In defence of the general validity of the Cha method of deriving rate equations

The importance of explicit recognition of the thermodynamic box in enzyme kinetics

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1. The suggestion by Segel & Martin [(1988) *J. Theor. Biol.* **135**, 445–453] that the well-known schematic method for the derivation of rate equations for combined quasi-equilibrium–steady-state models proposed by Cha [(1968) *J. Biol. Chem.* **243**, 820–825] may not be generally applicable was shown to be incorrect. By contrast, Cha's method was shown (a) to yield correct initial-rate equations that are exact and (b) not to require any constraints on the relative magnitudes of rate constants for slow steps outside the quasi-equilibrium segments of the kinetic model, including those suggested by Segel & Martin. 2. Examination of residual King–Altman patterns for the general modifier model of Botts & Morales [(1953) *Trans. Faraday Soc.* **49**, 696–707] revealed the reasons for the erroneous conclusions reached by Segel & Martin. The errors arise from the failure to take account of fluxes in parallel pathways that connect the two isolated groups of enzyme species existing in quasi-equilibrium with the modifier. 3. A similar failure to take explicit account of parallel pathways in a thermodynamic box that delayed proper appreciation of the form of pH-dependence of $k_{\text{cat.}}/K_m$ is briefly discussed.

INTRODUCTION

In principle the steady-state rate equation for any enzyme-catalysed reaction may be obtained by solution of the set of simultaneous equations resulting from the combination of the enzyme-conservation equation and the steady-state rate expressions for all the enzyme-containing intermediates. This may be achieved by the application of the determinant method (Cramer's rule), but this procedure is tedious and prone to human error for all but the simplest kinetic mechanisms when performed manually. The manual derivation of steady-state rate equations was greatly facilitated by the introduction of a schematic method by King & Altman (1956) and the publication a decade later of rule-based modification by Volkenstein & Goldstein (1966) using the theory of flow graphs. Several automated versions of the King–Altman method are now available (see, e.g., Lam & Priest, 1972; Cornish-Bowden, 1977; Lam, 1981; Olavarria, 1986).

The rigorous application of the steady-state assumption, however, often leads to the generation of initial-rate equations of such complexity as to be of limited value in cases of practical interest (see, e.g., Laidler, 1955). It was this that prompted Cha (1968) to propose a simplification of the full King–Altman method for the analysis of mechanisms in which some steps are considered as quasi-equilibria, whereas steady-state assumptions are retained for others. In outline, the Cha method treats each assembly of enzyme forms at quasi-equilibrium as a single form, and each rate constant (k_i) leading away from the composite form is weighted ($f_i k_i$) according to the fraction (f_i) of reactive molecules in the quasi-equilibrium mixture. If there is more than one reactive species within the composite form, then the rate constant is the sum of the reduced rate constants for the parallel reactions. This follows from rule (a) of Volkenstein & Goldstein (1966) which relates to the addition of two or more parallel branches interconverting a particular pair of enzyme forms. Cha's method may be applied to any kinetic model where there are steps that are considered to be at quasi-equilibrium. It is particularly useful in the analysis of pH-dependence (see, e.g.,

Brocklehurst & Dixon, 1976, 1977), since rapid-equilibrium assumptions may be justified for ionization reactions by the exceptionally high rate of hydron-transfer steps in buffered aqueous solutions (Jencks, 1969). Computer implementations of Cha's method have been described by Cornish-Bowden (1977), Lam (1981) and, more recently, by Ishikawa *et al.* (1988), where the algorithm is based on determinant expansion.

The Cha methodology is well-established and thus would not in itself merit further consideration were it not for the doubt

Scheme 1. The general modifier mechanism of Botts & Morales (1953)

Enzyme species enclosed within the two broken-line boxes may be treated as single composite species in the application of Cha's method if the binding of the modifier X to E and to ES are considered as quasi-equilibria.

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about its general validity suggested by Segel & Martin (1988). The doubt arises from their claim that the quasi-equilibrium binding assumption places restrictions on the ratios of rate constants for steps leading away from enzyme species, the fluxes around which are treated by using the steady-state assumption. Segel & Martin (1988) examined the general modifier mechanism of Botts & Morales (1953) in the absence of product (P) with the assumption that the binding of the modifier (X) to both the free enzyme (E) and the enzyme-substrate complex (ES) is maintained at quasi-equilibrium (Scheme 1). They concluded (a) that if the binding of X to E and ES is at quasi-equilibrium, this demands that $k'_{-1}k_{+2} = k_{-1}k'_{+2}$ and (b) that this equality is a necessary condition for quasi-equilibrium. This conclusion was based on their derivation of an initial-rate equation that was first degree in both [X] and [S] and their observation that the Cha-derived equation, which is first degree in [S], but second degree in [X], reduces to their simpler expression if $k'_{-1}k_{+2} = k_{-1}k'_{+2}$. The purpose of the present paper is to re-affirm the general validity of the Cha method and to demonstrate the flaw in the approach used by Segel & Martin (1988) to derive the simpler initial-rate expression [eqn. (38) of their paper]. More generally we point out that errors of this type which result from a failure to take into account fluxes between certain enzyme species are to be found elsewhere in the literature. Such errors are avoided by taking advantage of the Cha (1968) schematic method which ensures that thermodynamic boxes are treated correctly with all inter-conversions explicitly recognized.

ANALYSIS AND DISCUSSION

The graphical method of Cha (1968) may be applied to the general modifier mechanism of Botts & Morales (1953) by making the quasi-equilibrium assumption for the binding of X to E and to ES as indicated by the two broken-line boxes depicted in Scheme 1, with retention of the steady-state assumption for the fluxes around ES and EXS. The resulting expression for the initial-rate of reaction (v) as a function of the concentrations of substrate [S] and modifier [X] is eqn. (1):

$$v = \frac{(k_{+2} + k'_{+2}[X]/K'_x)[E]_T[S]}{1 + [X]/K'_x + \frac{K_m^s(1 + [X]/K_x)}{(1 + [X]/K'_x)} + [S]} \quad (1)$$

where $[E]_T$ is the total enzyme concentration, $K_x (= k_{-x}/k_{+x})$ and $K'_x (= k'_{-x}/k'_{+x})$ are the dissociation constants for the dissociation of modifier X from the binary (EX) and ternary (EXS) complexes respectively, and K_m^s , the ratio $[E][S]/[ES]$, is given by eqn. (2):

$$K_m^s = \frac{\frac{k_{-1}}{k_{+1}} \left[\left(\frac{k_{-1} + k_{+2}}{k_{-1}} \right) k_{+1} + \left(\frac{k'_{-1} + k'_{+2}}{k'_{-1}} \right) k'_{+1} \frac{[X]}{K_x} \right]}{\left(k_{+1} + k'_{+1} \frac{[X]}{K_x} \right)} \quad (2)$$

Eqn. (1) is not in dispute, and has been reported by Segel (1975), Segel & Martin (1988) and Topham (1990). Substitution of the expression in eqn. (2) for K_m^s in eqn. (1) and expansion leads to a rate equation of complex appearance. Because K_m^s is a function of [X] (eqn. 2), the rate equation contains terms in $[X]^2$ and $[S][X]^2$. If it is assumed *ab initio* that the binding of both S and X are at quasi-equilibrium, an initial-rate equation of the form of eqn. (1) is again obtained (Cornish-Bowden, 1976), but now $K_m^s (= k_{-1}/k_{+1})$ is no longer a function of [X] and consequently second-order terms in [X] are absent.

These higher-order terms may be eliminated also without any formal explicit assumption of quasi-equilibrium binding of either

X or S in a rigorous steady-state treatment of the Botts-Morales general modifier mechanism provided that $(1 + k'_{+2}/k'_{-1}) = (1 + k_{+2}/k_{-1})$ (Frieden, 1964; Whitehead, 1976). This identity, however, implicitly demands the quasi-equilibrium binding of X to E and ES. Thus the expressions for the ratios $[E][X]/[EX]$ and $[ES][X]/[EXS]$ reduce to K_x and K'_x respectively when $(1 + k'_{+2}/k'_{-1}) = (1 + k_{+2}/k_{-1})$. By contrast, the binding of S to E and EX need not necessarily be at quasi-equilibrium and, when the above identity holds, K_m^s collapses to the steady-state assembly, $(k_{-1} + k_{+2})/k_{+1}$. Clearly, therefore, the condition $((1 + k'_{+2}/k'_{-1}) = (1 + k_{+2}/k_{-1}))$ is sufficient to ensure that the binding of X is at quasi-equilibrium and that the resulting initial-rate equation is first degree in both [X] and [S].

It is instructive to distinguish between the various physical circumstances under which the identity is valid (see also Frieden, 1964).

(i) $k'_{+2}/k'_{-1} = k_{+2}/k_{-1}$ or $k'_{-1}k_{+2} = k_{-1}k'_{+2}$. This is referred to as the 'Frieden relationship' by Segel & Martin (1988) and describes the situation in which the modifier has no effect on the ratio of the rate constants for the breakdown of the ES complex to give E + S or E + P.

(ii) $k_{+2} = k_{-1}$ and $k'_{+2} = k'_{-1}$. This describes the circumstance where the rate constant for the process $ES \rightarrow E + S$ is equal to that for $ES \rightarrow E + P$ and the rate constant for the process $EXS \rightarrow EX + S$ equals that for $EXS \rightarrow EX + P$.

(iii) $k_{+2} = k'_{+2}$, $k_{-1} = k'_{-1}$, and $k_{+1} \neq k'_{+1}$. Here it is assumed that the modifier has no effect on the breakdown of the ES complex to give E + P or E + S. The inequality is needed to ensure that $K_x \neq K'_x$ because the modifier would have no effect on enzymic activity if $K_x = K'_x$ when $k_{+2} = k'_{+2}$ and $k_{-1} = k'_{-1}$. It should be noted that cases (ii) and (iii) are special cases of (i) and need not be considered further.

(iv) k'_{+2}/k'_{-1} and $k_{+2}/k_{-1} \ll 1$ or $k'_{+2} \ll k'_{-1}$ and $k_{+2} \ll k_{-1}$. This condition is equivalent to assuming quasi-equilibrium binding of S (i.e. $K_m^s = k_{-1}/k_{+1}$) and so E, EX, ES and EXS are at quasi-equilibrium with one another and the kinetics of the system can be accommodated by the treatment described by Cornish-Bowden (1976). More importantly in this context, condition (iv) satisfies the identity $(1 + k'_{+2}/k'_{-1}) = (1 + k_{+2}/k_{-1})$ and demands that the binding of X is at quasi-equilibrium, but is unconcerned with whether or not the modifier exerts an effect on the ratio of rate constants for the breakdown of the ES complex to give E + S or E + P [condition (i)]. In other words $k'_{-1}k_{+2} = k_{-1}k'_{+2}$ is a sufficient but not a necessary condition for the quasi-equilibrium binding of X. The claim by Segel & Martin (1988), namely that the quasi-equilibrium binding of X requires $k'_{-1}k_{+2} = k_{-1}k'_{+2}$, can therefore be refuted. It is immaterial to the argument that, unlike condition (i), condition (iv) demands the quasi-equilibrium binding of S, since no assumptions about the relative magnitudes of k'_{+2} and k'_{-1} and of k_{+2} and k_{-1} need to be made in order to apply the Cha method to the mechanism in Scheme 1.

It is not possible to reduce the Cha-derived initial-rate equation (eqn. 1) with K_m^s defined by eqn. (2), obtained on the basis of the sole assumption that the binding of X to E and ES is maintained at quasi-equilibrium, to one in which second-order terms in [X] do not appear, without the imposition of the additional assumption that $(1 + k'_{+2}/k'_{-1}) = (1 + k_{+2}/k_{-1})$. This identity may be satisfied in any of the four ways already discussed, but the quasi-equilibrium binding of X does not require it. Segel & Martin (1988), however, claimed that the assumption of quasi-equilibrium binding of X and the condition $k'_{-1}k_{+2} = k_{-1}k'_{+2}$ are interdependent. The specific example of quasi-equilibrium binding of substrate discussed above disposes of this assertion, but it is useful to consider the argument presented by Segel & Martin (1988) in order to understand how their erroneous conclusion arose.

Scheme 2. The four King-Altman patterns required for the steady-state treatment of the Botts-Morales general modifier mechanism

According to rule (a) of Volkenstein & Goldstein (1966), the parallel paths between E and ES and between EX and EXS in Scheme 1 can be condensed by the addition of appropriate pairs of rate constants $[(k_{-1} + k_{+2})$ and $(k'_{-1} + k'_{+2})$ respectively]. This results in a master pattern of a square, the corners of which represent the four enzyme species, and from which the four three-lined patterns shown are derived. Patterns 3 and 4 can be discarded if the binding of the modifier to both the free enzyme and enzyme-substrate complex is assumed to be maintained at quasi-equilibrium.

Segel & Martin (1988) correctly rearranged the Cha-derived equation in the form of eqn. (3) (their eqn. 17) shown below using the symbolism of Scheme 1:

$$\frac{v}{k_{+2}[E]_T} = \frac{\left(1 + \frac{k'_{+2}[X]}{k_{+2}K'_x}\right)[S]}{\left(\frac{k_{-1} + k_{+2}}{k_{+1}}\right) \cdot A \left(1 + \frac{[X]}{K_x}\right) + [S] \left(1 + \frac{[X]}{K'_x}\right)} \quad (3)$$

Where A is $k_{+1}/(k_{-1} + k_{+2}) \cdot K_m^s$, and K_m^s is given by eqn. (2). The first term in the denominator of the right-hand side of eqn. (3) represents the distribution of enzyme between E, EX and ES and collapses to the product of two terms: $K_m^s (= [E][S]/[ES])$, which accounts for distribution of enzyme between E and ES, and $(1 + [X]/K_x) [= ([E] + [EX])/[E]]$, which accounts for its distribution between E and EX. The argument used by Segel & Martin (1988) contains two incorrect assumptions: (i) that the distribution of enzyme between E and EX is given by $A(1 + [X]/K_x)$ rather than by $(1 + [X]/K_x)$ and (ii) that the distribution of enzyme between E and ES is given by $(k_{-1} + k_{+2})/k_{+1}$, i.e. the steady-state quantity to which K_m^s collapses in the absence of X, rather than by K_m^s . Given these incorrect assumptions, it would be necessary for A to be equal to unity if the term in the denominator of eqn. (3) under discussion is to equal $[(k_{-1} + k_{+2})/(k_{+1})] [1 + [X]/K_x]$, and A is indeed equal to unity if the relationship $k'_{-1}k_{+2} = k_{-1}k'_{+2}$ is assumed to apply.

Using an alternative approach to that of Cha (1968), Segel & Martin (1988) also derived an initial-rate expression by assuming a single quasi-equilibrium condition for the binding of X (i.e. either $k_{-x}[EX] = k_{+x}[E][X]$ or $k'_{-x}[EXS] = k'_{+x}[ES][X]$). This expression (eqn. 4) (eqn. 38 of Segel & Martin, 1988) is shown below in terms of the symbols used in Scheme 1:

$$\frac{v}{[E]_T} = \frac{\frac{k_{+1}k_{+2}[S]}{(k_{-1} + k_{+2})} + \frac{k_{+1}k'_{+2}k'_{+x}[S][X]}{k'_{-x}(k_{-1} + k_{+2})}}{1 + \frac{k_{+1}[S]}{(k_{-1} + k_{+2})} + \frac{k_{+x}[X]}{k_{-x}} + \frac{k_{+1}k'_{+x}[S][X]}{k'_{-x}(k_{-1} + k_{+2})}} \quad (4)$$

Inspection of eqn. (4) reveals the absence of terms in $[X]^2$ and $[S][X]^2$. Eqn. (4) may be more conveniently expressed in the form of eqn. (1), although in this case $K_m^s = (k_{-1} + k_{+2})/k_{+1}$ and thus is now independent of $[X]$. The discrepancy between eqn. (4) and eqn. (1) for K_m^s defined by eqn. 2) was taken by Segel & Martin (1988) to imply that $k'_{-1}k_{+2}$ must equal $k_{-1}k'_{+2}$ because this is the only way that the two expressions can simultaneously be true. It is demonstrated below, however, that there is a different explanation for the apparent requirement for $k'_{-1}k_{+2}$ to be equal to $k_{-1}k'_{+2}$, namely that eqn. (4) is incorrect! The error arises from the failure by Segel & Martin (1988) to take into account in their derivation the interconversion of free enzyme

and enzyme-substrate complex in both liganded ($EX \rightleftharpoons EXS$) and unliganded ($E \rightleftharpoons ES$) states.

A rigorous steady-state treatment of the Botts & Morales (1953) general modifier mechanism using the schematic method of King & Altman (1956) would require twelve patterns from the master pattern of Scheme 1. If the parallel paths between E and ES and between EX and EXS are added according to rule (a) of Volkenstein & Goldstein (1966), the master pattern becomes a square which requires only four patterns (Scheme 2). The assumption of rapid quasi-equilibrium binding of X to E and ES isolates two groups of enzyme species (E and EX) and (ES and EXS), as shown by the broken-line boxes of Scheme 1, connected to each other by slow steps. As a result, the King-Altman flow patterns 1 and 2 of Scheme 2 each incorporate one slow step, whereas the remaining pair of patterns each incorporate two slow steps. Comparison of the relative magnitudes of the fluxes, therefore, enables patterns 3 and 4 of Scheme 2 to be discarded. The remaining patterns (1 and 2) provide the fractional concentrations of each enzyme species in the absence of product (Ainsworth, 1977) as eqns. (5)–(8), where the denominator Σ is the same for each species, being the sum of all four numerators:

$$\frac{[E]}{[E]_T} = \frac{[k_{-x}k'_{-x}(k_{-1} + k_{+2}) + k_{-x}k'_{+x}(k'_{-1} + k'_{+2})[X]]}{\Sigma} \quad (5)$$

$$\frac{[ES]}{[E]_T} = \frac{(k_{-x}k'_{-x}k_{+1}[S] + k_{+x}k'_{-x}k'_{+1}[S][X])}{\Sigma} \quad (6)$$

$$\frac{[EX]}{[E]_T} = \frac{[k_{+x}k'_{-x}(k_{-1} + k_{+2})[X] + k_{+x}k'_{+x}(k'_{-1} + k'_{+2})[X]^2]}{\Sigma} \quad (7)$$

$$\frac{[EXS]}{[E]_T} = \frac{(k_{-x}k'_{+x}k_{+1}[S][X] + k_{+x}k'_{+x}k'_{+1}[S][X]^2)}{\Sigma} \quad (8)$$

The elimination of terms containing predetermined combinations of rate constants for slow steps has been used in automated procedures to generate initial-rate equations for mechanisms containing quasi-equilibrium segments from more complicated computer-derived expressions obtained by the strict application of steady-state assumptions (Kinderlerer & Ainsworth, 1976; Lam, 1981). The initial-rate of reaction (eqn. 1, with K_m^s given by eqn. 2) is then given in the normal way by substitution for $[ES]$ and $[EXS]$ from eqns. (5–8) into eqn. (9):

$$v = k_{+2}[ES] + k'_{+2}[EXS] \quad (9)$$

The method used by Segel & Martin (1988) to obtain eqn. (4) is flawed because the flux between EX and EXS has been effectively ignored in the derivation of the first set of expression for $[ES]$, $[EX]$ and $[EXS]$ in terms of $[E]$ (eqns. 21–28 of their paper). This is easily demonstrated by consideration of Scheme 2. If there is no flux between EX and EXS, the model may be represented by

a single King–Altman pattern (pattern 1 in Scheme 2). The fractional concentrations of the various enzyme species then become eqns. (10)–(13), where Σ is the sum of the four numerators.

$$\frac{[E]}{[E]_T} = [k_{-x}k'_{-x}(k_{-1} + k_{+2})]/\Sigma \quad (10)$$

$$\frac{[ES]}{[E]_T} = (k_{-x}k'_{-x}k_{+1}[S])/ \Sigma \quad (11)$$

$$\frac{[EX]}{[E]_T} = [k_{+x}k'_{-x}(k_{-1} + k_{+2})]/\Sigma \quad (12)$$

$$\frac{[EXS]}{[E]_T} = (k_{-x}k'_{+x}k_{+1}[S][X])/ \Sigma \quad (13)$$

Substitution for [ES] and [EXS] from eqns. (10)–(13) into eqn. (9) yields eqn. (4). In their derivation of eqn. (4), Segel & Martin (1988) assumed only that the $E + X \rightleftharpoons EX$ reaction was maintained at quasi-equilibrium. They concluded that the $ES + X \rightleftharpoons EXS$ reaction must also be at quasi-equilibrium, although no *a priori* assumptions were made about it. This view is an inevitable consequence of failing to consider the fluxes between EX and EXS. If the thermodynamic box is not closed between EX and EXS, EXS effectively becomes a dead-end complex and therefore is in quasi-equilibrium with ES.

Segel & Martin (1988) then proceeded to examine other possible ways in which [ES], [EX] and [EXS] may be expressed in terms of [E], again by setting $k_{+x}[E][X] = k_{-x}[EX]$ (eqns. 29–36 of their paper), but did so by neglecting the flux between E and ES. Now the model can be represented by King–Altman pattern 2 of Scheme 2, and the fractional concentrations of the enzyme species, after cancellation of common factor [X], become eqns. (14)–(17), where Σ is again the sum of the four numerators.

$$\frac{[E]}{[E]_T} = [k_{-x}k'_{+x}(k'_{-1} + k'_{+2})]/\Sigma \quad (14)$$

$$\frac{[ES]}{[E]_T} = (k_{+x}k'_{-x}k'_{+1}[S])/ \Sigma \quad (15)$$

$$\frac{[EX]}{[E]_T} = [k_{+x}k'_{+x}(k'_{-1} + k'_{+2})][X]/\Sigma \quad (16)$$

$$\frac{[EXS]}{[E]_T} = (k_{+x}k'_{+x}k'_{+1}[S][X])/ \Sigma \quad (17)$$

Thus, for example, eqn. (36) of Segel & Martin (1988) may be written as eqn. (18) or obtained directly from a combination of eqns. (14) and (17):

$$[EXS] = \frac{k_{+x}k'_{+1}[E][S][X]}{k_{-x}(k'_{-1} + k'_{+2})} \quad (18)$$

CONCLUDING COMMENTS

Despite suggestions to the contrary by Segel & Martin (1988), it is clear that the method of Cha (1968) (a) yields correct initial-rate equations that are exact and (b) does not require constraints on the relative magnitudes of rate constants for slow steps outside the quasi-equilibrium segments of the kinetic model. Such constraints may be applied additionally to Cha-derived initial-rate equations, of course, but they are not inherent in the method of derivation.

Examination of residual King–Altman patterns for the Botts–Morales (1953) general modifier model reveals the reason for the

erroneous conclusions reached by Segel & Martin (1988). The errors arise from the failure to take account of fluxes for the slow steps between EX and EXS and between E and ES that connect the two isolated groups of enzyme species existing in quasi-equilibrium with the modifier, X. This type of error in the derivation of initial-rate expressions for enzyme kinetic models is not unprecedented. Indeed, Roberts (1977) and Reinhart (1983) made similar errors to that made by Segel & Martin (1988) in their treatments of the same kinetic model. Two types of situation in which parallel pathways are not taken into account may be distinguished. One is when some of the pathways are explicitly excluded in the kinetic model as was done by Peller & Alberty (1959) in connection with analysis of pH-dependence (i.e. with H^+ as the modifier). The severe restrictions imposed by the somewhat implausible assumption of Peller & Alberty (1959) that direct interconversion of enzyme and enzyme–substrate complex occurs in only one ionization state were widely accepted or ignored until attention was drawn to them by Schmidt & Westheimer (1971) and by Plaut & Knowles (1972). The more serious type of situation, however, is when the kinetic model shows the parallel pathways but they are not taken into account in the analysis. A noteworthy example of this type of error is contained in an early attempt (Waley, 1953) to derive a steady-state rate equation for a two-step enzyme-catalysed reaction involving the reversible formation of an intermediate in three hydronic states (EH_2S , EHS , and ES), from the corresponding free enzyme states (EH_2 , EH , and E), only one of which (EHS) provides products in an irreversible step. The hydron-binding reactions (ionizations) were regarded as unperturbed equilibria, and a conventional steady-state treatment was apparently intended to apply to the fluxes through ES , EHS , and EH_2S . Waley's (1953) analysis, however, provided only the same simple bell-shaped dependence of k_{cat}/K_m on pH characterized by free-enzyme pK_a values as was obtained later by Peller & Alberty (1959) by specifically assuming that EH and EHS were the only interconvertible forms of enzyme and enzyme–substrate complex (i.e. by using a kinetic model that did not contain any closed thermodynamic cycles). The complexity that exists in the pH-dependence of k_{cat}/K_m when due recognition is given to parallel pathways in the model defined by Waley (1953) is revealed by the analysis reported by Brocklehurst & Dixon (1976, 1977). The present paper serves to emphasize the importance of giving due consideration to the existence of parallel pathways in thermodynamic boxes in the treatment of kinetic models. For models of any complexity, the schematic method of Cha (1968) is probably less prone to procedural error than conventional algebraic manipulation.

C. M. T. is supported by Nederlandse Unilever. K. B. thanks the Science and Engineering Research Council for Project Grants and Earmarked Studentships involving kinetic analysis of enzyme reactions.

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Received 21 June 1991/8 August 1991; accepted 30 August 1991