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# Steady State Kinetics of Consecutive Enzyme Catalysed Reactions Involving Single Substrates: Procedures for the Interpretation of Coupled Assays

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Sets of differential rate equations are written describing a linear sequence of reactions occurring in solution each catalysed by a control enzyme or one of the Michaelis–Menten type. It is shown that the solutions of these equations may be formulated as a set of Maclaurin polynomials, expressing the concentration of each reactant and of final product as a function of time. From arrays of such polynomials, general expressions are induced for the first non-zero term of the series. These are used to formulate a procedure (illustrated with an example simulated by numerical integration) by which results of coupled enzymic assays may be analysed in terms of maximal velocities and apparent Michaelis constants: correlation is made with other established methods for conducting coupled assays. The present procedure assumes a steady state of enzyme–substrate complexes but not of intermediate reactants.

## 1. Introduction

Considerable interest has been shown in the kinetic behaviour of consecutive reactions catalysed by enzymes in solution (e.g. Garfinkel, Garfinkel, Pring, Green & Chance, 1970; Tschudy & Bonkowsky, 1972) and immobilized on supporting matrices (Goldman & Katchalski, 1971). The reasons for this interest are twofold. First, metabolic pathways considered as linear sequences of reactions or cycles may be examined in terms of the concentrations of intermediate compounds as a function of time (e.g. Garfinkel & Hess, 1964) or of initial substrate concentration (London, 1966). Both endeavours utilized a set of differential rate equations and associated kinetic parameters formulated on the basis of kinetic studies of individual enzyme catalysed reactions, the former proceeding by numerical integration and the latter

by assuming a steady state of intermediate reactants as well as of enzyme-substrate complexes.

The second area of interest concerns the interpretation of results obtained in coupled enzymic assays: it is related to the first in that such assays performed with both enzymes operating together are the simplest examples of a linear sequence of reactions and because they may provide the only convenient way of establishing a rate equation and kinetic parameters describing the first enzyme catalysed reaction, which may be useful in simulating a pathway including this reaction. Again numerical integration of rate equations, written either in terms of the steady state parameters (Michaelis constants and maximal velocities) or individual rate constants, may be used to simulate plots of the change of concentration of final product with time for various initial substrate concentrations. However, such numerical examples do not provide general expressions to guide the experimenter in interpreting results of the same type. On the other hand, if such expressions could be obtained and used to evaluate kinetic parameters, numerical simulations do offer the potential of confirming or refining these estimates.

The major purpose of the present work is to formulate mathematical expressions for the concentrations of reactants and of final product in a linear sequence of reactions catalysed by enzymes of either the Michaelis–Menten or control types. These expressions are used to discuss the interpretation of coupled enzymic assays, comparisons being made with other established procedures (McClure, 1969; Easterby, 1973). The present treatment is restricted to reactions in homogeneous solution involving single substrates in a linear sequence and it assumes a steady state of all enzyme–substrate complexes but not of intermediate reactants. It is general in the sense that it is applicable to a sequence involving more than two enzymes and is potentially adaptable to more complicated mechanisms than those used herein to illustrate the approach.

## 2. Illustration of the Approach with Michaelis–Menten Mechanisms

Consider first a linear sequence of reactions occurring in solution each catalysed by a Michaelis–Menten enzyme, leading to the formation of the final product, $S_p$

$$S_1 \xrightarrow{E_1} S_2 \xrightarrow{E_2} \ldots \xrightarrow{E_{p-1}} S_p.$$

The rates of change of the molar concentrations of substrates and of the final product are taken to be,

$$\mathrm{d}[S_1]/\mathrm{d}t = -V_1 K_1 [S_1]/(1 + K_1 [S_1]) \qquad (1a)$$

$$d[S_i]/dt = \frac{V_{i-1}K_{i-1}[S_{i-1}]}{1+K_{i-1}[S_{i-1}]} - \frac{V_iK_i[S_i]}{1+K_i[S_i]} \qquad (1b)$$

$$d[S_p]/dt = V_{p-1}K_{p-1}[S_{p-1}]/(1+K_{p-1}[S_{p-1}]) \qquad (1c)$$

where $i$ denotes the consecutive reactants, $S_i$ ($i = 2, —, p-1$) and $V_i$ and $K_i$ are respectively the maximal velocity and reciprocal of the conventional Michaelis constant of the reaction whose substrate is $S_i$. The formulation of equation (1) implies that there is no inhibition of any kind nor interaction between enzymes and that there is a steady state of all enzyme-substrate complexes, though not necessarily of each intermediate reactant, $S_i$. It would be possible to formulate a more extensive set of rate equations than given in equation (1) if account were taken of the presteady state formation of enzyme–substrate complexes and of their breakdown. This set would involve $3(p-1)$ individual rate constants; but, although sets of similar kind written for protein unfolding (Elson, 1972) have been solved in terms of sums of exponentials (Eigen & de Maeyer, 1963), the analysis in these terms of steady state kinetic results (the concern of this work) is prohibitively difficult when so many unknowns are involved.

Since the concentration of each species $S_j$ ($j = 1$, all $i$ and $p$) is a continuous and differentiable function of $t$, it may be expressed as a power series in $t$ by means of a Taylor expansion: indeed, this is the basis of numerical integration methods used to solve equation (1) (e.g. Chance, Garfinkel, Higgins & Hess, 1960; McCracken & Dorn, 1964). In this instance, expansion is made about the origin ($t = 0$, $[S_j]_t = [S_j]_0$) thereby expressing $[S_j]_t$ by a Maclaurin polynomial, justification of this procedure being given later.

$$[S_j]_t = [S_j]_0 + [S_j]'_0 t + ([S_j]''_0 t^2/2!) + \ldots + ([S_j]^{\langle n\rangle}_0 t^n/n!) + \ldots \qquad (2)$$

where $[S_j]_0 = 0$ for all $j \neq 1$. The value of $[S_1]_0$ used in evaluating $[S_1]'_0$ directly from equation (1a) will be visualized as the concentration of the first reactant prior to the commencement of the reaction, implying as is frequently and reasonably done that the amount of $S_1$ bound to $E_1$ is small and that a negligible amount of $S_2$ is formed during the first pre-steady state period of extremely short duration.

Expressions for other first derivatives $[S_j]'_0$ are also directly obtainable from equation (1), while those for higher order derivatives are evaluated by successive differentiation with respect to time. This is facilitated by noting that equation (1) involves only terms of the form $V_jK_j[S_j]/(1+K_j[S_j])$, whereupon expressions for $[S_j]''$ and $[S_j]'''$, for example, involve only the respective terms,

$$\frac{V_jK_j[S_j]'}{(1+K_j[S_j])^2} \quad \text{and} \quad \frac{V_jK_j\{[S_j]''(1+K_j[S_j]) - 2K_j([S_j]')^2\}}{(1+K_j[S_j])^3}.$$

TABLE 1

*Expressions in terms of* $[S_1]_0$ *and* $\alpha = (1+K_1[S_1]_0)$ *for successive derivatives of equation* (2) *for the coupled assay system* $S_1 \rightarrow S_2 \rightarrow S_p$ *involving two enzymes of the Michaelis–Menten type*

| $j$ | $[S_j]_0$ | $[S_j]'_0$ | $[S_j]''_0$ |
|---|---|---|---|
| 1 | $[S_1]_0$ | $-V_1K_1[S_1]_0/\alpha$ | $V_1^2K_1^2[S_1]_0/\alpha^3$ |
| 2 | 0 | $V_1K_1[S_1]_0/\alpha$ | $-V_1K_1[S_1]_0(V_1K_1 + V_2K_2\alpha^2)/\alpha^3$ |
| $p$ | 0 | 0 | $V_1K_1V_2K_2[S_1]_0/\alpha$ |

The problem of obtaining expressions for the coefficients in equation (2) therefore becomes one of successive substitution. Table 1 summarizes expressions for $[S_j]_0$, $[S_j]'_0$ and $[S_j]''_0$, so obtained, for the case of a coupled assay system comprising two enzymes (i.e. $p = 3$). It follows from the first line of Table 1 and equation (2) that,

$$[S_1]_t = [S_1]_0 - \frac{V_1K_1[S_1]_0 t}{(1+K_1[S_1]_0)} + \frac{V_1^2K_1^2[S_1]_0 t^2}{2(1+K_1[S_1]_0)^3} \tag{3}$$

where the alternating series has been truncated after the third term. Numerical examples have shown that equation (3) closely describes, in the early stages of the reaction, the depletion of the initial substrate with time, calculable from the definite integral (Dixon & Webb, 1958) of equation (1a). Indeed, the fit is improved and extended to longer times as more terms are included in the power series. This provides the first justification for the use of equation (2) in that it shows that the series converges. It could be noted that the definite integral being an implicit function of $[S_1]_t$ is not in a form suitable for direct use in relation to equation (1). However, expansion of the logarithmic term in the definite integral led to a polynomial in $[S_1]_t$ and the solution of the quadratic arrived at by truncation of this polynomial gave equation (3). This provides further justification in the formulation of the problem for the use of the Maclaurin polynomial, which also offers the advantage that higher order terms than those shown in equation (3) may readily be evaluated. Table 1 also permits the formulation of expressions for $[S_2]_t$ and $[S_p]_t$, from which it may be induced that the first non-zero term of each expression is given by,

$$\frac{[S_j]_0^{\langle j-1\rangle} t^{(j-1)}}{(j-1)!} = \frac{\left\{\prod_{k=2}^{j} V_{k-1}K_{k-1}\right\}[S_1]_0 t^{(j-1)}}{(j-1)!(1+K_1[S_1]_0)}, \quad j \geqslant 2 \tag{4}$$

where $\langle j-1\rangle$ denotes the $(j-1)$th derivative. This general expression was verified with an array more extensive than shown in Table 1, obtained with

larger values of $p$ and for higher order derivatives. It is dimensionally correct since the products $K_1[S_1]_0$ and $V_jK_jt$ are dimensionless.

The practical use of equation (4) may be illustrated immediately in relation to a coupled enzymic assay, for which the experimenter has obtained a plot of $[S_p]_t$ versus $t$ with both enzymes operating together. For this system, equation (4) may be written as,

$$[S_p]_t = V_1K_1V_2K_2[S_1]_0t^2/2(1+K_1[S_1]_0). \tag{5a}$$

Although equation (5a) is an approximation applying at time small enough to permit the concentration of the final product to be represented by the first non-zero term in a Maclaurin power series of $t$, it follows rigorously that,

$$\lim_{t\to 0}\{\mathrm{d}[S_p]_t/\mathrm{d}(t^2)\} = V_1K_1V_2K_2[S_1]_0/2(1+K_1[S_1]_0). \tag{5b}$$

The slope, defined by the left-hand side of equation (5b) and obtained experimentally from a plot of $[S_p]_t$ versus $t^2$, together with the corresponding value of $[S_1]_0$, forms one point on a rectangular hyperbola described by equation (5b). Such data accumulated from a series of experiments conducted at different values of $[S_1]_0$ and plotted in double-reciprocal form will yield a straight line with abscissa intercept $-K_1$ and ordinate intercept $2/V_1V_2K_2$. Since the second enzyme is chosen because its final product may readily be assayed, values of $V_2$ and $K_2$ are available from independent studies on this enzyme alone using $S_2$ as substrate: it follows that $V_1$ and $K_1$ pertaining to the reaction catalysed by the first enzyme may now be evaluated. For systems involving more than two enzymes in the reaction mixture, an entirely similar procedure of analysis is possible based on a plot of $[S_p]_t$ versus $t^{(p-1)}$. Although this analysis does not utilize the more familiar $v(=[\mathrm{d}S_p]_t/\mathrm{d}t)$ versus $[S_1]_0$ plot (since $v_{t\to 0} = 0$), it is clear from equation (4) that such a plot would closely approximate the form of a rectangular hyperbola, provided values of $v_t$ ($t \neq 0$) for each $[S_1]_0$ were determined at a fixed time within the domains over which plots of $[S_p]_t$ versus $t^{(p-1)}$ were approximately linear. If so determined in time domains for which equation (4) applied, this rectangular hyperbola would differ from that pertaining to the first reaction only by the dimensionless ordinate scaling factor

$$\left(\prod_{k=3}^{p} V_{k-1}K_{k-1}\right) t^{(p-2)}/(p-2)!$$

The question arises as to the forms of kinetic plots when the mechanisms are more complicated than the simple Michaelis–Menten type stipulated in equation (1). Of these, the most interesting in relation to metabolic control pertain to systems where individual kinetic plots for certain enzymes are sigmoidal.

### 3. Extension of the Approach to Control Enzymes

Again a linear sequence of reactions is considered where the substrate of a reaction is the product of the preceding reaction, the chain being initiated with species $S_1$ of initial concentration $[S_1]_0$ and terminated with $S_p$. A set of rate equations analogous to equation (1) is selected for examination, the general expression being given by,

$$d[S_i]/dt = \frac{V_{i-1}K_{i-1}[S_{i-1}](1+K_{i-1}[S_{i-1}])^{(y_{i-1}-1)}}{(1+K_{i-1}[S_{i-1}])^{y_{i-1}}+X_{i-1}} - \frac{V_iK_i[S_i](1+K_i[S_i])^{y_i-1}}{(1+K_i[S_i])^{y_i}+X_i}. \quad (6)$$

The form of each term of equation (6) is based on a model for allosteric enzymes proposed by Monod, Wyman & Changeux (1965) and Nichol, Jackson & Winzor (1967) and extended to the kinetic situation by Dalziel (1968). It involved the binding of substrate at $y_j$ equivalent and independent sites on one isomeric form of the enzyme coexisting in equilibrium with an inactive isomeric state (isomerization constant, $X$). It could be noted that each term on the right-hand side of equation (6) is a ratio of polynomials in $[S_j]$ as are other expressions formulated to describe allosteric effects on the basis of ligand-induced conformational changes (Koshland & Neet, 1968). With $i = 1$, equation (6) gives an expression describing the rate of depletion of initial substrate, which may be analytically integrated to yield,

$$-V_1K_1t = K_1\{[S_1]_t-[S_1]_0\}+(1+X_1)\ln\{[S_1]_t/[S_1]_0\} - \left\{X_1\ln\frac{1+K_1[S_1]_t}{1+K_1[S_1]_0}\right\}y_1 \geqslant 2 + X_1\sum_{\substack{k=1\\ y_1\geqslant 3}}^{y_1-2}\frac{1}{k}\left\{\frac{1}{(1+K_1[S_1]_t)^k}-\frac{1}{(1+K_1[S_1]_0)^k}\right\} \quad (7)$$

where the first two terms apply when $y_1 = 1$; the first three, when $y_1 = 2$; the first four, when $y_1 = 3$ and so on. Equation (7) has been used in numerical examples to show that for this system also the use of equation (2) as a converging power series is justified.

On the basis of equation (2) and proceeding as before it may be shown that the first non-zero term in the polynomials of $[S_j]_t$ is given by,

$$\frac{[S_j]_0^{\langle j-1\rangle}t^{(j-1)}}{(j-1)!} = \left\{\prod_{k=3}^{j}\frac{V_{k-1}K_{k-1}}{1+X_{k-1}}\right\} \times \frac{V_1K_1[S_1]_0(1+K_1[S_1]_0)^{y_1-1}t^{(j-1)}}{(j-1)!\{(1+K_1[S_1]_0)^{y_1}+X_1\}}, \quad j \geqslant 2. \quad (8)$$

If all $X$ equal zero, the model simplifies to one involving a sequence of reactions each catalysed by an enzyme of the Michaelis–Menten type and equation (8) becomes equation (4). In the case of a coupled enzymic assay ($p = 3$) involving two control enzymes of the type specified, equation (8) may be used to write an expression for the concentration of final product

$$[S_p]_t = \frac{V_2 K_2 V_1 K_1 [S_1]_0 (1 + K_1 [S_1]_0)^{(y_1 - 1)} t^2}{2(1 + X_2)\{(1 + K_1 [S_1]_0)^{y_1} + X_1\}}. \tag{9}$$

Although equation (9) is an approximation, it follows that,

$$\lim_{t \to 0} \frac{\mathrm{d}[S_p]_t}{\mathrm{d}(t^2)} = \frac{V_2 K_2 V_1 K_1 [S_1]_0 (1 + K_1 [S_1]_0)^{(y_1 - 1)}}{2(1 + X_2)\{(1 + K_1 [S_1]_0)^{y_1} + X_1\}}. \tag{10}$$

In relation to the use of equation (10), two cases are of particular interest. The first involves placing $X_1 = 0$ and $X_2 \neq 0$, which specifies that the first enzyme is of the Michaelis–Menten type while the second is a control enzyme. Equation (10) simplifies to the form of a rectangular hyperbola and from the corresponding linear double-reciprocal plot, values of $-K_1$ and $2(1 + X_2)/V_2 K_2 V_1$ may be obtained from the abscissa and ordinate intercepts, respectively. Independent studies with the second enzyme offer the opportunity of fitting the associated sigmoidal kinetic curve with values of the parameters $X_2$, $V_2$ and $K_2$. This could proceed in a manner analogous to that previously employed to obtain binding parameters relevant to a system (aspartate transcarbamylase–succinate) which exhibits the essential features of the model chosen to form equation (6) (Changeux & Rubin, 1968; Nichol, Smith & Winzor, 1969). It follows that values of $V_1$ and $K_1$ may be estimated. The second case of interest involves placing $X_1 \neq 0$ and $X_2 = 0$, specifying that a reaction catalysed by a control enzyme is being monitored with the use of an enzyme of the Michaelis–Menten type. In this case equation (10) describes a sigmoidal curve ($y_1 > 1$ and $X_1$ large), which with independent knowledge of $V_2$ and $K_2$, permits application of the above-mentioned fitting procedures to estimate $X_1$, $V_1$ and $K_1$.

## 4. General Discussion

One established method for the conduct of a coupled enzymic assay proceeds in two stages, a denaturing agent being introduced to terminate the first reaction prior to the adjustment of environmental conditions and introduction of the second enzyme. The procedure is laborious, introduces dilution steps and requires controls to ensure that complete conversion of the first product to the final product has been achieved. There are obvious advantages in employing a reaction mixture in which both enzymes operate together. Several points

in relation to the present treatment which permits this joint operation may be conveniently discussed with the aid of the numerical example shown in Fig. 1. Figure 1(a) depicts the time course of changes in concentration of $S_1$, $S_2$ and $S_p$ obtained by the numerical integration of equation (1), with $p = 3$, by the improved Euler and predictor-corrector method (McCracken

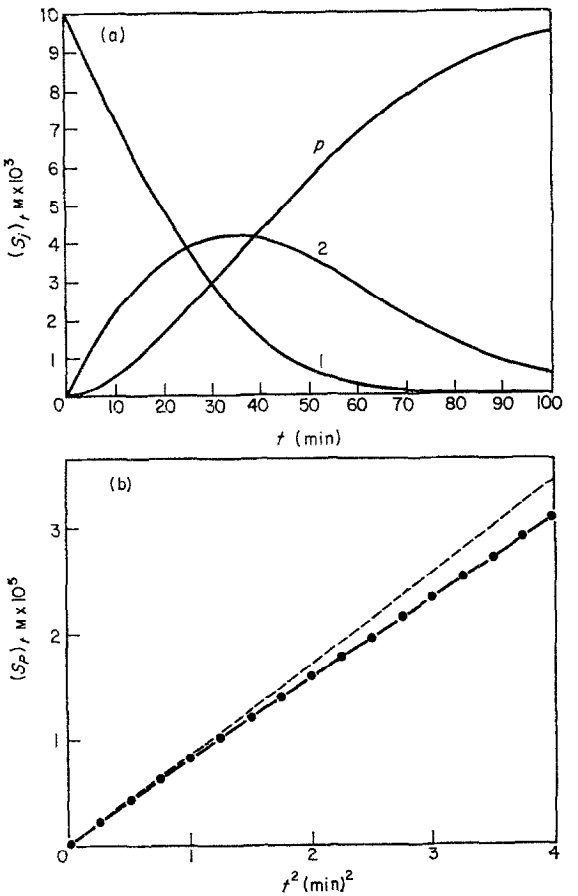


FIG. 1. Numerical results referring to a coupled enzyme system of the type $S_1 \rightarrow S_2 \rightarrow S_p$ where each reaction is catalysed by an enzyme of the Michaelis–Menten type. (a) The variation of the molar concentration of $[S_j]_t$ ($j = 1$, 2 and $p$) with time, obtained by numerically integrating equation (1) with

$$V_1 = 0{\cdot}4 \times 10^{-3} \text{ M min}^{-1}; \quad K_1 = 250 \text{ M}^{-1};$$
$$V_2 = 0{\cdot}3 \times 10^{-3} \text{ M min}^{-1}; \quad K_2 = 200 \text{ M}^{-1}.$$

(b) The corresponding plot of molar concentration of final product $[S_p]_t$ versus $t^2$ (●). The broken line was computed using equation (5a).

& Dorn, 1964). Values of $V_1$, $V_2$, $K_1$ and $K_2$ used are reported in the caption of Fig. 1 and were selected arbitrarily but in realistic ranges. It is clear from Fig. 1 that the concentration of the intermediate compound, $S_2$, is not in a steady state in the experimentally accessible time domain over which a plot of $[S_p]_t$ versus $t^2$ is approximately linear. In Fig. 1(b) the solid points were calculated directly from the values given in Fig. 1(a), while the broken line was plotted according to equation (5a). Clearly, the value of $[\mathrm{d}[S_p]_t/\mathrm{d}(t^2)]_{t\to 0}$ obtained from the curve described is appropriate for use in equation (5a), as previously described, to estimate the kinetic parameters of the first reaction.

Comparison is now possible with the second established method for conducting a coupled enzymic assay, which utilizes a relatively large concentration of the second enzyme in an attempt to ensure that the concentration of $S_2$ is low and in an approximate steady state after the termination of a period termed the transient time (McClure, 1969; Easterby, 1973). When $[S_2]$ = constant, differentiation of the mass conservation equation (assuming that the amounts of substrates bound to the enzymes are small) yields $\mathrm{d}[S_p]_t/\mathrm{d}t = -\mathrm{d}[S_1]_t/\mathrm{d}t$, a relation evidently obeyed at the position of the maximum in the curve describing $S_2$ in Fig. 1(a). In this example, however, $\mathrm{d}[S_1]_t/\mathrm{d}t$ thus determined does not equal $(\mathrm{d}[S_1]_t/\mathrm{d}t)_{t\to 0}$ appropriate to equation (1a) in the evaluation of $V_1$ and $K_1$ with the necessary substitution $[S_1] \sim [S_1]_0$. The effect of increasing $[E_2]_0$ and hence $(V_2)$ holding other parameters fixed is to shorten the transient time and lower the steady state concentration of $S_2$ so that $\mathrm{d}[S_p]/\mathrm{d}t$, determined after the termination of the transient time, more closely approximates the required $(\mathrm{d}[S_1]/\mathrm{d}t)_{t\to 0}$: the procedure is potentially valuable, but since $V_1$ and $K_1$ are unknown, there is little to guide the selection of $[E_2]_0$ to ensure a steady state of $S_2$. The present treatment makes no assumption concerning a steady state of $S_2$ and, indeed, equation (5a) describes $[S_p]_t$ over a time range prior to the achievement of such a state (Fig. 1). It is therefore applicable regardless of the value of the ratio $V_2K_2/V_1K_1$, although the determination of the slope of the limiting tangent to the curve, $[S_p]_t$ versus $t^2$ may become less accurate as the ratio becomes very large (cf. Easterby, 1973) or very small. It would be of particular value in the study of systems where a single enzyme or complex catalyses consecutive reactions (e.g. Koch, Shaw & Gibson, 1971, 1972), prohibiting the arbitrary alteration of the ratio $V_2K_2/V_1K_1$. Finally, in the discussion of equation (5a), it is noted that a description of $[S_p]_t$ as a function of time is also given by equation (4) of Easterby (1973), who used it (in conjunction with an extrapolation procedure to determine the transient time) to elucidate the kinetic properties of the first enzyme in a coupled assay. He noted that the procedure is only valid when certain implicit

assumptions are fulfilled, notably that $V_2K_2/V_1K_1$ is large. However, expansion of the exponential term in equation (4) of Easterby leads on truncation after the $t^2$ term to equation (5a). This consistency arises because the assumptions ($[S_2] \ll 1/K_2$ and $d[S_1]/dt$ = constant) made by Easterby are reasonably fulfilled for all coupled systems as $t \to 0$ regardless of the value of $V_2K_2/V_1K_1$ and supports the present contention based on the use of Maclaurin polynomials that analysis of coupled assay results may proceed without the extrapolation procedure suggested by Easterby with its inherent restriction on the value of $V_2K_2/V_1K_1$.

In summary, the essential difference between the approach suggested in this work and those given previously for the interpretation of coupled assay results is the recommendation that plots be constructed of $[S_p]_t$ versus $t^2$ for various values of $[S_1]_0$. It is clear from equations (5b) and (10) that the resulting initial slopes are functions of $[S_1]_0$ and the characteristic parameters of the two enzymes. These functions are those describing the kinetics of the first enzyme-catalysed reaction (e.g. rectangular hyperbolic or sigmoidal in form) multiplied by $V_2K_2/2(1+X_2)$, $X_2 = 0$ or $X_2 \neq 0$. The approach could be regarded as complementary to established procedures (Chance *et al.*, 1960; Chance & Curtis, 1970) involving numerical integration of the basic differential equations, such as (1) and (6), and fitting of plots of $[S_j]_t$ versus $t$ so obtained to experimental results. Thus, the possibility of obtaining in this way a unique fit to results of this type (which may well be affected by end-product inhibition or other effects at long times) is remote without knowledge of the initial velocity parameters, available from the present method of analysis. Finally, it is clear that the derivation of equations such as (5b) and (10) follows directly from equation (2), use of which merely requires specification of functions (and their derivatives) describing the rate of change of substrate concentrations of each enzyme-catalysed reaction in the sequence. It follows that the approach may be extended to other systems, in which, for example, one step of the sequence involves two substrates.

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