditions for which it has been possible to show that the forward reaction will conform to the Theorell-Chance mechanism over a wide range of substrate concentrations, the reverse reaction will also conform to that mechanism.

Although mechanisms of this type, with alternative pathways, do not lend themselves readily to experimental evaluation, these considerations of a simple case emphasize the fact that, under less restrictive conditions than are assumed in the equilibrium treatment, such mechanisms may conform to the linear reciprocal initial rate eqn. (2), and provide a possible explanation of complex types of deviation at extremes of substrate concentration.

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