The Interpretation of Kinetic Data for Enzyme-Catalysed Reactions involving Three Substrates

By K. DALZIEL

Department of Chemistry, University of Oxford, OX1 3QU

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The analysis and interpretation of initial-rate data for reactions involving three substrates, obtained in suitably designed experiments, are discussed. Possible mechanisms for such reactions are classified, the rate equations are compared and the extent to which they can be distinguished experimentally is considered.

Initial-rate equations for several possible mechanisms for enzyme reactions involving three substrates have been derived by various authors (Frieden, 1959; Cleland, 1963; Keleti, 1965; Keleti & Batke, 1965; Fromm, 1967). The use of competitive inhibitors to distinguish between several mechanisms has been considered in detail by Fromm (1967). However, there has been no systematic and comprehensive treatment of the analysis and interpretation of rate data of the kind that has proved useful for studies of the mechanisms of two-substrate reactions (Alberty, 1953; Dalziel, 1957; Cleland, 1963). As regards discrimination between possible mechanisms, initial-rate studies are more penetrating with two-substrate reactions than with single-substrate reactions; similarly the additional experimental variable in a three-substrate reaction permits still greater discrimination between plausible mechanisms, provided that, as with two-substrate reactions (Dalziel, 1957), the concentrations of all the substrates are varied simultaneously. The time and labour involved is by no means prohibitive if continuous or automated methods for recording progress curves are used. Such detailed studies are essential if adequate basic information on the catalytic properties of an enzyme is to be obtained, and are a necessary adjunct to other investigations such as inhibition effects, including product inhibition, pre-steady-state studies and isotope-exchange experiments. So far only one such detailed kinetic study of a three-substrate enzyme, that of glyceraldehyde 3-phosphate dehydrogenase by Keleti & Batke (1965), has been reported.

There are two main groups of synthetic reactions involving three substrates. The first comprises the oxidative phosphorylation and reductive carboxylation and amination reactions catalysed by certain nicotinamide nucleotide-linked dehydrogenases, such as glyceraldehyde 3-phosphate dehydrogenase,

isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, 'malic' enzyme and glutamate dehydrogenase. In these cases only two substrates (excluding water and the H^+ ion) are involved in the reverse reactions. The second main group consists of the reactions catalysed by ligases, in which the linkage of two molecules is accompanied by the fission of ATP, so that three substrates are involved in both directions. The first group is perhaps more easily subjected to the necessary detailed study, including rate measurements with low concentrations of all three substrates, because of the availability of a sensitive fluorimetric method for recording the oxidation and reduction of nicotinamide nucleotides (cf. Dalziel, 1962). The handling and interpretation of experimental data for this group of enzymes (P. C. Engel, J. C. Londesborough, R. H. Villet & K. Dalziel, unpublished work) provided the immediate stimulus for the theoretical treatment given in this paper, which can readily be extended to include product inhibition or an essential activator such as a metal ion. Information about the mechanisms of ligases has come largely from isotope-exchange studies, but initial-rate data from properly designed experiments could provide important confirmatory and supplementary evidence.

EXPERIMENTAL DETERMINATION OF INITIAL-RATE PARAMETERS

If an enzyme-catalysed reaction:

shows Michaelis-Menten behaviour with respect to each substrate, i.e. if plots of the reciprocal initial rate against the reciprocal of the concentration of each substrate, with fixed (but not necessarily saturating) concentrations of the other two, are linear over wide ranges of concentration, then over

these ranges the initial-rate behaviour can be described by the empirical equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_C}{[C]} + \frac{\phi_{AB}}{[A][B]} + \frac{\phi_{AC}}{[A][C]} + \frac{\phi_{BC}}{[B][C]} + \frac{\phi_{ABC}}{[A][B][C]} \quad (1)$$

where e is the concentration of active centres and v_0 is the initial rate. This general initial-rate equation is analogous to that for two-substrate reactions (Dalziel, 1957), and an equation of this form is obtained by equilibrium treatment of a random-order quaternary-complex mechanism, as shown below. Initial-rate equations derived for other mechanisms, by steady-state or equilibrium treatment, can be considered as special cases of eqn. (1), in which there may be relations between some of the parameters, or in which one or more of the parameters may be zero.

The parameters or kinetic coefficients, ϕ_0, ϕ_A etc., are of course functions of rate or dissociation constants in the various mechanisms. As with two-substrate reactions, this form of initial-rate equation has some advantages over alternative formulations in terms of the maximum rate and Michaelis constants. The kinetic coefficients are obtained directly as slopes and intercepts of suitable plots. Ratios of kinetic coefficients may have the dimensions of dissociation constants, but not necessarily their physical significance. The kinetic coefficients are usually simpler functions of velocity constants than are Michaelis constants, and indeed in some cases are the reciprocals of single velocity constants. Finally, their variations with temperature and pH are simpler to interpret than those of Michaelis constants.

ϕ_0 is of course the reciprocal of the maximum rate with unit enzyme concentration. In general, the concentration of one substrate that gives half the saturation rate varies with the fixed concentrations of the other substrates. As with two-substrate reactions, it is convenient to define the

K_m for each substrate as the concentration that gives half the true maximum rate in the presence of

infinitely large concentrations of the other substrates. Thus the K_m values for substrates A, B and C are $\phi_A/\theta_0, \phi_B/\phi_0$ and ϕ_C/ϕ_0 respectively. Michaelis constants for each substrate, and half-saturation values with any finite concentrations of the other two substrates, are best obtained from experimental values for the kinetic coefficients.

To evaluate all eight parameters in eqn. (1), or to show that some of them are zero, initial-rate measurements are required with several concentrations of each substrate in all possible combinations. From primary plots of e/v_0 against $1/[A]$ (say), intercepts and slopes can be obtained for the various fixed values of $[B]$ and $[C]$. Secondary plots of these slopes and intercepts against either $1/[B]$ or $1/[C]$ again yield slopes and intercepts, expressions for which are given in Table 1. These slopes and intercepts can then be plotted against $1/[C]$ or $1/[B]$ in ternary plots illustrated in Fig. 1, from which the kinetic coefficients can be obtained directly. From one such set of plots two estimates of each parameter are obtained, and the tertiary plots are conveniently plotted in pairs with common intercepts or slopes (Fig. 1). Obviously the primary plots could equally well be made against $1/[B]$ or $1/[C]$, thereby grouping the initial-rate measurement in different ways, and yielding in all six estimates of each parameter.

If any one of the parameters in eqn. (1) is zero this will be reflected in two of the secondary plots: there will be no change of slope or intercept with the concentration of the third substrate, so that the secondary plots for different concentrations of the third substrate will be either parallel or will intersect on the ordinate (cf. Table 1).

Table 1. *Intercepts, I, and slopes, S, of primary and secondary plots of initial-rate data that satisfy the general form of initial-rate equation*

The general equation is: $\frac{e}{v_0} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_C}{[C]} + \frac{\phi_{AB}}{[A][B]} + \frac{\phi_{AC}}{[A][C]} + \frac{\phi_{BC}}{[B][C]} + \frac{\phi_{ABC}}{[A][B][C]}$			
Primary plots of $\frac{e}{v_0}$ against $\frac{1}{[A]}$:	$I = \phi_0 + \frac{\phi_B}{[B]} + \frac{\phi_C}{[C]} + \frac{\phi_{BC}}{[B][C]}$	$S = \phi_A + \frac{\phi_{AB}}{[B]} + \frac{\phi_{AC}}{[C]} + \frac{\phi_{ABC}}{[B][C]}$	
Secondary plots of I and S against $\frac{1}{[B]}$:	$I_1 = \phi_0 + \frac{\phi_C}{[C]}$	$S_1 = \phi_B + \frac{\phi_{BC}}{[C]}$	$I_2 = \phi_A + \frac{\phi_{AC}}{[C]} \quad S_2 = \phi_{AB} + \frac{\phi_{ABC}}{[C]}$
Secondary plots of I and S against $\frac{1}{[C]}$:	$I_3 = \phi_0 + \frac{\phi_B}{[B]}$	$S_3 = \phi_C + \frac{\phi_{BC}}{[B]}$	$I_4 = \phi_A + \frac{\phi_{AB}}{[B]} \quad S_4 = \phi_{AC} + \frac{\phi_{ABC}}{[B]}$

Fig. 1. Illustration of the determination of the kinetic coefficients in eqn. (1) by tertiary plots of the slopes and intercepts of secondary plots, defined in Table 1. The kinetic coefficients are equal to the slopes or intercepts of the tertiary plots, as indicated.

CLASSIFICATION OF MECHANISMS

The mechanisms considered may be divided into two main types, analogous to the single-displacement (or ternary-complex) and double-displacement (enzyme-substitution or 'ping-pong') mechanisms for group transfer in two-substrate reactions. These two types are:

(I) Quaternary-complex mechanisms, in which all three substrates must be bound to the enzyme before any product is formed.

(II) Enzyme-substitution mechanisms, in which, for example, phosphorylated, carboxylated or reduced enzyme is formed, together with a product, by reaction of the enzyme with only one or two of the three substrates. The group is then transferred from the enzyme to another substrate in a separate step, forming another product and the original, un-substituted enzyme.

It may be recalled that, with two-substrate reactions, the enzyme-substitution or double-displacement mechanism can be distinguished from ternary-complex mechanisms by the absence of a term $\phi_{AB}/[A][B]$ in the initial-rate equation (Dalziel,

1957), as well as by isotope-exchange experiments. Similarly, for three-substrate reactions, all mechanisms of type II are characterized by the absence of the term $\phi_{ABC}/[A][B][C]$, i.e. $\phi_{ABC} = 0$ in eqn. (1), as shown below.

Type I mechanisms may be subdivided according to whether there is an ordered or random combination of substrates with the enzyme to form the quaternary complex. Type II mechanisms may also be subdivided according to the way in which the intermediate substituted enzyme is formed. The various mechanisms will be considered in turn, and the initial-rate equations are listed in Table 2, where the initial-rate parameters in eqn. (1) are expressed in terms of rate constants or dissociation constants in the mechanisms.

(I) Quaternary-complex mechanisms

(a) *Equilibrium random-order mechanism.* If each of the three substrates can form active binary complexes with the enzyme, so that there are alternative pathways for the formation of the quaternary complex (Fig. 2), then the reciprocal

Table 2. *Kinetic coefficients in the initial-rate equations for various mechanisms, expressed as functions of dissociation constants of enzyme complexes and velocity constants in the mechanism*

For details of the mechanisms see the text. C.O. and E.R.O. denote compulsory and equilibrium random order of addition of the two substrates in the concerted-enzyme-substitution step, as described in the text.

Mechanism	ϕ_0	$\frac{\phi_A}{K_{BCA}}$	$\frac{\phi_B}{K_{ACB}}$	$\frac{\phi_C}{K_{ABC}}$	$\frac{\phi_{AB}}{K_{ACB}K_{CA}}$	$\frac{\phi_{AC}}{K_{ABC}K_{BA}}$	$\frac{\phi_{BC}}{K_{ABC}K_{AB}}$	$\frac{\phi_{ABC}}{K_A K_{AB} K_{BC}}$	$\frac{K_0}{\phi_{PQR}}$
I(a)	$\frac{1}{\bar{k}}$								$\frac{\phi_{PQR}}{\phi_{ABC}}$
I(b)	$\frac{1}{\bar{k}'} + \frac{1}{\bar{k}'-2} + \frac{1}{\bar{k}} + \frac{k'}{\bar{k}\bar{k}'-2}$	$\frac{1}{\bar{k}_{+1}}$	$\frac{1}{\bar{k}_{+2}}$	$\frac{Y^*}{\bar{k}_{+3}}$	$\frac{k_{-1}}{\bar{k}_{+1}\bar{k}_{+2}}$	0	$\frac{k_{-2}Y^*}{\bar{k}_{+2}\bar{k}_{+3}}$	$\frac{k_{-1}k_{-2}Y^*}{\bar{k}_{+1}\bar{k}_{+2}\bar{k}_{+3}}$	$\frac{\phi_{PQR}}{\phi_{ABC}}$
I(c)	$\frac{1}{\bar{k}}$	$\frac{1}{\bar{k}_{+1}}$	$\frac{K_{ACB}}{\bar{k}}$	$\frac{K_{ABC}}{\bar{k}}$	0	0	$\frac{K_{ABC}K_{AB}}{\bar{k}}$	$\frac{k_{-1}K_{ABC}K_{AB}}{\bar{k}_{+1}\bar{k}}$	$\frac{\phi_{PQR}}{\phi_{ABC}}$
II(a)	$\frac{1}{\bar{k}_3} + \frac{1}{\bar{k}_7} + \frac{1}{\bar{k}_{11}}$	$\frac{k_2 + k_3}{\bar{k}_1\bar{k}_3}$	$\frac{k_6 + k_7}{\bar{k}_5\bar{k}_7}$	$\frac{k_{10} + k_{11}}{\bar{k}_9\bar{k}_{11}}$	0	0	0	0	$\frac{\phi_P\phi_Q\phi_R}{\phi_A\phi_B\phi_C}$
II(b) C.O.	$\frac{1}{\bar{k}_5} + \frac{1}{\bar{k}_9} + \frac{1}{\bar{k}_{11}}$	$\frac{1}{\bar{k}_1}$	$\frac{k_4 + k_5}{\bar{k}_3\bar{k}_5}$	$\frac{k_8 + k_9}{\bar{k}_7\bar{k}_9}$	$\frac{k_2(k_4 + k_5)}{\bar{k}_1\bar{k}_3\bar{k}_5}$	0	0	0	$\frac{\phi_P\phi_Q\phi_R}{\phi_{AB}\phi_C}$
II(b) E.R.O.	$\frac{1}{\bar{k}_5} + \frac{1}{\bar{k}_9} + \frac{1}{\bar{k}_{11}}$	$\frac{K_{BA}}{\bar{k}_5}$	$\frac{K_{AB}}{\bar{k}_5}$	$\frac{k_8 + k_9}{\bar{k}_7\bar{k}_9}$	$\frac{K_A K_{AB}}{\bar{k}_5}$	0	0	0	$\frac{\phi_P\phi_Q\phi_R}{\phi_{AB}\phi_C}$
II(c) C.O.	$\frac{1}{\bar{k}_5} + \frac{1}{\bar{k}_7} + \frac{1}{\bar{k}_{11}}$	$\frac{1}{\bar{k}_1}$	$\frac{k_4 + k_5}{\bar{k}_3\bar{k}_5}$	$\frac{k_{10} + k_{11}}{\bar{k}_9\bar{k}_{11}}$	$\frac{k_2(k_4 + k_5)}{\bar{k}_1\bar{k}_3\bar{k}_5}$	0	0	0	$\frac{\phi_Q\phi_R\phi_P}{\phi_{AB}\phi_C}$
II(c) E.R.O.	$\frac{1}{\bar{k}_5} + \frac{1}{\bar{k}_7} + \frac{1}{\bar{k}_{11}}$	$\frac{K_{BA}}{\bar{k}_5}$	$\frac{K_{AB}}{\bar{k}_5}$	$\frac{k_{10} + k_{11}}{\bar{k}_9\bar{k}_{11}}$	$\frac{K_A K_{AB}}{\bar{k}_5}$	0	0	0	$\frac{\phi_Q\phi_R\phi_P}{\phi_{AB}\phi_C}$
II(d) C.O.	$\frac{1}{\bar{k}_3} + \frac{1}{\bar{k}_9} + \frac{1}{\bar{k}_{11}}$	$\frac{k_2 + k_3}{\bar{k}_1\bar{k}_3}$	$\frac{1}{\bar{k}_5}$	$\frac{k_8 + k_9}{\bar{k}_7\bar{k}_9}$	0	0	$\frac{k_6(k_8 + k_9)}{\bar{k}_5\bar{k}_7\bar{k}_9}$	0	$\frac{\phi_P\phi_Q\phi_R}{\phi_{BC}\phi_A}$
II(d) E.R.O.	$\frac{1}{\bar{k}_3} + \frac{1}{\bar{k}_9} + \frac{1}{\bar{k}_{11}}$	$\frac{k_2 + k_3}{\bar{k}_1\bar{k}_3}$	$\frac{K_{CB}}{\bar{k}_5}$	$\frac{K_{BC}}{\bar{k}_9}$	0	0	$\frac{K_B K_{BC}}{\bar{k}_9}$	0	$\frac{\phi_P\phi_Q\phi_R}{\phi_{BC}\phi_A}$

$$*Y = \frac{k_{-3}k' + k_{-3}k'-2 + kk'-2}{kk'-2}$$

Fig. 2. Random-order mechanism for the reaction:

The dissociation constants in the mechanism are defined by:

$$K_A = [E][A]/[EA], \quad K_{AB} = [EA][B]/[EAB], \quad K_{ABC} = [EAB][C]/[EABC] \quad \text{etc.}$$

The initial-rate equation for the rapid-equilibrium case is given in Table 2.

initial-rate equation derived by the steady-state assumption does not take the simple linear form of eqn. (1). In the special case that all the enzyme complexes are in rapid equilibrium with the substrates and products, and the intermolecular reaction of the quaternary reactant complex to yield a quaternary product complex is rate-limiting, the initial rate equation is of the form of eqn. (1). The initial rate is $k[EABC]$ (Fig. 2) and $[EABC]$ may be expressed in terms of total enzyme and substrate concentrations by equilibrium relations (Frieden, 1959). The kinetic coefficients in eqn. (1) are functions of k and the dissociation constants of the various complexes (Fig. 2 and Table 2).

Corresponding to the six alternative pathways from E to EABC, there are six alternative expressions for ϕ_{ABC} , since $K_A K_{AB} K_{ABC} = K_A K_{AC} K_{ACB} = K_B K_{BA} K_{ABC}$ etc. In other words, there are five thermodynamic relations between the 12 dissociation constants in the mechanism, and therefore only seven of the latter are independent. All the

dissociation constants, together with the velocity constant k , can therefore be calculated from experimental values for the eight kinetic coefficients.

The Michaelis constants, previously defined, are the three dissociation constants of the quaternary complex EABC, i.e. K_m for A = $\phi_A/\phi_0 = K_{BCA}$, for B = $\phi_B/\phi_0 = K_{ACB}$ and for C = $\phi_C/\phi_0 = K_{ABC}$. The two dissociation constants for each of the ternary complexes EAB, EAC and EBC are given by $K_{AB} = \phi_{BC}/\phi_C$, $K_{BA} = \phi_{AC}/\phi_C$, $K_{BC} = \phi_{AC}/\phi_A$ etc. Finally, the dissociation constants for the binary complexes are $K_A = \phi_{ABC}/\phi_{BC}$, $K_B = \phi_{ABC}/\phi_{AC}$ and $K_C = \phi_{ABC}/\phi_{AB}$.

For the general case of this mechanism, in which the binding of each substrate may affect the affinity of the enzyme for the other, there are no relationships between the eight kinetic coefficients themselves. The coefficient ϕ_{ABC} , and the corresponding initial-rate parameter for the reverse reaction, are related to the overall equilibrium constant, K_0 , for reaction (1) by the Haldane relation $K_0 = \phi_{PQ}/\phi_{ABC}$ if there are two substrates in the reverse reaction,

and $K_0 = \phi_{PQR}/\phi_{ABC}$ if there are three. (It is readily shown that this is true whether the reverse reaction occurs by a compulsory-order mechanism or by an equilibrium random-order mechanism, although in the former case ϕ_0 will be a function of k and of velocity constants in the product phase of the reaction.)

Independent binding of the substrates gives rise to various special cases, which may be detected by

may be difficult in practice to be sure whether a term such as $\phi_{AC}/[A][C]$ is really absent from the rate equation or whether it is merely small relative to other terms over the accessible ranges of substrate concentrations. It is important, therefore, that there are relations between the other initial-rate parameters which must be satisfied if this mechanism applies.

The mechanism is:

Mechanism I(b)

relations between some of the initial-rate parameters, or, less objectively, by the point of intersection of the secondary plots. For example, if the binding of B to the enzyme does not affect its affinity for C, so that $K_{BC} = K_C$ (and $K_{CB} = K_B$), then $\phi_{AC}/\phi_A = \phi_{ABC}/\phi_{AB}$. Then the slopes of primary plots of v/v_0 against $1/[A]$, when plotted against $1/[B]$ (or $1/[C]$), will intersect on the $1/[B]$ (or $1/[C]$) axis, as can readily be shown from Table 1. The other possibilities of independent binding may be easily characterized, most objectively by comparing the values of all the dissociation constants derived from estimates of the initial-rate parameters, and need not be elaborated. The same Haldane relation, $K = \phi_{PQ(R)}/\phi_{ABC}$, applies to all these special cases as well as to the general case, but as a result of the relationships between some of the kinetic coefficients there are additional Haldane relations for the various special cases. The foregoing considerations are quite analogous to the random-order mechanism for a two-substrate reaction, and as in that case the additional Haldane relations are a less reliable test of independent binding than are the relations between the coefficients that give rise to them (Dalziel, 1957).

(b) *Compulsory-order mechanism.* If the quaternary complex EABC is formed by only one ordered combination of the three substrates, the initial-rate equation derived by the steady-state assumption is of the form of eqn. (1), except that one binary concentration term is absent. For example, if B is the second substrate to add, then the term $\phi_{AC}/[A][C]$ is missing from the initial-rate equation. In contrast with the situation for two-substrate reactions therefore, a compulsory-order mechanism can in principle be distinguished from an equilibrium random-order mechanism by initial-rate studies alone (Frieden, 1959). It should be emphasized that this distinction can only be made if rate measurements are made with small concentrations of all three substrates, so that all eight kinetic coefficients can be evaluated as described above. Moreover, it

The kinetic coefficients in the general initial-rate eqn. (1) for this mechanism are shown as functions of the velocity constants in Table 2. It is assumed for simplicity that there are only two products; if there are three, the initial-rate equation retains the same form.

The following experimental criteria are predicted for this mechanism:

(1) $\phi_{AC} = 0$.

(2) $\phi_{ABC}/\phi_{BC} = \phi_{AB}/\phi_B (= k_{-1}/k_{+1}$, the dissociation constant of EA). This relationship between four initial-rate parameters is shared by the special case of a random-order mechanism in which A and C bind to the enzyme independently of one another.

(3) $\phi_{ABC}/\phi_{BC}\phi_A = \phi_{AB}/\phi_A\phi_B = k_{-1}$ and $\phi_{ABC}/\phi_{AB}\phi_C = \phi_{BC}/\phi_B\phi_C = k_{-2}$. Since these rate constants must be greater than or equal to the maximum specific rate of the reverse reaction ($1/\phi'_0$), the following relations analogous to the 'maximum rate relation' for a two-substrate reaction (Dalziel, 1957) must be satisfied:

$$\phi'_0\phi_{AB}/\phi_A\phi_B \geq 1 \text{ and } \phi'_0\phi_{BC}/\phi_B\phi_C \geq 1$$

The equalities will apply in the special case of a compulsory-order mechanism in which the rate of dissociation of A from EA, or of B from EAB, is the rate-limiting step of the reverse reaction under maximum rate conditions, analogous to a Theorell-Chance mechanism for a two-substrate reaction.

(4) ϕ_A and ϕ_B are the reciprocals of individual velocity constants. If the enzyme can use alternative substrates in the same mechanism, ϕ_B should be independent of the nature of C, and ϕ_A of the nature of B and C. It may also be possible to estimate these individual velocity constants directly by stoichiometric studies of the reaction of the enzyme with A in the absence of B and C, or of EA with B in the absence of C, by using rapid reaction techniques.

(5) This mechanism shares the same Haldane relation as a random-order mechanism (Table 2), but there are also additional Haldane relations

two of the three substrates have been proposed on the basis of isotope-exchange studies of several ligases, which catalyse the fission of ATP to ADP or AMP coupled with bond formation between the other two substrates. These mechanisms are distinguished from quaternary-complex mechanisms by the absence of the last term of eqn. (1) from their rate equations, i.e. $\phi_{ABC} = 0$. Distinctions within this group of mechanisms may be made by the absence of other terms also.

(a) *Triple-transfer mechanism.* The enzyme may react with each substrate in turn with the formation of a product and two substituted enzyme species (E' and E''). For simplicity only one enzyme-substrate complex will be postulated in each of the three steps, but the form of the rate equation is unchanged if two or more isomeric complexes are included.

Mechanism II(a)

The initial-rate equation derived by steady-state analysis consists of only four terms (Fromm, 1967), the three binary and the ternary concentration terms of eqn. (1) being absent (Table 2). Consequently, Lineweaver-Burk plots with any one of the three substrates as variable, and several fixed concentrations of the other two, will be parallel. The mechanism is symmetrical, and the initial-rate equation for the reverse reaction has the same form. It is analogous to the double-displacement mechanism for two-substrate reactions (Doudoroff, Barker & Hassid, 1947; Alberty, 1953), and has been proposed for γ -glutamylcysteine synthetase by Webster & Varner (1954) from isotope-exchange studies:

(b) *Concerted-substitution mechanism.* By a concerted reaction of the enzyme with two of the three substrates, a single substituted enzyme may be formed that then reacts with the third substrate in a separate step. The enzyme-substitution step may occur by either ordered or random addition of the first two substrates; and either one or two products may be formed in this step.

If only one product is liberated in the enzyme-substitution step the mechanism will be symmetrical, and is that suggested by Berg (1956) for acetyl-CoA synthetase. Assuming that the first two substrates react in an ordered manner, the mechanism is:

Mechanism II(b)

In the Berg mechanism, for example, A and B are ATP and acetate, P and Q are AMP and acetyl-CoA, C and R are CoA and pyrophosphate, and E is the acetyl-AMP compound of the enzyme. The rate equation obtained by steady-state treatment (Fromm, 1967) is of the same form as eqn. (1), but lacks two of the binary concentration terms and the ternary concentration term. The kinetic coefficients are given in Table 2. If the enzyme-substitution step occurs by random addition of A and B to the enzyme, and if k_5 is small so that the steady-state concentration of EA, EB and EAB can be derived by the equilibrium assumption, the rate equation retains the same form with the kinetic coefficients shown in Table 2 in terms of dissociation constants and rate constants. This mechanism can therefore be distinguished from all those considered above, and the two substrates involved in the concerted step may be identified. The differences between the compulsory-order and equilibrium-random-order cases are the same as for a two-substrate reaction (Dalziel, 1957) and need not be elaborated; for example, there is a maximum rate relation for the compulsory-order mechanism. There are also special cases of a Theorell-Chance mechanism and, for the random-order case, of independent binding of A and B.

If two products are liberated in the concerted-enzyme-substitution step, the mechanism will be unsymmetrical:

Mechanism II(c)

Such a mechanism has been proposed for propionyl-CoA carboxylase (Kaziro & Ochoa, 1964),

for which A and B are ATP and bicarbonate, R and Q are phosphate and ADP, E' is the enzyme-CO₂ compound, and C and P are propionyl-CoA and methylmalonyl-CoA. The initial-rate equation for the forward reaction obtained by steady-state analysis of Mechanism II(c) (Fromm, 1967) and that obtained by equilibrium treatment of the analogous mechanism in which A and B combine randomly are of the same form as for Mechanism II(b), as shown in Table 2.

For consistency, it is convenient to represent the reverse of Mechanism II(c) as follows:

Mechanisms of this kind, in which A and R are ATP and ADP, and E' is phosphorylated enzyme, have been suggested for succinyl-CoA synthetase (Kaufman, 1955) and for formyltetrahydrofolate synthetase (Jaenicke & Brode, 1961). The initial-rate equations for both the compulsory-order (Fromm, 1967) and equilibrium-random-order cases are again of the same form as for Mechanism II(b).

Thus the concerted enzyme-substitution mechanisms cannot be distinguished from one another by initial-rate studies alone, but they can be distinguished from the triple-transfer mechanism and from quaternary-complex mechanisms, and the two substrates involved in the concerted step can be identified.

DISCUSSION

The main objectives of this work were to attempt a classification of likely reaction sequences for reactions involving three substrates, to outline a simple practical method for treating initial-rate data and evaluating empirical parameters that will describe the data, and consider the extent to which such investigations will help to define a reaction sequence. As with two-substrate systems, the reliability with which the constants can be evaluated will depend in any particular case on their relative values and restrictions on the useful ranges of concentrations of the substrates imposed by the sensitivity of the method of measurement or the occurrence of substrate inhibition or activation at high concentrations. Nevertheless, as is now well recognized for two-substrate systems, it is preferable to attempt

a complete analysis by simultaneous variation of the concentrations of all the substrates than to base conclusions about mechanism on more limited data. In principle, five types of mechanisms can be distinguished experimentally: three quaternary-complex mechanisms, namely fully-compulsory-order and rapid-equilibrium cases of a fully-random and a partially-random mechanism with an obligatory leading substrate; and two types of enzyme-substitution mechanism, the triple-transfer and concerted-substitution mechanisms.

In the first instance these mechanisms can be distinguished by the absence from the rate equation of one or more of the eight terms that appear in the most general form of rate equation, applicable to an equilibrium-random-order mechanism. The individual rate equations indicate further means by which a tentative mechanism may be tested. These have not been elaborated in full, but are well known from studies of two-substrate systems. For example, for compulsory-order mechanisms there are relations between initial-rate parameters for forward and reverse reactions, and some initial-rate parameters are reciprocals of individual rate constants and should be unchanged when alternative substrates are used (Dalziel, 1957, 1962; Dalziel & Dickinson, 1966). Product inhibition has not been considered, but would obviously be of value for distinguishing between random and compulsory-order mechanisms, and also between the different concerted-enzyme-substitution ('ping-pong') mechanisms that give the same forms of initial-rate equation (Alberty, 1958; Cleland, 1963). Studies of isotope exchange at equilibrium that have proved valuable for two-substrate systems (Silverstein & Boyer, 1964) might also be applied. Fromm (1967) has shown how the kinetic effects of competitive inhibitors for each substrate might be used to distinguish between some of the mechanisms considered, without evaluating all the initial-rate parameters in the absence of inhibitors. However, suitable inhibitors may not always be available, and it is also difficult to predict the mechanism of action of a particular inhibitor. This is indeed usually deduced from the effects of an inhibitor on each of the initial-rate parameters.

Other quantitative studies that are of particular value in association with initial-rate investigations include the direct determination of dissociation constants of enzyme-substrate compounds, and measurement of the rates of partial reaction with stoichiometric amounts of enzyme by rapid reaction techniques.

Isotope-exchange studies have so far provided most of the available evidence on the enzyme-substitution mechanisms of ligases (cf. Dixon & Webb, 1964). However, the qualitative demonstration of exchange reactions does not establish that they

are steps in the overall catalytic reaction, and misleading results can also be obtained if the enzyme preparation contains unsuspected traces of substrates. For these reasons the characteristic initial-rate behaviour predicted for the enzyme-substitution mechanisms would prove valuable corroborative evidence of their validity.

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