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A Generalized Theoretical Treatment of the Transient-state Kinetics of Enzymic Reaction Systems Far from Equilibrium

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The transient-state kinetics of enzyme systems far from equilibrium have been analyzed by a theoretical treatment of the generalized King-Altman mechanism for enzyme reactions. Conditions are defined under which the kinetic differential equations for this generalized mechanism can be analytically solved, and the structure of the analytical solution is characterized. The practical implications of the theoretical results are discussed with reference to the planning, evaluation, and interpretation of transient-state kinetic experiments performed by stopped-flow techniques.

Enzyme kinetics is the most important and powerful technique available for the study of enzymic reaction mechanisms. Early investigations of the kinetics of enzymically catalyzed reactions were directed mainly towards the steady-state phase of catalysis. Steady-state kinetic data for enzyme reactions are evaluated and interpreted in terms of analytically derived rate equations, and the corresponding kinetic theory has been treated in considerable detail.[1-3] Methods are available for the derivation of steady-state rate equat ons for any enzyme reaction conforming to the generalized mechanism defined by King and Altman.[4] The general structure of such rate equations has been characterized by Wong *et al.* in their analysis of the steady-state rate behaviour inherent in the King-Altman mechanism,[5] and generalized statistical methods for the determination of empirical rate equations have been described.[6,7]

During the last 20 years, the application of rapid-reaction techniques for examination of the transient-state kinetics of enzyme reactions has become widespread. Stopped-flow techniques have proven to be of particular informative value in this respect, and are now routinely applied by enzymologists. Enzyme kinetic data obtained by stopped-flow techniques are only exceptionally evaluated and interpreted in terms of analytically derived relationships, however. The reason for this is that stopped-flow kinetic experiments are carried out in reaction systems far from equilibrium, and the transient-state rate behaviour of such systems has not been well-characterized from a theoretical point of view. The mathematical analysis of kinetic systems far from equilibrium is complex, and an exact analytical solution of the corresponding kinetic differential equations cannot be obtained even for the simplest possible Michaelis-Menten mechanism.[8] Approximate solutions of practical utility have been derived for some specific enzyme mechanisms of comparatively low complexity,[9-13] but it is not known to what extent and under which conditions such solutions may be obtained in the general case.

Since stopped-flow techniques are now extensively used in enzyme kinetic studies, a generalized treatment of the transient-state kinetic theory for enzyme systems far from equilibrium seems highly desirable. The present investigation provides such a treatment in the form of a theoretical analysis of the transient-state kinetic properties of the generalized King-Altman mechanism for enzyme reactions. Conditions are defined under which the pre-steady-state time-dependence of concentration variables in this mechanism can be analytically described, and the structure of the analytical solution is characterized. The practical implications of the results obtained are discussed.

RESULTS

Kinetic differential equations for the generalized King-Altman mechanism. Let us consider an enzyme mechanism involving $n+1$ enzyme-containing species EX_0, EX_1,...EX_n (including free enzyme), all of which are treated as convertible into one another. Reaction steps in the mechanism are assumed to be either monomolecular isomerizations of enzyme-containing species [eqn. (1)] or bimolecular reactions

$$EX_i \xrightarrow{k_{ij}} EX_j \qquad \varkappa_{ij} = k_{ij} \tag{1}$$

between an enzyme-containing species and a non-enzymic reactant R [eqn. (2)]. This mechanism is basically identical with the one discussed by King and Altman in their generalized discussion of steady-state rate equations for enzyme reactions.[4] Following the convention of these authors, we define kappas for the reaction steps as indicated in eqns. (1) and (2). To simplify the mathematical formalism we further define $\varkappa_{ii} = 0$.

$$EX_i + R \xrightarrow{k_{ij}} EX_j \qquad \varkappa_{ij} = k_{ij}[R] \tag{2}$$

The bimolecular reaction steps will be specified in more detail. Let us assume that there are s different kinds of non-enzymic reactants R_1, R_2, $\cdots$ R_s (substrates, products, inhibitors, or activators). The arbitrarily chosen reactant R_k is assumed to be involved in d_k bimolecular reaction steps; d_k represents the "degree" of the mechanism with respect to R_k in the terminology of Wong *et al.*[5] The time dependence of the concentration variable $[R_k]$ is then given by eqn. (3), summation being performed

$$\frac{d[R_k]}{dt} = \sum_{pq} (\varkappa_{qp}[EX_q] - \varkappa_{pq}[EX_p]) \tag{3}$$

over the d_k combinations of subscripts p and q which represent reaction steps involving association of R_k to a species EX_p with formation of a species EX_q. Equations similar to (3) are obtained for all non-enzymic reactants, *i.e.* for $k = 1, 2, \ldots s$.

The time dependence of concentrations of enzyme-containing species in the mechanism is governed by a set of $n+1$ differential equations [eqn. (4)]. These differential equations exhibit

$$\begin{aligned}
\frac{d[EX_0]}{dt} &= -\sum_{j=0}^{n} \varkappa_{0j}[EX_0] + \varkappa_{10}[EX_1] + \cdots + \varkappa_{n0}[EX_n] \\
\frac{d[EX_1]}{dt} &= \varkappa_{01}[EX_0] - \sum_{j=0}^{n} \varkappa_{1j}[EX_1] + \cdots + \varkappa_{n1}[EX_n] \\
&\vdots \\
\frac{d[EX_n]}{dt} &= \varkappa_{0n}[EX_0] + \varkappa_{1n}[EX_1] + \cdots - \sum_{j=0}^{n} \varkappa_{nj}[EX_n]
\end{aligned} \tag{4}$$

the linear dependence given in eqn. (5).

$$\sum_{i=0}^{n} \frac{d[EX_i]}{dt} = 0 \tag{5}$$

Integration of eqn. (5) gives the stoichiometric relationship for the total concentration (c_E) of enzyme [eqn. (6)], which can be used for

$$\sum_{i=0}^{n} [EX_i] = c_E \tag{6}$$

elimination of one of the concentration variables (say $[EX_0]$) in eqn. (4), giving a set of n linearly independent differential equations [eqn. (7)].

$$\begin{aligned}
\frac{d[EX_1]}{dt} &= \varkappa_{01}c_E - (\varkappa_{01} + \sum_{j=0}^{n} \varkappa_{1j})[EX_1] + \\
&+ (\varkappa_{21} - \varkappa_{01})[EX_2] + \cdots + (\varkappa_{n1} - \varkappa_{01})[EX_n] \\
\frac{d[EX_2]}{dt} &= \varkappa_{02}c_E + (\varkappa_{12} - \varkappa_{02})[EX_1] - \\
&- (\varkappa_{02} + \sum_{j=0}^{n} \varkappa_{2j})[EX_2] + \cdots + (\varkappa_{n2} - \varkappa_{02})[EX_n] \\
&\vdots \\
\frac{d[EX_n]}{dt} &= \varkappa_{0n}c_E + (\varkappa_{1n} - \varkappa_{0n})[EX_1] + \\
&+ (\varkappa_{2n} - \varkappa_{0n})[EX_2] + \cdots - (\varkappa_{0n} + \sum_{j=0}^{n} \varkappa_{nj})[EX_n]
\end{aligned} \tag{7}$$

All differential equations in eqn. (3), and at least some of those in eqn. (7), are non-linear, *i.e.* they include terms containing products $[R_k][EX_i]$ of various concentration variables. This means that there is no exact general solution to the set of differential equations governing the kinetics of the generalized enzyme mechanism.

A solution accounting for the steady-state rate behaviour of the system can be obtained by introduction of the approximations $d[EX_i]/dt = 0$, $i = 1, 2, \ldots n$. This solution has been

treated in detail by Wong *et al.*[5] and will not be considered here. Solutions accounting for the transient-state rate behaviour of the system can be obtained by the introduction of approximations leading to linearization of the kinetic differential equations, as will now be shown.

Linearization of the kinetic differential equations. Non-linearity resulting from the presence of terms containing the product $[R_k][EX_i]$ can be neglected when changes of either $[R_k]$ or $[EX_i]$ (or both) are negligibly small during the period of time considered in the transient-state kinetic experiments. In the kinetic systems near equilibrium examined by relaxation techniques (*e.g.* temperature-jump spectrometry), changes $\Delta[R_k]$ and $\Delta[EX_i]$ of all concentration variables can be kept small by a proper choice of perturbation conditions (*e.g.* small jumps in temperature). Under such conditions contributions from terms containing the product $\Delta[R_k]\Delta[EX_i]$ are negligibly small, and the kinetic differential equations can be transformed into a linear form without imposing additional restrictions on the system investigated. The corresponding solution of the differential equations has been described and discussed by Hammes *et al.*[8] and will not be considered here.

In systems far from equilibrium, however, several concentration variables may vary considerably in magnitude before steady-state conditions are attained. Linearization of the differential equations then requires the introduction of restrictions ensuring that pseudo first-order conditions prevail, *i.e.* ensuring that either $[R_k]$ or $[EX_i]$ remains essentially constant in case the differential equations contain the product of these concentration variables. The present treatment will be confined to the case where concentrations of the non-enzymic reactants $[R_k]$ are kept approximately constant. This case represents the more general one from a theoretical point of view, and serves well to illustrate all essential features of the transient-state kinetic behaviour inherent in enzyme systems operating far from equilibrium.

For this reason, we will use the approximations in eqn. (8) for linearization of the kinetic

$$[R_k]=[R_k]_0 \ (k=1,2,\ldots s) \tag{8}$$

differential equations, where $[R_k]_0$ denotes the initial ($t=0$) concentration of R_k. Application of eqn. (8) over the short periods of time considered in transient-state kinetic experiments is justified when initial concentrations of reactants are such that

$$[R_k]_0 \gg c_E \tag{9}$$

for all reactants being initially present in the reaction solution. Where R_k stands for a product and reactions are carried out in the initial absence of this product, the additional assumption has to be made that the amount of product accumulated during the short transient reaction phase is too small to cause any kinetically significant reversion of the step of product formation (kappas for the bimolecular reaction steps involving the product are assumed to remain negligibly small).

Analytical solution for the generalized King-Altman mechanism. When the approximations expressed by eqn. (8) are valid, all kappas can be regarded as constants. This means that eqn. (7) can be solved by standard methods of linear algebra.[14] The solution is of the form given in eqn. (10), where $[EX_i]_{ss}$ is the time-

$$[EX_i]=[EX_i]_{ss}+\sum_{j=0}^{n} A_{ij}e^{-r_j t} \ (i=0,1,\ldots n) \tag{10}$$

independent steady-state concentration of the enzyme-containing species EX_i. A_{ij} and r_j denote amplitude and rate parameters, respectively, for the exponential transients governing the kinetics of the system. When $t=0$, eqn. (10) reduces to eqn. (11), where $[EX_i]_0$ stands for

$$[EX_i]_0=[EX_i]_{ss}+\sum_{j=1}^{n} A_{ij} \ (i=0,1,..n) \tag{11}$$

the initial concentration of EX_i. This means that eqn. (10) can be written alternatively as in eqn. (12).

$$[EX_i]=[EX_i]_0+\sum_{j=1}^{n} A_{ij}(e^{-r_j t}-1) \ (i=0,1,\ldots n) \tag{12}$$

Although concentration changes of the non-enzymic reactants have been assumed to be sufficiently small to justify application of eqn. (8), they might be large enough to be experimentally observed. Insertion of eqn. (10) into eqn. (3) shows that the kinetic differential equation for the non-enzymic reactant R_k may be written as eqn. (13), where the constants v_k

$$\frac{d[R_k]}{dt}=v_k+\sum_{j=1}^{n} M_{kj}e^{-r_j t} \tag{13}$$

and M_{kj} are given by eqns. (14) and (15);

$$v_k = \sum_{pq} (\varkappa_{qp}[\mathrm{EX}_q]_{ss} - \varkappa_{pq}[\mathrm{EX}_p]_{ss}) \quad (14)$$

$$M_{kj} = \sum_{pq} (\varkappa_{qp}A_{jj} - \varkappa_{pq}A_{pj}) \quad (15)$$

summations over p and q being performed as in eqn. (3). An analytical solution for the time dependence of $[\mathrm{R}_k]$ can thus be obtained by integration of eqn. (13), which yields eqn.

$$[\mathrm{R}_k] = [\mathrm{R}_k]_0 + v_k t + \sum_{j=1}^{n} B_{kj}(e^{-r_j t} - 1) \quad (16)$$

(16), where amplitudes B_{kj} of the exponential transients are given by

$$B_{kj} = -\frac{M_{kj}}{r_j} \quad (17)$$

Since R_k in eqn. (3) represents an arbitrarily chosen non-enzymic reactant, solutions of the general form indicated by eqns. (14)–(17) are obtained for all non-enzymic reactants. By definition, and in consistence with eqn. (14), v_k equals the steady-state reaction velocity v when R_k stands for a product in a catalytic reaction mechanism. Similarly, we have $v_k = -v$ when R_k is a substrate and $v_k = 0$ when R_k is an inhibitor or an activator of a catalytic reaction. If the reaction mechanism considered does not involve any catalytic conversion of substrate(s) into product(s), v_k equals zero independently of the nature of the non-enzymic reactant.

Experimentally observed transients. The transient-state kinetics of enzyme systems far from equilibrium are usually studied by observation of an experimental parameter A (*e.g.* absorbance) which is linearly related to the concentrations of enzyme-containing species and non-enzymic reactants. Such a relationship can be expressed as in eqn. (18), where ε_i and ε_i' denote

$$A = \sum_{i=0}^{n} \varepsilon_i[\mathrm{EX}_i] + \sum_{k=1}^{s} \varepsilon_k'[\mathrm{R}_k] \quad (18)$$

the proportionality constants (*e.g.* absorption coefficients) for the dependence of A on the respective concentration variables.

Insertion of eqns. (12 and (16) into eqn. (18) gives eqn. (19), where A_0 stands for the initial value of A, and where the constants α and β_j are given by eqns. (20) and (21).

$$A - A_0 = \alpha t + \sum_{j=1}^{n} \beta_j(\mathrm{e}^{-r_j t} - 1) \quad (19)$$

$$\alpha = \sum_{k=1}^{s} \varepsilon_k' \cdot v_k \quad (20)$$

$$\beta_j = \sum_{i=0}^{n} \varepsilon_i A_{ij} + \sum_{k=1}^{s} \varepsilon_k' B_{kj} \quad (21)$$

Eqns. (19)–(21) provide an analytical expression for the time-dependence of the experimentally observed variable A under conditions where application of the approximations in eqn. (8) is justified.

Rate parameters for the exponential transients. Rate parameters r_j for the exponential transients governing the kinetics of the generalized enzyme mechanism represent the roots of the nth degree secular equation corresponding to eqn. (7). This secular equation will be written in the form given in eqn. (22) and can, according

$$r^n - p_{n-1}r^{n-1} + p_{n-2}r^{n-2} - \cdots + (-1)^{n-1}p_1 r + (-)^n p_0 = 0 \quad (22)$$

to the coefficient matrix of eqn. (7), be expressed as in eqn. (23).

$$\begin{vmatrix} r - (\varkappa_{01} + \sum_{j=0}^{n} \varkappa_{1j}) & \varkappa_{21} - \varkappa_{01} & \cdots & \varkappa_{n1} - \varkappa_{01} \\ \varkappa_{12} - \varkappa_{02} & r - (\varkappa_{02} + \sum_{j=0}^{n} \varkappa_{2j}) & \cdots & \varkappa_{n2} - \varkappa_{02} \\ \vdots & & & \\ \varkappa_{1n} - \varkappa_{0n} & \varkappa_{2n} - \varkappa_{0n} & \cdots & r - (\varkappa_{0n} + \sum_{j=0}^{n} \varkappa_{nj}) \end{vmatrix} = 0 \quad (23)$$

The coefficient p_0 in eqn. (22) is given by the determinant [eqn. (24)] obtained by putting $r = 0$ in the determinant in eqn. (23).

$$p_0 = \begin{vmatrix} -(\varkappa_{01} + \sum_{j=0}^{n} \varkappa_{1j}) & \varkappa_{21} - \varkappa_{01} & \cdots & \varkappa_{n1} - \varkappa_{01} \\ \varkappa_{12} - \varkappa_{02} & -(\varkappa_{02} + \sum_{j=0}^{n} \varkappa_{2j}) & \cdots & \varkappa_{n2} - \varkappa_{02} \\ \vdots & & & \\ \varkappa_{1n} - \varkappa_{0n} & \varkappa_{2n} - \varkappa_{0n} & \cdots & -(\varkappa_{0n} + \sum_{j=0}^{n} \varkappa_{nj}) \end{vmatrix} \quad (24)$$

Expansion of this determinant shows that p_0 can be expressed as a sum of products of n different kappas, such that all terms are positive and no term contains two directly opposing kappas (*e.g.* $\varkappa_{ij}$ and $\varkappa_{ji}$). We will use the abbre-

viated notation in eqn. (25) to express these characteristics of p_0. Subdeterminants obtained

$$p_0 = \sum^{n} \Pi \varkappa_{ij} \quad (25)$$

by expansion of the determinant in eqn. (24) by its diagonal elements exhibit the same basic characteristics, which means that

$$p_t = \sum^{n-t} \Pi \varkappa_{ij} \ (t=0,1,...n-1) \quad (26)$$

Eqn. (26), for $t=n-1$, states that p_{n-1} is given by a sum of kappas. This can be readily verified by examination of the determinant in eqn. (23). Expansion of this determinant by its diagonal elements shows that each term containing r^{n-1} must be a product of the r of $n-1$ of the diagonal elements and the constant part of the remaining diagonal element. This means that p_{n-1} is given by the sum of the constant parts of the diagonal elements, *i.e.* we

$$p_{n-1} = \sum_{i=1}^{n} (\varkappa_{0i} + \sum_{j=0}^{n} \varkappa_{ij}) = \sum_{i=0}^{n} \sum_{j=0}^{n} \varkappa_{ij} \quad (27)$$

have eqn. (27), which shows that p_{n-1} is actually given by the sum of all kappas in the mechanism. The elementary relationships between roots and coefficients in eqn. (22), further, prescribe that

$$p_{n-1} = \sum_{j=1}^{n} r_j \quad (28)$$

Eqns (27) – (28) confirm and provide proof for the supposition of Laidler *et al.*[12] that the sum of rate parameters r_j for the exponential transients appearing in enzyme reactions equals the sum of kappas in the reaction mechanism.

Kappa products in eqn. (26) may contain varying numbers of the $[R_k]$-containing kappas defined by the mechanism. Coefficients p_t in the secular equation, therefore, will take the form of polynomials in $[R_k]$ [eqn. (29)]. Since

$$p_t = \sum_{s=0}^{dt'} a_{ts} [R_k]^s \ (t=0,1,...n-1) \quad (29)$$

no kappa appears twice in any term of eqn. (26), the degree d_t' of the polynomial will equal or be less than the total number of $[R_k]$-containing kappas, which is given by the degree d_k of the mechanism with respect to R_k. Further, it is obvious that d_t' cannot exceed the number $(n-t)$ of kappas present in each kappa product of eqn. (26), *i.e.* we have eqn. (30). It follows

$$d_t' \leq \min (d_k, n-t) \quad (30)$$

from the definition of the symbolism in eqn. (26) that all coefficients a_{ts} in eqn. (29) are positive, and that no term in a_{ts} is negative or contains two directly opposing kappas.

It can be similarly shown that coefficients a_{ts} in eqn. (29) are polynomials in concentrations of non-enzymic reactants other than R_k (if present). If R_g $(g \neq k)$ denotes such a reactant, we thus have

$$a_{ts} = \sum_{m=0}^{dts'} a_{tsm} [R_g]^m \quad (31)$$

where

$$d_{ts}' \leq \min (d_g, n-t) \quad (32)$$

Approximate solution to the secular equation. Although eqn. (22) can be explixitely solved for $n<5$, the exact solutions obtained for $n>1$ contain root expressions and are inconvenient to handle. Approximate solutions of greater practical interest can be obtained under certain conditions. To illustrate this, we will assume that roots of the secular equation are real and differ considerably in magnitude (we arbitrarily define $r_1 < r_2 < \cdots < r_n$). The approximations in eqns. (31) and (32) are then valid according to the elementary relationships between roots and coefficients in eqn. (22). Eqns. (31) and (27)

$$r_n = p_{n-1} \quad (31)$$

$$r_j = \frac{p_{j-1}}{p_j} \ (j=1,2,\cdots n-1) \quad (32)$$

state that the rate parameter r_n for the fastest transient can be approximated as the sum of all kappas in the mechanism. Eqns. (32) and (26) state that other rate parameters are given by eqn. (33), *i.e.* they can be approximated as

$$r_j = \frac{\sum^{n-j+1} \Pi \varkappa_{ij}}{\sum^{n-j} \Pi \varkappa_{ij}} \ (j=1,2,\cdots n-1) \quad (33)$$

the quotient between two sums of kappa products (two polynomials in $[R_k]$), terms in the numerator containing one more kappa than those in the denominator.

The approximate solution for the rate parameter r_1 of the slowest transient can be anticipated to be of particular interest from a practical point of view. This solution is given

$$r_1 = \frac{p_0}{p_1} = \frac{\sum \prod^{n} \varkappa_{ij}}{\sum \prod^{n-1} \varkappa_{ij}} = \frac{\sum_{s=0}^{d_0'} a_{0s}[\mathrm{R}_k]^s}{\sum_{s=0}^{d_1'} a_{1s}[\mathrm{R}_k]^s} \quad (34)$$

by eqn. (34) and is valid as soon as $r_1 \ll r_j$ $(j=2,3,\ldots n)$, *i.e.* as soon as the rate of the slowest transient is much lower than rates of the remaining transients. It may be observed that the structure of eqn. (34) agrees closely with that of the steady-state rate equation for the generalized enzyme mechanism,[5] except that coefficients in the numerator polynomial of the steady-state rate equation in some cases may be negative whereas all terms in eqn. (34) are positive.

Amplitudes of the exponential transients. Eqn. (7) can be written more concisely as in eqn.

$$\frac{\mathrm{d}[\mathrm{EX}_i]}{\mathrm{d}t} = \varkappa_{0i} c_{\mathrm{E}} + \sum_{\substack{p=0 \\ p\neq i}}^{n} (\varkappa_{pi} - \varkappa_{0i})[\mathrm{EX}_p] - (\varkappa_{0i} + \sum_{t=0}^{n} \varkappa_{it})[\mathrm{EX}_i] \quad (i=1,2,\ldots n) \quad (35)$$

(35). Differentiation of eqn. (10) with respect to time gives the identical relationship in eqn.

$$\frac{\mathrm{d}[\mathrm{EX}_i]}{\mathrm{d}t} = -\sum_{j=1}^{n} r_j A_{ij} \mathrm{e}^{-r_j t} \quad (i=1,2,\ldots n) \quad (36)$$

(36). Insertion of eqn. (10) on the right-hand side of eqn. (35) and identification of coefficients for the exponential terms $(j=1,2,\ldots n)$ of the resulting expression with those in eqn. (36) shows that

$$\sum_{\substack{p=0 \\ p\neq i}}^{n} (\varkappa_{pi} - \varkappa_{0i}) A_{pj} + [r_j - (\varkappa_{0i} + \sum_{t=0}^{n} \varkappa_{it})] A_{ij} = 0 \quad (i=1,2,\ldots n;\ \ j=1,2,\ldots n) \quad (37)$$

Since r_j represents a root of eqn. (23), the n equations corresponding to any particular value of j in eqn. (37) are linearly dependent (the coefficient determinant for this set of equations equals zero). This means that any one of these n equations, say the one obtained for $i=1$, can be considered as "superfluous". The remaining $n(n-1)$ equations obtained by putting $i=2,3,\ldots n$ and $j=1,2,\ldots n$ in eqn. (37), together with the n equations obtained by putting $i=1,2,\ldots n$ in eqn. (12), constitute a set of n^2 linearly independent simultaneous equations which can be solved for the n^2 "unknowns" represented by the amplitudes A_{ij} $(i=1,2,\ldots n;\ j=1,2,\ldots n)$.

The generalized solution for A_{ij} can be explicitly expressed by the use of determinants, but this expression will not be considered here. It may suffice to point out that an explicit expression for each A_{ij} can be derived in the general case, and that this expression shows that $A_{i,}$ is dependent on kappas in the mechanism, as well as on initial conditions [the initial values of $[\mathrm{EX}_i]$ according to eqns. (11) and (12)]. The dependence on kappas is partly direct, and partly indirect through a dependence on rate parameters r_j for the transients [eqn. (37)].

Limiting amounts of non-enzymic reactants. Up to this point we have considered only the case where linearization of the kinetic differential equations is effected by introduction of the approximations in eqn. (8), *i.e.* where enzyme is used in limiting amounts to ensure that pseudo first-order conditions may prevail. In mechanisms where a non-enzymic reactant (say R_1) interacts with a single enzyme-containing species (say EX_0) linearization of the differential equations can be similarly effected using the approximations in eqns. (38) and (39).

$$[\mathrm{EX}_0] = [\mathrm{EX}_0]_0 \quad (38)$$

$$[\mathrm{R}_k] = [\mathrm{R}_k]_0 \quad (k=2,3,\ldots s) \quad (39)$$

Application of eqns. (38)–(39) requires that R_1 is used in limiting amounts, *i.e.* that other non-enzymic reactants and enzymes are present in large excess to R_1. Depending on the detailed nature of the reaction mechanism investigated, some additional restrictions may have to be imposed on the reaction system. For example, certain non-enzymic reactants may have to be used in saturating amounts to justify the application of eqn. (38).[13]

Solutions of the linear differential equations obtained using eqns. (38) and (39) are structurally analogous to those derived using eqn. (8) and will not be explicitly considered. It may suffice to point out that non-cycling conditions are obtained when a non-enzymic reactant is used in limiting amounts. This means that the analytical relationships derived

in the latter case usually take a less complex form than those obtained for cycling systems.

Non-general analytical solutions. Linearization of the kinetic differential equations is not necessarily required to obtain analytical solutions describing the transient-state kinetics of enzymic reaction systems. Other restrictions may be introduced to obtain solutions of an entirely different structure (solutions which do not involve exponential transients) in certain systems of low complexity. For example, analytical relationships for the binding of a single ligand R to a single enzymic site have been derived with the restrictive assumption that $c_R = c_E$.[15] Such specific analytical solutions are of little general interest and will not be considered here.

DISCUSSION

Almost all enzymic reaction mechanisms discussed in the literature represent special cases of the generalized King-Altman mechanism, as defined in the present investigation. The present results establish that an analytical description of the transient-state kinetics inherent in this generalized enzyme mechanism is possible as soon as the kinetic differential equations for the mechanism become approximately linear. In reaction systems far from equilibrium, linearization of the differential equations requires a proper choice of the initial concentrations of reactants. Either the enzyme or a non-enzymic reactant must be used in limiting amounts, small enough to ensure that pseudo first-order conditions prevail. Since the corresponding analytical solutions of the kinetic differential equations are structurally analogous, the present discussion will be confined to the more general case where enzyme concentration is limiting. It will, furthermore, be assumed that the enzymic reaction is monitored by stopped-flow spectrophotometry; extension of the discussion to other observation techniques is straight-forward.

The present investigation shows that the kinetic differential equations for the generalized enzyme mechanism can be analytically solved by application of the approximations expressed in eqn. (8). The validity of eqn. (8) is dependent upon the assumption that initial concentrations of non-enzymic reactants added to the reaction solution greatly exceed the total concentration of enzyme. If the reaction examined leads to the formation of a product that is not initially present in the reaction solution, the additional assumption is required that accumulation of this product may be neglected over the short period of time considered in transient-state kinetic experiments. These assumptions are identical with those required to justify the application of conventional steady-state rate equations to enzyme kinetic data.[1,2] Hence it may be concluded that the transient-state kinetic relationships derived in the present paper are of the same general validity and applicability as conventional steady-state rate equations. It should, in principle, be possible to carry out most transient-state kinetic studies of enzyme systems far from equilibrium under such conditions that an analytical description and treatment of the experimental data are justified.

It is, therefore, of interest to examine the structural characteristics of the analytical solution for the generalized enzyme mechanism considered in the present investigation. This solution is of the form indicated in eqns. (12) and (16), and prescribes that the time-dependence of each concentration variable in an enzyme mechanism involving $n+1$ enzyme-containing species is governed by n exponential transients with rate parameters $r_1, r_2, \ldots r_n$ which are the same for all concentration variables. The amplitude of a certain (the jth) transient, however, may attain different values (A_{ij}, $i = 0, 1, \ldots n$ and B_{ij}, $k = 1, 2, \ldots s$) for different concentration variables. This means that the extent to which a certain transient contributes to the time-dependence of different concentration variables may vary, but the rate of the transient will always be the same. In case the reaction examined involves a catalytic conversion of substrate(s) into product(s), the transient concentration changes will be superimposed on the steady-state rates of substrate consumption and product formation [eqn. (16)]. It follows from these structural properties of the analytical solution that the absorbance of the reaction solution must be dependent on n exponential transients with the same rates as those observed for the concentration variables, transient absorbance changes being super-

imposed on the steady-state absorbance changes [eqn. (19)].

The latter observation is of great practical interest, since it implies that transient-state kinetic data for enzyme reactions can be analytically evaluated in terms of a regression function of the unique form indicated in eqn. (19). Theoretically, the regression function should include the total number (n) of exponential transients defined by the mechanism ($n+1$ enzyme-containing species). The number of experimentally detectable transients, however, may be considerably lower. Some transients may be too rapid and/or exhibit too low amplitudes to be of kinetic significance under the actual experimental conditions. The minimal number of exponential transients required for an adequate description of the experimental data can be objectively determined by standard statistical methods, *e.g.* by non-linear regression analysis followed by analysis of variance or statistical tests of the significance of amplitude estimates obtained.[7,16] Such an analysis will provide estimates, also, of rates (r_j) and amplitudes (β_j) of the kinetically significant transients.

The analytical solution derived in the present paper provides generalized relationships for the interpretation of the transient-state kinetic parameters r_j and β_j. These relationships show that r_j and β_j are known (in principle) functions of kappas in the reaction mechanism. Determinations of r_j and β_j as a function of concentrations of non-enzymic reactants, therefore, can be used to obtain quantitative information about rate constants in the mechanism. Amplitudes β_j (in contrast to rates r_j) are dependent also on initial concentrations of the enzyme-containing species, as well as on absorption coefficients for both enzyme-containing species and non-enzymic reactants [eqn. (21)].

The specific information content of the amplitude parameters is utilized in several enzymological applications of transient-state kinetic techniques. For example, estimates of amplitudes can be used for determination of the active-site concentration of enzyme when absorption coefficients for reactants contributing to the transients are known. Conversely, determinations of amplitudes at different wavelengths may provide information about the spectral properties of transiently appearing reaction intermediates. Determination of the sign of the amplitudes (burst or lag kinetics), and examination of the effect of variation of the pre-mixing conditions, can be similarly used to obtain valuable information about the reaction mechanism. A detailed treatment of these specific applications of rapid-reaction techniques is beyond the scope of the present investigation. Obviously, however, the interpretation of such experiments is simplified by the availability of analytical expressions for the amplitude parameters considered. The present paper indicates how such expressions may be derived in the general case.

On the other hand, the large information content of the amplitude parameters reduces their informative value for the purpose of determination of rate constants in the reaction mechanism. Such interpretations of amplitude estimates require a detailed knowledge about absorption coefficients for all enzymic and non-enzymic reactants in the mechanism and should always be made with great caution. The corresponding interpretation of rate parameters r_j is more straight-forward. Irrespectively of the magnitude of absorption coefficients for reactants and of the enzyme concentrations, pre-mixing conditions, and wavelength used for observation of the transients, rate parameters can be uniquely identified as roots of the secular equation defined by the reaction mechanism. The present investigation shows that an analytical expression [eqns. (22) and (29)] for the secular equation can be obtained for any postulated reaction mechanism of the King-Altman type, and interpretation of the rate parameters can be made in view of this expression.

If several closely overlapping transients are observed, interpretations of the rate parameters r_j may have to be made implicitly, *e.g.* by numerical solution of the secular equation. Such a situation, however, has not been frequently encountered in the stopped-flow kinetic studies hitherto reported in the literature. As a rule, single-exponential processes have been observed. In systems where multiple transients appear. these transients usually have been found to be well-separated in time, at least over certain ranges of concentrations of non-enzymic reactants. In the latter situations, it might be justified to interprete the rate parameters explicitely in terms of the approximate rela-

tionships in eqns. (31) and (32) for roots of the secular equation. These relationships have a structure similar to that of conventional steady-state rate equations and can be analytically handled in a manner familiar to all enzymologists.

Hammes *et al.*, in a frequently cited review on the transient-state kinetics of enzyme reactions,[8] expressed a very pessimistic view on the practical utility of analytical solutions relating to the transient state of systems far from equilibrium. The approximate theoretical treatments that can be made were claimed to involve so many restrictions and to give so complex relationships that detailed kinetic information is sacrificed. This was in contrast to the more obvious practical utility of the analytical relationships routinely derived for and used in relaxation kinetic studies of systems near equilibrium.

The present investigation provides no support for such a pessimistic view. The pseudo first-order assumptions required to justify an analytical treatment of the transient-state kinetics of systems far from equilibrium cannot be considered as unreasonably restrictive, since they agree with those required to justify the application of conventional steady-state rate equations. As for the complexity of the analytical relationships, it may be noted that any treatment of the transient-state kinetics of mechanisms of the King-Altman type must be based on the differential equations for the concentrations of enzyme-containing species (eqn. (35) for $i=1,2,\ldots n$) and non-enzymic reactants [eqn. (3) for $k=1,2,\ldots s$] in the mechanism. In systems near equilibrium, these differential equations can be solved without imposing any restrictions on the relative magnitudes of the concentration variables. The more general solution thus obtained prescribes that the time-dependence of concentration variables is governed by $n+s$ exponential transients, the rates of which represent roots of the $(n+s)$th degree secular equation defined by the coefficient matrix for the differential equations.[8] Under pseudo first-order conditions, this secular equation reduces approximately to an nth degree secular equation which is identical with the one [*e.g.* eqn. (23)] defining rate parameters for the exponential transients appearing under the same conditions in systems far from equilibrium. Consequently, the analytical relationships derived for rate parameters in a system far from equilibrium cannot possibly be more complex (but might, due to the restrictions introduced, be considerably less complex) than those obtained for the near-equilibrium state of the same system.

The same argument is valid as concerns the complexity of amplitude parameters in the two cases. Furthermore, amplitudes of the transients observed in relaxation experiments are dependent on certain thermodynamic properties of the system which do not affect the amplitudes observed in stopped-flow kinetic experiments.[8] For these reasons, it may be concluded that the analytical treatment of the transient-state kinetics of systems far from equilibrium (when possible) must lead to relationships that are of much lower complexity than those which have to be considered in relaxation studies of the corresponding systems. The kinetic advantage of relaxation techniques resides mainly in the better time-resolution obtained by such techniques.

The practical utility of stopped-flow kinetic techniques for the purpose of estimation of rate constants in enzymic reaction mechanisms appears well-established.[17] Stopped-flow experiments of this kind have often been evaluated in terms of apparent first-order processes (*i.e.* in terms of exponential transients), the rates of which have been more or less intuitively assumed to reflect the rate of some particular reaction step in the mechanism. Such an approach does not seem appropriate in view of the present results. Evaluation of transient-state kinetic data in terms of exponential transients is justified only when the kinetic differential equations for concentration variables in the system can be considered as linear. Under such conditions the differential equations can be analytically solved, and there is no need for intuitive guesses. The informative value of transient-state rate parameters determined by stopped-flow techniques, furthermore, should not be overestimated. As was pointed out by Hammes *et al.*,[8] the interpretation of such rate parameters may be quite complex, and this is true also in the simplest situation where application of the approximate relationships in eqns. (31)–(34) is justified. The latter relationships establish that the rate parameter r_j, at best,

can be assumed to represent a sum of kappas in the mechanism. In general, the rate parameter must be expected to be given by the quotient of two sums of kappa products, *i.e.* by the quotient of two polynomials in concentrations of the non-enzymic reactants. Any claim that (the limiting value of) a certain transient-state rate parameter equals the rate constant for some particular reaction step should be supported by evidence justifying a corresponding reduction of the implicite [eqn. (22)] or explicite [eqns. (31) and (32)] analytical expression for the rate parameter.

An alternative common approach for the quantitative interpretation of stopped-flow kinetic experiments has been to apply computer techniques for numerical integration of the differential equations governing the kinetics of the system investigated.[8] This approach certainly is justified under conditions where the transient-state kinetics of the system cannot be analytically treated (non-linear differential equations) or where the validity of the approximate analytical relationships applied may be questioned. Computer simulation of the reaction kinetics, however, requires detailed assumptions about the magnitude of all rate constants in the mechanism, as well as about the magnitude of absorption coefficients for all enzymic and non-enzymic reactants involved. These shortcomings drastically reduce the practical utility and reliability of simulation techniques for the purpose of estimation of rate constants in the mechanism. An analytical approach to such problems is clearly preferable, and should always be attempted. The present investigation indicates along which lines such attempts can be generally made in transient-state kinetic studies of enzyme systems far from equilibrium.

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