Studies on the Mechanism of Enzyme-Catalyzed Oxidation Reduction Reactions. II. Methods for Characterization of the Mechanism for Two-Substrate Systems*

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Received August 11, 1961

A least-squares treatment of kinetic data, including error calculations, suitable for programming on the IBM-650 computer and designed for two stoichiometric and one catalytic component, is presented. This treatment is applicable to a variety of enzyme-catalyzed reactions involving a single enzyme and substrate plus either an inhibitor or a co-substrate or coenzyme. Methods suitable for the selection of a most-likely mechanism among a group which gives rise to the Dalziel initial-rate equation $v_0/E_0 = \varphi_0 + \varphi_1/A + \varphi_2/B + \varphi_{12}/AB$ are evaluated. Two new sets of criteria, one involving kinetic isotope effects and another the use of continuous steady state rate equations, have been developed. The latter required the formulation of the appropriate expressions for both the general equilibrium and steady state cases which give rise to the Dalziel equation.

Bisubstrate Kinetics

Equation (1), the fundamental relation between the observed initial rate of an enzyme-catalyzed reaction (v_0) and the concentration of a single substrate, was first derived by Henri (1903) and

$$v_0 = V_{max.}/(1 + K_M/S) \qquad (1)$$

Michaelis and Menten (1913). It was transformed into a variety of linear forms, more suitable for the treatment of experimental data and for the evaluation of the two kinetic parameters, K_M and $V_{max.}$, by Lineweaver and Burk (1934), Eadie (1952), Augustinsson (1948), and Hofstee (1956). For a more complete discussion of the utility and limitations of single-substrate enzyme kinetics and its implications with regard to mechanism, the reader is referred to the contributions by King (1956), Dixon and Webb (1958), Laidler (1958), Segal (1959), Alberty (1959), Hearon *et al.* (1959), and Reiner (1959).

The treatment of these authors has been extended to substrate plus modifier and two-substrate systems by Hunter and Downs (1945), Ingraham and Makower (1945), Segal *et al.* (1952), Botts and Morales (1953), Alberty (1953), Friedenwald and Maengwyn-Davies (1954), Laidler (1956, 1958), King and Altman (1956), Dalziel (1957), and Hearon *et al.* (1959). The formulation of the initial-rate equation by Dalziel (1957) is particularly simple, and its interpretation in terms of a number of allowed mechanisms has been dissussed *in extenso* by that investigator. It is shown here as equation (2), where v_0 is again the observed initial velocity, E_0 is the original stoichiometric enzyme concentration, A and B are the stoichiometric concentrations of the two substrates added initially, and the φ_i values are constants analogous to Michaelis' K_M. Graphical methods of determining the constants have been proposed by several investigators, but only in the methods of Alberty (1953) and Dalziel (1957) are all four constants evaluated.

$$E_0/v_0 = \varphi_0 + \varphi_1/A + \varphi_2/B + \varphi_{12}/AB \qquad (2)$$

It seemed desirable to us to place the treatment of data for those enzyme-catalyzed reactions which obey equation (2) on the firmer basis provided by a regression analysis (Snedecor, 1946; Mood, 1950; Fisher, 1958). In this manner it is possible to obtain, free of any possible bias, results which are more precise and reliable than those available by graphical methods, together with an estimate of the errors inherent in the calculation of any of the derived parameters. While this investigation was in progress a short communication by Abrash *et al.* (1960) appeared dealing with a program for the Datatron 220 digital computer of a least-squares treatment of equation (1).

Equation (2) was programmed for Indiana University's IBM-650 computer so that data can be fed into it in the following manner (Baker, 1960): Experimentally, initial rates, v_{ij}, are determined at a constant enzyme concentration, E_0, over a range of substrate concentrations, A_i and B_j. Up to ten values of both A_i and B_j may be utilized, producing the matrix, Table I. If n is the total number of A's and m the total number of B's used, it is apparent that $n + m \geq 2$, but there is no requirement that $n = m$. Taking reciprocals of the various concentration terms, we define the relation-

TABLE I

MATRIX OF SUBSTRATE CONCENTRATIONS FOR INITIAL RATE MEASUREMENTS

	A_1	A_2	A_3	→	A_{10}
B_1	$v_{1,1}$	$v_{1,2}$	$v_{1,3}$	→	$v_{1,10}$
B_2	$v_{2,1}$	$v_{2,2}$	$v_{2,3}$	→	$v_{2,10}$
B_3	$v_{3,1}$	$v_{3,2}$	$v_{3,3}$	→	$v_{3,10}$
↓	↓	↓	↓		↓
B_{10}	$v_{10,1}$	$v_{10,2}$	$v_{10,3}$	→	$v_{10,10}$

* For paper I, see Mahler and Douglas (1957).

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‡ Supported in part by a program grant of the American Cancer Society, Inc.

§ Contribution No. 1028.

TABLE II
COMPUTATION OF PARAMETERS FOR EQUATIONS (4) AND (5)

	φ_0	φ_1
C	$\sum_{j=1}^{m} Y_j \cdot \sum_{i=1}^{n} X_i \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i \cdot Y_j$	$-N \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i \cdot Y_j$
D	$\sum_{i=1}^{n} X_i^2 \cdot \sum_{j=1}^{m} Y_j^2 \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji}$	$-\sum_{i=1}^{n} X_i \cdot \sum_{j=1}^{m} Y_j^2 \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji}$
F	$-\sum_{i=1}^{n} X_i \cdot \sum_{j=1}^{m} Y_j^2 \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i$	$N \sum_{j=1}^{m} Y_j^2 \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i$
G	$-\sum_{i=1}^{n} X_i^2 \cdot \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot Y_j$	$\sum_{i=1}^{n} X_i \cdot \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot Y_j$
H	$\sum_{i=1}^{n} X_i^2 \cdot \sum_{j=1}^{m} Y_j^2$	$N \cdot \sum_{j=1}^{m} Y_j^2$

	φ_2	φ_{12}
	$-M \sum_{i=1}^{n} X_i \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i \cdot Y_j$	$M \cdot N \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i \cdot Y_j$
	$-\sum_{i=1}^{n} X_i^2 \cdot \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji}$	$\sum_{i=1}^{n} X_i \cdot \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji}$
	$\sum_{i=1}^{n} X_i \cdot \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i$	$-N \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot X_i$
	$M \sum_{i=1}^{n} X_i^2 \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot Y$	$-M \cdot \sum_{j=1}^{m} Y_j \cdot \sum_{j=1}^{m} \sum_{i=1}^{n} E_{ji} \cdot Y_j$
	$M \cdot \sum_{i=1}^{n} X_i^2$	$N \cdot M$

ships shown as (3); the solutions for the various φ's of equation (2) then are of the type shown as equation (4).

$$X_i \equiv 1/A_i, \quad Y_j \equiv 1/B_j, \quad E_0/v_{ij} = E_{ij} \qquad (3)$$

$$\varphi_p = \frac{C_p + D_p + F_p + G_p}{\text{denominator}} \qquad (4)$$

If we assume that the only experimental error is in the measurement of the velocities v_{ij} and that there is none in the determination of the stoichiometric quantities E_0, X_i, and Y_j, the variances (the square of the standard error) are given by equation (5).

$$\sigma^2\varphi_p = \frac{\sigma^2 E \cdot H_p}{\text{denominator}} \qquad (5)$$

where $$\sigma^2 E = \frac{\Sigma[E_{ij(obs.)} - E_{ij(calc.)}]^2}{nm - 2}$$

The denominators in equations (4) and (5) are identical and given by equation (6).

$$\text{denominator} = \left[n \sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i \right)^2 \right] \cdot \left[m \sum_{j=1}^{m} y_j^2 - \left(\sum_{j=1}^{m} y_j \right)^2 \right] \qquad (6)$$

The values of C_p, D_p, F_p, G_p, and H_p used are summarized in Table II. The time for a complete computation on the IBM-650 is $5(n + m)$ seconds.

INHIBITION KINETICS

The kinetic equations for inhibition of single-substrate systems are in general to be regarded as modifications of the simple model first proposed by Michaelis and Pechstein (1914). Suitable mechanisms have been formulated in various ways by a number of different authors (Hunter and Downs, 1945; Segal *et al.*, 1952; Botts and Morales, 1953; Friedenwald and Maengwyn-Davies, 1954; Ogston, 1955; Dixon and Webb, 1958; Laidler, 1958; Reiner, 1959; Segal, 1959; Hearon *et al.*, 1959, Alberty, 1959). One of a number of possible sequences involving two binding sites on the enzyme, one for S, the substrate, and one for I, the inhibitor, is indicated in equations (7) (a) through (i).

$$\text{(a)}\quad E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (7)$$

$$\text{(b)}\quad E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI$$

$$\text{(c)}\quad ES + I \underset{k_7}{\overset{k_6}{\rightleftharpoons}} ESI$$

$$\text{(d)}\quad EI + S \underset{k_9}{\overset{k_8}{\rightleftharpoons}} ESI$$

$$\text{(e)}\quad ESI \xrightarrow{k_{10}} E + I + P$$

(f) $K_s' = (k_2 + k_3)/k_1$

(g) $K_i' = k_5/k_4$

(h) $K_i'' = k_7/k_6$

(i) $K_s'' = k_9/k_8$

Provided that quasi-equilibrium conditions (Laidler 1958; Hearon *et al.*, 1959) obtain or that, less

stringently, $K_s'/K_s'' = K_i'/K_i''$, the rate law for v_i, the initial velocities in the presence of inhibitor, is given by equation (8),

$$v_i = \frac{E_0(k_3S/K_s' + k_{10}IS/K_i''K_s')}{1 + I/K_i' + S/K_s' + SI/K_i''K_s'} \qquad (8)$$

where S and I are the total stoichiometric concentrations of substrate and inhibitor respectively. If we assume that k_{10} is either zero or $\ll k_3$, equation (8) can be simplified to yield equation (9).

$$E_0v_i = 1/k_3 + K_s'/k_3S + I/k_3K_i'' + IK_s'/k_3K_i'S \qquad (9)$$

The basic similarity of equations (9) and (2) is self-evident (see also Dalziel, 1957). Thus inhibition data can be treated in a manner entirely analogous to two-substrate data. Then, having computed the φ values of equation (9) [and thus explicitly the K's of equation (7) (f-i)] with their respective errors, an objective characterization of inhibition type becomes possible by use of the criteria set forth in Table III.

TABLE III

CHARACTERIZATION OF INHIBITION TYPE

Inhibition Type	Characteristic
Competitive	$K_s'' = K_i'' = \infty$; $\varphi_1 \simeq 0$
Competitive-noncompetitive transition	$K_s'' > K_s'$ and $K_i'' > K_i'$
Noncompetitive	$K_s'' = K_s'$ and $K_i'' = K_i'$
Noncompetitive-uncompetitive transition	$K_s'' < K_s'$ and $K_i'' < K_i'$
Uncompetitive	$K_i' = \infty$; $\varphi_{12} \simeq 0$

CHARACTERIZATION OF MECHANISM

As indicated earlier, a variety of possible mechanisms ("homeomorphs") generate a rate law identical with equation (2) susceptible to treatment in the manner indicated. Means must therefore be available to the investigator to permit him to select a single most likely, or at least a subclass of several most likely, mechanisms from the large class of possible ones. Among the more important mechanisms enumerated by Dalziel (1957) are those summarized in Table IV, all of which give rise to a rate expression in which the φ values of equation (2) are non-zero.

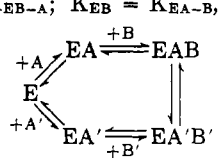
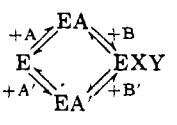
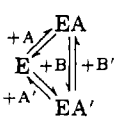
It is of some interest that in all of the six mechanisms shown in Table IV, the dissociation constants for at least one each of the binary enzyme-reactant and enzyme-product complexes may be obtained from the φ values. In both mechanisms of type I, all pertinent dissociation constants are obtained readily. If a type II mechanism is operative, the dissociation constants in question involve the complexes with the "leading" substrate (*i.e.*, the coenzyme in most coenzyme-requiring reactions). The appropriate dissociation constants for the complexes EA and EA′ are then given (Frieden, 1957) by the ratios φ_{12}/φ_2 and φ_{12}'/φ_2'. In this case it is possible, furthermore, to compute the individual rate constants for the binding and dissociation of the two binary complexes between the "leading" substrate (or product) with the enzyme (Dalziel, 1957).

If the reaction in question can be studied in both directions a total of eight φ values may be evaluated. From their values and certain relationships between them a choice between mechanisms is possible based on the following criteria.

"Haldane Relationships" (Alberty, 1953; Dalziel, 1957).—(a) The equilibrium constant for the over-all reaction determined independently must equal $\varphi_{12}'/\varphi_{12}$ in all cases. (b) Using all eight φ

TABLE IV

HOMEOMORPHIC MECHANISMS GENERATING THE RATE LAW OF EQUATION (2)
(AFTER DALZIEL, 1957)

Mechanism	Type	Characteristic[a]
Ia	Equilibrium "dependent" case	$E + A = EA$; $EA + B = EAB$; $E + B = EB$; $EB + A = EAB$ } rapid and reversible; $EAB \rightleftharpoons EA'B'$ (rate limiting); $EA'B' = EA' + B'$; $EA' = E + A'$; $EA'B' = EB' + A$; $EB' = E + B'$ } rapid and reversible; $K_{EA} \neq K_{EB-A}$; $K_{EB} \neq K_{EA-B}$, etc.
b	Equilibrium "independent" case	mechanism as before, but $K_{EA} = K_{EB-A}$; $K_{EB} = K_{EA-B}$, etc.
IIa	Steady state with compulsory binding order (A, A′ = leading substrates) two binary, two ternary complexes	
b	as before, but only one ternary complex is kinetically significant	
c	as before, but K_{diss} for EXY in either direction $\gg K_{diss}$ of either EA or EA′, respectively	as before but V_{max} (forward) $= K_{diss}$ EA′; V_{max} (reverse) $= K_{diss}$ EA
d	as before, but only the two binary complexes are kinetically significant, Theorell-Chance (1951) mechanism	

[a] Unprimed parameters are characteristic of the forward, primed of the reverse reaction.

values, case Ib and the group (IIc or IId) may be distinguished from each other and from all other mechanisms. Distinction between Ia, IIa, and IIb is not possible.

"Dalziel Relationships" (Dalziel, 1957).—From each set of four φ values (forward or reverse) independently a distinction is possible between case Ib, the group (IIa or IIb), and the group (IIc or IId). Case Ia is indeterminate.

"Product Inhibition."—Both Alberty (1958) and Dalziel (1957) have expanded the initial-rate equations for the two-substrate case to incorporate product inhibition. Alberty has proposed obtaining initial rates for the following cases, *i.e.*, by varying reactant concentration and adding one of the products at a known and constant concentration.

$$\begin{aligned} &\text{(a)}\quad A + B + E \longrightarrow A' \\ &\text{(b)}\quad A + B + E \longrightarrow B' \\ &\text{(c)}\quad A' + B' + E \longrightarrow A \\ &\text{(d)}\quad A' + B' + E \longrightarrow B \end{aligned} \qquad (10)$$

where A and A′ represent the "leading" substrate or coenzyme and all the species shown are added at zero-time. Under these conditions the initial-rate equation (2) is modified to equation (11)

$$E_0/v_0 = \Delta_0\varphi_0 + \Delta_1\varphi_1/A + \Delta_2\varphi_2/B + \Delta_{12}\varphi_{12}/AB \qquad (11)$$

where the Δ values are mechanism-dependent constants defined by equation (12), and A and B again represent the concentrations of the respective substrates.

$$\varphi_p\,(\text{inhibited})/\varphi_p\,(\text{uninhibited}) = \Delta_p \qquad (12)$$

The mechanism-dependence of the Δ values is shown in Table V.

TABLE V
MECHANISM CHARACTERIZATION BY PRODUCT INHIBITION

Mechanism	Inhibitor Added	Identities	Equal to Unity	Not Equal to Unity
Ia and Ib	A′ or B′		Δ_0, Δ_1, Δ_2	Δ_{12}
IIa, IIb, and IIc	A′	$\Delta_1 = \Delta_{12}$	Δ_0 and Δ_2	None
	B′	$\Delta_2 = \Delta_{12}$	Δ_1	Δ_0
IId	A′	$\Delta_1 = \Delta_{12}$	Δ_0 and Δ_2	None
	B′	$\Delta_2 = \Delta_{12}$	Δ_0 and Δ_1	None

This method of selection is capable of distinguishing between the groups shown and permits a choice between cases IIc and IId, which cannot be distinguished by any other criterion. At best, however, the method perhaps requires excessive material and time.

From the discussion just presented it is clear that, at least in principle, an application of the three criteria cited should permit a choice between five of the six mechanisms allowed by adherence to equation (2) (no distinction between IIa and IIb is possible by any of the criteria described here). These characterizations depend on the use of groups of φ values (usually four or more in number) and the evaluation of certain equalities and inequalities between them. Since it has been our experience that the φ values generally have statistical errors ranging from 5 to 10%, it is a difficult task to set up meaningful and valid comparisons between groups (*i.e.*, quotients or products) of numbers with total errors as high as 30%. Therefore, we deemed it fruitful to devise methods of mechanism characterization which could be used in lieu of or in conjunction with the published techniques and which would be more specific and require fewer computed parameters in their application.

"Isotope Effect."—One such system is based on the kinetic isotope effect (Wiberg, 1955; Mahler and Douglas, 1957; Streitwieser, 1957; Shiner *et al.*, 1960; Melander, 1960), *i.e.*, a study of the effect on the various φ values of substituting deuterium for hydrogen in certain reactants at critical and specific positions. As an example of this method we may examine its application to a reaction which obeys the Theorell-Chance (1951) mechanism, set forth explicitly as equation (13).

$$E + DPNH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot DPNH \qquad (13)$$

$$E\cdot DPNH + H^+ + CH_3CHO \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot DPN^+ + CH_3CH_2OH$$

$$E\cdot DPN^+ \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + DPN^+$$

In the forward direction:

$$\varphi_0 = 1/k_5;\ \varphi_1 = 1/k_1;\ \varphi_2 = 1/k_3;\ \text{and}\ \varphi_{12} = k_2/k_3k_1 \qquad (14)$$

and in the reverse direction:

$$\varphi_0' = 1/k_2;\ \varphi_1' = 1/k_6;\ \varphi_2' = 1/k_4;\ \text{and}\ \varphi_{12}' = k_5/k_4k_6 \qquad (15)$$

Making no assumption whatever as to the actual magnitude of the kinetic isotope effects to be expected as a result of specific deuterium substitution except the obvious one, that in the absence of such substitution there can be no isotope effect, the following relations are obtained:

$$\text{By definition: } \varphi_p\,(\text{deuterated})/\varphi_p\,(\text{hydrogenated}) = \bar{\varphi}_p \qquad (16)$$

$$\bar{\varphi}_1' = 1,\ \bar{\varphi}_0 = 1,\ \bar{\varphi}_2' = \bar{\varphi}_{12}',\ \text{and}\ \bar{\varphi}_1\bar{\varphi}_2/\bar{\varphi}_{12} = \bar{\varphi}_0' \qquad (17)$$

The derivation of (17) is straightforward: For both $\bar{\varphi}_1'$ and $\bar{\varphi}_0$, the respective determinant rate constants k_5 and k_6 can show no isotope effect since the deuterium must have already been transferred in an earlier step. In $\bar{\varphi}_2'$, the rate k_4 is probably isotope-dependent, but the same effect is also present in $\bar{\varphi}_{12}'$ and to the same extent. Finally, $\bar{\varphi}_0'$ is dependent on a possible isotope effect on rate k_2, but this effect would be equal to that for the ratio $\bar{\varphi}_1\bar{\varphi}_2/\bar{\varphi}_{12}$. Therefore, the Theorell-Chance mechanism is characterizad by a very specific type of isotope effect on φ_p.

In a similar manner, the isotope effects on the φ_p for other mechanisms for reversible oxidation-reduction reactions involving pyridine nucleotides and analogous hydrogen-transfer reactions may be derived and are tabulated in Table VI.

"Continuous Steady State Rate Equations."—The evaluations of data discussed up to this point are all dependent on initial rate measurements, *i.e.*,

TABLE VI

MECHANISM CHARACTERIZATION BY ISOTOPE EFFECT

Mechanism	Identities	Equal to Unity
Ia and Ib	$\bar{\varphi}_0' = \bar{\varphi}_1'$; $\bar{\varphi}_2' = \bar{\varphi}_{12}'$	None
	$\bar{\varphi}_0 = \bar{\varphi}_2$; $\bar{\varphi}_1 = \bar{\varphi}_{12}$	
IIa and IIb	$\bar{\varphi}_2' = \bar{\varphi}_{12}'$	$\bar{\varphi}_1'$
IIc and IId	$\bar{\varphi}_2' = \bar{\varphi}_{12}'$; $\bar{\varphi}_1\bar{\varphi}_2/\bar{\varphi}_{12} = \bar{\varphi}_0'$	$\bar{\varphi}_1'$, $\bar{\varphi}_0$

the measurement is made in such a way that the presence of product can be dealt with in a particularly simple manner, either as being virtually constant and zero or as being virtually constant and identical to the actual concentration added initially. Yet in the collection of initial rate data, at least as performed by us, *i.e.*, by recording spectrophotometry, the experimenter selects but one velocity (and an extrapolated velocity at that) from an arbitrarily large number of measured instantaneous velocities produced throughout the course of the reaction as the concentrations of the various reactants approach their equilibrium value. In principle the proper use of this continuum of velocity measurements should permit the derivation of all requisite kinetic parameters from a single, continuous rate run and thus provide all the information ordinarily requiring a whole series of the more conventional initial rate measurements. The analytical expression describing this continuum is known as the complete or continuous steady state rate equation. Its use and relation to the commonly used initial steady state velocity v_0, in the simple one-substrate system, has been discussed by Alberty (1959).

Such complete rate equations were derived independently by the use of the steady state approximation (King and Altman, 1957) for each of the six mechanisms previously mentioned. It was soon found, however, that four of these equations, along with the thirty-six initial rate equations cited by Alberty (1953) and Dalziel (1957), could all be derived[1] from the reversible rate equations [equations (19) and (21)] for the general mechanisms Ia and IIa shown in equations (18) and (20).

Mechanism Ia (18)

$$\begin{array}{llll} E + A \rightleftharpoons EA & K_1 & EB' \rightleftharpoons E + B' & K_5 \\ E + B \rightleftharpoons EB & K_2 & EA' \rightleftharpoons E + A' & K_6 \\ EA + B \rightleftharpoons EAB & K_3 & EA'B' \rightleftharpoons EA' + B' & K_7 \\ EB + A \rightleftharpoons EAB & K_4 & EA'B' \rightleftharpoons EB' + A' & K_8 \end{array}$$

$$EAB \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA'B'$$

Continuous Steady State Rate Equation (19)

$$v/E_0 = [k_1AB/(AB + K_4B + K_3A + K_1K_3 + K_1K_3A'/K_6 + K_1K_3B'/K_5 + K_1K_3A'B'/K_6K_7)] - [k_2A'B'/(A'B' + K_8B' + K_7A' + K_4K_7 + K_6K_7A/K_1 + K_6K_7B/K_2 + K_6K_7AB/K_1K_3)]$$

where A, B, A′, and B′ are now the *instantaneous* concentrations of the various species at any time, t, characterized by the velocity v.

Mechanism IIa (20)

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \qquad EA' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E + A'$$

$$EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB \underset{k_8}{\overset{k_5}{\rightleftharpoons}} EA'B' \underset{k_5}{\overset{k_7}{\rightleftharpoons}} EA' + B'$$

Continuous Steady State Rate Equation (21)

$$v/E_0 = \frac{C_1 - C_2}{C_3 + C_4 + C_5 + C_6 + C_7 + C_8 + C_9 + C_{10} + C_{11} + C_{12} + C_{13}}$$

where:

$$\begin{aligned} C_1 &= k_1k_3k_5k_7k_9AB \\ C_2 &= k_2k_4k_6k_8k_{10}A'B' \\ C_3 &= k_2k_5(k_4k_8 + k_4k_7 + k_5k_7) \\ C_4 &= k_3k_5k_7k_9B \\ C_5 &= k_1A(k_4k_8k_9 + k_4k_7k_9 + k_5k_7k_9) \\ C_6 &= k_8Dk_2k_4k_6 \\ C_7 &= k_{10}C(k_2k_4k_6 + k_2k_4k_7 + k_2k_5k_7) \\ C_8 &= k_1k_4k_6k_8AB' \\ C_9 &= k_1k_3AB(k_6k_9 + k_7k_9 + k_8k_9 + k_5k_7) \\ C_{10} &= k_3k_5k_7k_{10}A'B \\ C_{11} &= k_8k_{10}A'B'(k_4k_6 + k_2k_6 + k_2k_4 + k_2k_8) \\ C_{12} &= k_3k_6k_{10}BA'B'(k_8 + k_5) \\ C_{13} &= k_1k_3k_8ABB'(k_5 + k_6) \end{aligned}$$

with A, B, A′, and B′ having the same meaning as described for equation (19). An equivalent form of this expression has been derived by Reiner (1959, p. 11).

In order to test these relationships a general formulation suitable for a description of the instantaneous velocity, v, as a function of the initial concentrations (A_0, B_0; A_0', B_0') and extent of reaction is required. This is easily accomplished by the use of the relations shown in equations (22) and (23) derived on the assumption that $A_0' = B_0' = 0$.

$$\text{If } A = A_0 - x,\ B = B_0 - x, \quad A' = x, \text{ and } B' = x, \text{ then} \tag{22}$$

$$\frac{dx}{dt} = v/E_0 = \frac{a_1x^2 + a_2(A_0 + B_0)x + A_0B_0}{b_1x^3 + b_2x^2 + b_3x + b_4} \tag{23}$$

The coefficients a_1, a_2, b_1, b_2, b_3, and b_4 are made up of large groups of rate of equilibrium constants. They are difficult to evaluate, but their composition is mechanism-dependent.[2] More important, however, is the fact that b_1 and b_2 may actually be zero in particular mechanisms[3] (relation 24).

Mechanisms	b values zero	b values finite and non-zero
IIa, IIb, IIc	...	b_1, b_2, b_3, b_4
Ia, Ib, IId	b_1	b_2, b_3, b_4

(24)

Thus it is possible to use instantaneous steady state rates and continuous rate equations to (a) provide an arbitrarily large number of points for the evaluation of kinetic parameters, (b) test

[1] By dividing the terms of equation (21) by those rate constants which are assumed to be very fast (and set equal to infinity), and applying Hospital's rule, the continuous steady state rate equations for the simpler mechanisms IIb, IIc, and IId are obtained. From these expressions the various initial rate equations may be obtained by setting the concentrations of appropriate products equal to zero.

[2] The generalized steady state rate equation for *all* two-substrate systems in terms of polynominals similar to equation (23) and its utility in the selection of specific mechanisms has been treated comprehensively and independently by Wong and Hanes (1962 and private communication).

[3] In one of the mechanisms described by Dalziel but not discussed here, for instance, $b_1 = b_2 = 0$ (Dalziel's mechanism III).

postulated mechanisms under conditions other than those of the usual initial rate measurements, and (c) aid in the selection of an appropriate set of possible mechanisms.

Acknowledgements

The authors wish to thank Prof. V. J. Shiner, Jr., for many helpful discussions and for advice in the preparation of the manuscript, and Prof. E. L. King for his suggestions. Mrs. Margaret Hanes provided capable technical assistance in many phases of this investigation.

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