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THE ANALYSIS OF PROGRESS CURVES FOR ENZYME-CATALYSED REACTIONS BY NON-LINEAR REGRESSION

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Summary

A procedure, based on the Gauss-Newton method for non-linear regression, has been developed to obtain enzyme kinetic constants from the analysis of progress curve data. Rules are presented which greatly simplify the derivation of the necessary equations. The method has been applied to the reactions catalysed by prephenate dehydratase, acid phosphatase and lactate dehydrogenase and has yielded values for kinetic parameters which agree well with those obtained from steady-state rate measurements.

Introduction

There has been a growing interest in the utilisation of progress curve data to evaluate the kinetic constants for enzyme-catalysed reactions. There are several advantages of such an approach and a few are worthy of special mention: (1) The need to differentiate the data (i.e. measure rates) is eliminated. This procedure can be subjective and inexact, particularly when the product concentration is not a linear function of time due to substrate exhaustion or inhibition by the accumulated products. (2) More of the available information is utilised and thus the number of experiments to be performed is reduced, with a resultant saving of time and materials. (3) The formation, during the course of the reaction, of pure products obviates the necessity to add these compounds in order to determine product inhibition constants. This is particularly useful when products are unstable or difficult to prepare, or when they may be obtained only as a mixture of several isomers.

The most severe practical limitation of progress curve analysis occurs when the enzyme, substrates or products show a significant degree of instability under the chosen assay conditions. Under these circumstances the integrated

Abbreviations: MOPS, morpholinopropane sulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid.

rate equation is not an adequate representation of the reaction progress curve and more favourable assay conditions must be sought.

There have been several different approaches to progress curve analysis. Bates and Frieden [1,2] use a numerical integration approach, in which a simulated curve is compared with the experimental data, and the rate constants are adjusted until a good visual fit is obtained. While this approach is simple to apply to a variety of enzyme mechanisms, it gives no indication of the uniqueness of the values of the rate constants, or any measure of their reliability, Other investigators have proposed a variety of methods which are based on the solution of linear equations [3—8] but such methods give no, or at best biased, estimates of the standard errors of the parameters. These methods are not of general applicability to complex enzyme mechanisms owing to the rather restrictive assumptions made in order to apply the procedures. Darvey et al. [9] have suggested a procedure for non-linear regression of the progress curves of a reversible one-substrate-one-product system, while Fernley [10] and Nimmo and Atkins [11] have described a non-linear regression procedure for an irreversible one-substrate enzyme showing no product inhibition. These non-linear methods appear to be the most promising for the analysis of progress curve data as they give an estimate of both the uniqueness and the reliability of the kinetic constants. Further, they can in principle be applied to complex enzyme mechanisms.

The aim of the present work was to extend the non-linear regression method to more complex cases than those considered in earlier studies [10,11] and this communication reports a general procedure for the non-linear fitting of progress curve data for any reaction which can be approximated as a one-substrate irreversible system.

Theory

The principles of the Gauss-Newton method of non-linear regression will be familiar to most enzyme kineticists because of the work of Johansen and Lumry [12], Wilkinson [13] and Cleland [14]. However, to facilitate the discussion of the complexities which arise in the application of this method to integrated rate equations, it will be outlined briefly.

For a model such as

$$y = f(\theta_1, \theta_2 \ldots \theta_p; x_1, x_2 \ldots x_n) \tag{1}$$

where θ is a non-linear parameter associated with the model, and x is an independent variable, the first step is to obtain initial estimates ($^0\theta$) of the parameters. This is often done by transformation of the model into a form for which a set of linear parameters ($^T\theta$) may be defined. The transformed data are fitted to this linear equation and then $^0\theta$ is calculated from $^T\theta$. Alternatively, $^0\theta$ may be obtained from visual inspection of the data, external considerations, or guesswork. These initial estimates are then refined using a first-order approximation of a Taylor series, by fitting (with weighing if necessary) to the linear equation

$$(y - \hat{y}) = \Delta\theta_1 \cdot \left[\frac{dy}{d\theta_1}\right] + \Delta\theta_2 \cdot \left[\frac{dy}{d\theta_2}\right] \ldots + \Delta\theta_p \cdot \left[\frac{dy}{d\theta_p}\right] \tag{2}$$

where $\hat{y}$ is the expected value of y calculated for all $\theta = {}^0\theta$, $dy/d\theta$ is the partial derivative of the function with respect to θ (which is evaluated at all $\theta = {}^0\theta$ and is treated as an independent variable) and $\Delta\theta$ is a correction term which is obtained by regression. Refined estimates of the parameters (${}^1\theta$) are then obtained from

$${}^1\theta = {}^0\theta + \Delta\theta \tag{3}$$

These refined estimates are then utilised in a second iteration to give ${}^2\theta$ and so on, until the correction terms become negligible. At this point, iteration is terminated, and the standard errors of the parameters are obtained from the equality of the variances.

$$\mathrm{var}(\theta) = \mathrm{var}(\Delta\theta) \tag{4}$$

The approach outlined above cannot be applied to progress curve data in a straightforward fashion. The expected amount of product formed during reaction (designated z) cannot be expressed as a explicit function * of the parameters and the independent variables and therefore cannot be calculated directly. This difficulty may be overcome by employing the Newton-Raphson procedure to calculate the expected values of z, viz. $\hat{z}$. One of the equations for this procedure, as well as the partial derivatives necessary for non-linear regression, may be obtained by integration of the rate equation with respect to time followed by differentiation with respect to the dependent variable, or with respect to the parameters. These manipulations are not only formidable, but are also circular to some extent. Methods have been devised which greatly simplify the derivation of these equations.

The Newton-Raphson procedure and the derivation of partial derivatives

The general form of the rate equation for most enzymes catalysing irreversible reactions is

$$\frac{dz}{dt} = \frac{V}{\Delta} = \frac{V}{f(z)} \tag{5}$$

Integration of Eqn. 5 gives

$$V \cdot t = \int_0^t f(z) \cdot dz \tag{6}$$

which does not, in general, yield an explicit expression for z. The calculation of a theoretical value for z is equivalent to finding the appropriate root of Eqn 7.

$$F(z) = \int_0^t f(z) \cdot dz - V \cdot t = 0 \tag{7}$$

* An explicit function is one for which may be written an equation of the type $y = f(x)$, where y is the dependent variable, and $f(x)$ is an expression which does not involve y.

The basis of the Newton-Raphson procedure for finding roots of equations is that given an estimate of $\hat{z}$ (^0z), then a refined estimate (^1z) may be calculated [15] from

$$^1z = {}^0z - \left[\frac{F(z)}{F'(z)}\right]_{z={}^0z} \tag{8}$$

where $F'(z)$ is the first derivative of $\equiv(z)$ with respect to z. Using ^1z, a further refinement to give ^2z is performed. Iteration is continued until $^{(n+1)}z \simeq {}^nz$. It is easily shown (see Appendix) that provided the rate equation is expressed in the form of Eqn. 5, then

$$F'(z) = f(z) \tag{9}$$

Eqn. 5 may be expanded as

$$\frac{dz}{dt} = \frac{V}{\Delta} = \frac{V}{f_\theta(\theta) \cdot f_z(z) + g_z(z)} \tag{10}$$

where $f_\theta(\theta)$ is a function of a particular parameter θ, but not of z, and $f_z(z)$ and $g_z(z)$ are functions of z but not of θ. It is shown in Appendix that

$$\frac{dz}{dV} = \frac{t}{\Delta} \tag{11}$$

and

$$\frac{dz}{d\theta} = -\left[\frac{d[f_\theta(\theta)]}{d\theta} \cdot \int_0^t [f_z(z)] \cdot dz\right]/\Delta \tag{12}$$

Application of method

Consider a Uni Uni irreversible enzyme-catalysed reaction which exhibits competitive product inhibition. The rate equation for this mechanism is

$$\frac{dz}{dt} = \frac{V}{1 + \frac{K_a}{A_t}\left(1 + \frac{P_t}{K_p}\right)} \tag{13}$$

where A_t and P_t represent the concentrations of substrate and product, respectively, at time t. Substitution of $A_t = A_0 - z$ and $P_t = P_0 + z$, followed by rearrangement gives

$$\frac{dz}{dt} = \frac{V}{\Delta} \tag{14}$$

where

$$\Delta = 1 - \frac{K_a}{K_p} + \frac{K_a}{(A_0 - z)} \cdot \left(1 + \frac{(A_0 + P_0)}{K_p}\right) \tag{15}$$

Integrating and rearranging in the form of Eqn. 7, we get

$$F(z) = \left(1 - \frac{K_a}{K_p}\right) \cdot z - K_a \cdot \left(1 + \frac{(A_0 + P_0)}{K_p}\right) \cdot \ln\left(1 - \frac{z}{A_0}\right) - V \cdot t \tag{16}$$

From Eqn. 9

$$F'(z) = \Delta = 1 - \frac{K_a}{K_p} + \frac{K_a}{(A_0 - z)} \cdot \left(1 + \frac{(A_0 + P_0)}{K_p}\right) \tag{17}$$

Eqns. 16 and 17 are sufficient to obtain theoretical values of z for a particular set of values for V, K_a and K_p, using the Newton-Raphson procedure.

In order to obtain the partial derivative with respect to K_a, Eqn. 14 is written in the form of Eqn. 10 to give

$$\frac{dz}{dt} = \frac{V}{K_a\left[\frac{-1}{K_p} + \frac{1}{(A_0 - z)} \cdot \left(1 + \frac{(A_0 + P_0)}{K_p}\right)\right] + 1} \tag{18}$$

from which

$$f_{K_a}(K_a) = K_a \tag{19}$$

$$f_z(z) = \frac{-1}{K_p} + \frac{1}{(A_0 - z)} \cdot \left(1 + \frac{(A_0 + P_0)}{K_p}\right) \tag{20}$$

and

$$g_z(z) = 1 \tag{21}$$

Applying Eqn. 12 we get

$$\frac{dz}{dK_a} = \left[\frac{z}{K_p} + \left(1 + \frac{(A_0 + P_0)}{K_p}\right) \cdot \ln\left(1 - \frac{z}{A_0}\right)\right]/\Delta \tag{22}$$

Similarly, the partial derivative with respect to K_p may be calculated.

$$f_{K_p}(K_p) = \frac{1}{K_p} \tag{23}$$

and

$$f_z(z) = K_a\left(-1 + \frac{(A_0 + P_0)}{(A_0 - z)}\right) \tag{24}$$

Hence

$$\frac{dz}{dK_p} = -K_a\left[z + (A_0 + P_0) \cdot \ln\left(1 - \frac{z}{A_0}\right)\right]/(\Delta \cdot K_p^2) \tag{25}$$

Finally, the partial derivative with respect to V is given by Eqn. 11. Thus in Eqns. 11, 22 and 25 we have all the necessary relationships for non-linear regression.

This procedure may be applied to almost any rate equation which may be written in the form of Eqn. 5. However, the algebra is considerably simpler if the system has, or behaves as if it has, only one substrate and discussion will be limited to such cases. Such a restriction does not introduce any great practical obstacle as most two-substrate systems may be manipulated to behave as one-substrate systems.

Linear transformation to obtain initial estimates of parameters

There are a number of ways in which integrated rate equations may be cast in a linear form in order to obtain initial estimates of parameters. The transformation used in the present work is one in which the integrated equation is divided by t and then z/t is treated as the dependent variable. For example, with the Uni Uni case considered earlier, this transformation leads to the equation

$$\frac{z}{t} = V + K_a \cdot \left[\frac{1}{t} \cdot \ln\left(1 - \frac{z}{A_0}\right)\right] + \frac{K_a}{K_p} \cdot \left[\frac{z}{t} + \frac{(A_0 + P_0)}{t} \cdot \ln\left(1 - \frac{z}{A_0}\right)\right] \tag{26}$$

The data are then fitted to this equation by multiple linear regression treating the terms in square brackets as the independent variables, and V, K_a and K_a/K_p as the parameters to be estimated. From this analysis, initial estimates of V, K_a and K_p are readily obtained.

Certain types of rate equation yield, on integration, equations which are intrinsically non-linear. That is, there exists no transformation for which it is possible to define a set of linear parameters which will allow a unique set of initial estimates to be calculated. In such cases, it is usually possible to use the same transformation as that oulined above, but to assume a value for one of the parameters. The remaining parameters are then obtained by regression.

Progress curves with unknown origins

Atkins and Nimmo [7] have pointed out that there may often be some uncertainty as to the origin of a progress curve (i.e. the coordinates of the curve at the moment that the reaction is started). This is particularly true when monitoring of the initial portion of the reaction is impractical. Generally speaking, this uncertainty will be confined to an unknown displacement on the concentration axis, with no significant uncertainty as to the origin of the time axis. Cornish-Bowden [16] has considered some aspects of this problem and has noted the unreliability of polynomials in time in estimating either the initial slope or the origin of a progress curve. Nimmo and Atkins [11] have shown that, in a simple system, non-linear fitting to a progress curve with an unknown origin could be achieved by including the displacement term as another parameter to be estimated. This approach has been extended to more complex cases.

It has been established that any progress curve with a molecularity of Uni Bi or less may be described by the general equation

$$\frac{dz'}{dt} = \frac{R_1}{1 + R_2(z' - R_4) + R_3/(A_0 - (z' - R_4))} \tag{27}$$

where R_1, R_2 and R_3 are constants which are functions of the parameters and the initial conditions; z' is the apparent amount of product formed by reaction and includes the displacement term; R_4 is the amount by which the origin is displaced on the concentration axis. The Newton-Raphson equations and partial derivatives may be obtained as described earlier, with the exception of dz'/dR_4, which is equal to unity. Any single progress curve may be fitted to this equation, and the origin determined. z' is then corrected by subtraction of R_4 to give z, the product formed by reaction. If required, the initial slope (v) may

be calculated from the relationship

$$v = \frac{R_1}{1 + R_3/A_0} \tag{28}$$

The advantage of this technique is that the form of the equation to which the data are fitted is an exact representation of the expected shape of a progress curve. A polynomial, on the other hand, is at best an approximation and holds the twin dangers of using too few terms, which leads to a poor approximation, and too many terms which leads to a "fitting away" of random fluctations in the data. In either case, an unreliable extrapolation is to be expected.

Materials and Methods

Potato acid phosphatase and muscle lactate dehydrogenase were purchased from Calbiochem. Chorismate mutase-prephenate dehydratase was purified from *Escherichia coli*, strain JP492, by the method of Gething [17]. Sodium prephenate was prepared from chorismic acid [18,19]. Other chemicals were high purity preparations from commercial sources.

Enzyme assays

Acid phosphatase was assayed at 25°C with *p*-nitrophenyl phosphate as substrate in 0.1 M sodium MOPS/0.1 M NaCl (pH 7.20) using either a Cary 118 or a Varian 635 spectrophotometer. The formation of *p*-nitrophenol was followed at 478 nm for progress curves and at 410 nm for steady-state velocity measurements. Product concentrations at these two wavelengths were calculated using effective molar extinction coefficients which were determined to be 86.7 and 9090, respectively.

Prephenate dehydratase was assayed at 37°C in a medium containing 0.10 M imidazole, 0.054 M TES, 1.0 mM EDTA, 1.0 mM dithiothreitol and 0.10 mg/ml bovine serum albumin (pH 7.50). Samples were removed at intervals and the reaction stopped by the addition of NaOH to a final concentration of 0.7 M. Phenylpyruvate was estimated at 320 nm, using a molar extinction coefficient determined to be 15 400.

Muscle lactate dehydrogenase was assayed at 27°C in 0.2 M sodium phosphate buffer (pH 7.65). The oxidation of NADH in the presence of 4.0 mM pyruvate was followed at 340 nm using a Cary 118 spectrophotometer. Absorbance data were automatically converted to digital form and collected on magnetic tape using a device which was built for this laboratory by D.P.C.E. (Canberra, A.C.T., Australia) and which was interfaced to a Texas Instruments Silent 700 data terminal. The molar extinction coefficient of NADH was taken to be 6220.

Data processing

Preliminary processing of data collected on magnetic tape was performed using programs written in FOCAL, and run on a Digital Equipment Corporation PDP-8/I. Data analysis programs were written in FORTRAN, and run on a Univac 1100/42 computer. The programs used for the analysis of steady-state velocity data were based on those of Cleland [14]. Weighting factors used in

these analyses were chosen on the basic of estimated or experimentally determined variances. Programs for the analysis of progress curves were written in this laboratory. In cases where there was uncertainty as to the origin of a progress curve, a preliminary fit was made to the integrated form of Eqn. 27, and the appropriate correction applied to the data. Listings of and a Users Manual for progress curve analysis programs are available on request.

Results

Kinetic studies on prephenate dehydratase

Chorismate mutase-prephenate dehydratase from *E. coli* is a bifunctional enzyme which catalyses two successive reactions in the biosynthesis of phenylalanine: viz, the dismutation of chorismate to prephenate, and the dehydration of prephenate to give phenylpyruvate. The assay procedure for prephenate dehydratase (cf. Materials and Methods) proved to be unsuitable for studies of the product inhibition by phenylpyruvate. Therefore advantage was taken of the methods developed for the analysis of progress curve data to determine the inhibition constant for phenylpyruvate. Data from five progress curves (nine points per curve), using prephenate concentrations from 0.225 to 2.7 mM, were collected and fitted to the integrated form of Eqn. 13 which assumes that phenylpyruvate is a competitive inhibitor. V and K_a were also determined from steady-state rate measurements. The values obtained for the kinetic parameters from both types of analyses are given in Table I while the good agreement between the progress curve data and the fitted curves is illustrated in Fig. 1. When the progress curve data were fitted to the integrated form of the equation which allows for non-competitive inhibition by phenylpyruvate, the value for the intercept inhibition constant was found to be high and negative. Thus the inhibition of the enzyme by phenylpyruvate is probably competitive.

Kinetic studies on acid phosphatase

For studies on potato acid phosphatase, data were collected from eight progress curves (24—36 points per curve), with the initial *p*-nitrophenyl phosphate concentration ranging from 1.0 to 3.0 mM. Although not strictly necessary,

TABLE I

KINETIC CONSTANTS OF PREPHENATE DEHYDRATASE FROM *E. COLI*

Prephenate dehydratase was assayed as described in Materials and Methods. Progress curve data were collected at initial prephenate concentrations of 2.70, 0.90, 0.45, 0.30 and 0.225 mM, and fitted to the integrated form of Eqn. 13, using a weighting factor of $1/A_0$. Steady-state rate data were fitted to Eqn. 13, assuming equal variance of rates. K_a and K_p denote the Michaelis constants for prephenate and phenylpyruvate, respectively.

Kinetic constant	Method of analysis	
	Progress curves	Steady-state rates
V (units/mg)	18.0 ± 0.2	16.9 ± 0.4
K_a (mM)	0.472 ± 0.008	0.470 ± 0.028
K_p (mM)	4.59 ± 0.62	n.d.

n.d., not determined.

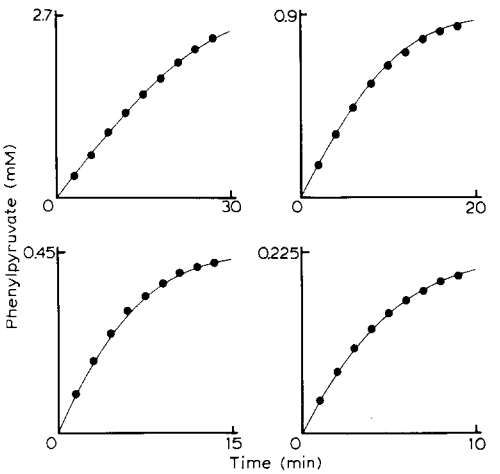

Fig. 1. Time course for the conversion of prephenate to phenylpyruvate, catalysed by chorismate mutase-prephenate dehydratase from *E. coli*. The symbols represent the experimental data, while the solid lines are theoretical curves calculated from the kinetic parameters given in Table I. The initial concentration of prephenate in each case is indicated on the ordinate.

some experiments were performed in the presence of added inorganic phosphate, up to a maximum of 0.73 mM. Hsu et al. [20] have also investigated the kinetic properties of potato acid phosphatase but the experimental conditions differed from those used for the present studies. Therefore steady-state rate measurements were made under the same conditions as those used for the progress curve investigations, except that concentrations of inorganic phosphate ranged up to 0.9 mM. The results (Table II) indicate that similar values for the kinetic parameters are obtained by the two procedures, and that the values of the parameters associated with *p*-nitrophenyl phosphate and inorganic phos-

TABLE II

KINETIC CONSTANTS FOR POTATO ACID PHOSPHATASE

Acid phosphatase was assayed as described in Materials and Methods. Data were collected from eight progress curves under the following initial conditions, expressed as *p*-nitrophenyl phosphate concentration followed by phosphate concentration in parentheses: 0.988 mM (0.010 mM); 1.976 mM (0.019 mM); 2.964 mM (0.029 mM); 0.988 mM (0.195 mM); 1.976 mM (0.391 mM); 2.964 mM (0.725 mM); 0.988 mM (0.010 mM); 2.964 mM (0.725 mM). The phosphate concentrations include the amount which is present as a contaminant of *p*-nitrophenyl phosphate. Progress curve data were fitted to the integrated form of Eqn. 13, using a weighting factor of $1/A_0$. Steady-state rate data were fitted to Eqn. 13, using a weighting factor of $1/v^2$. K_a denotes the Michaelis constant for *p*-nitrophenyl phosphate, and K_p denotes $K_{i(slope)}$ for inorganic phosphate.

Kinetic constant	Method of analysis		
	Progress curves	Steady-state rates	
		Present work	Hsu et al. [20]
V (units/mg)	117 ± 8	104 ± 3	—
K_a (mM)	2.12 ± 0.20	1.57 ± 0.09	2.0 —2.5
K_p (mM)	0.262 ± 0.011	0.219 ± 0.008	0.24—0.25

phate agree well with those reported by Hsu et al. [20]. Values of the inhibition constants for *p*-nitrophenol could not be determined from the experimental data.

Kinetic studies on lactate dehydrogenase

Although the reaction catalysed by muscle lactate dehydrogenase follows a sequential Bi Bi rate law, it may be treated as a Uni Bi reaction under conditions where the pyruvate concentration is high relative to both its K_m value and the concentration of NADH. The rate equation [21] then reduces to

$$\frac{dz}{dt} = \frac{V}{1 + \frac{K_a}{A_t} \cdot \left[1 + \frac{Q_t}{K_{iq}} \cdot \left(1 + \frac{P_t}{K_p^*}\right)\right] + \frac{P_t}{K_{ip}}} \tag{29}$$

where $K_p^* = \frac{K_a K_{ib} K_p}{K_{ia} K_b}$ and A_t, P_t and Q_t represent the concentrations of NADH, L-lactate, and NAD, respectively.

Integration of Eqn. 29 shows that in order for all five kinetic constants to be uniquely determined by progress curve analysis, data must be collected at several substrate concentrations and that some progress curves must be conducted in the presence of at least one of the products. For studies on this enzyme, lactate was selected as the product to be added because, under the chosen experimental conditions lactate, unlike NAD, was produced in amounts which were low relative to its inhibition constants.

Progress curve data under ten different sets of conditions (50—80 points per curve) with NADH concentrations ranging from 0.025 to 0.15 mM, and lactate concentrations in the range from zero to 50 mM were collected and fitted to the integrated form of Eqn. 29. For each initial condition, two experiments were performed at different enzyme concentrations. This procedure permitted

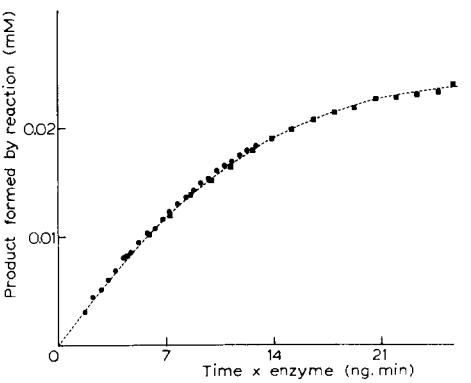

Fig. 2. Time course for the oxidation of NADH as catalysed by muscle lactate dehydrogenase in the presence of pyruvate. The symbols represent the experimental data, collected at enzyme concentrations of 5.2 (●) and 13.8 (■) ng/ml. Every third data point is plotted. The initial concentrations of NADH and lactate were 0.0247 and 50.0 mM, respectively. The line is a theoretical curve calculated from the kinetic parameters given in Table III.

TABLE III

KINETIC CONSTANTS FOR MUSCLE LACTATE DEHYDROGENASE

Lactate dehydrogenase was assayed as described in Materials and Methods. Data were collected from progress curves obtained under the following initial conditions, expressed as NADH concentration followed by lactate concentration in parentheses: 0.151 mM (0.0 mM); 0.148 mM (50.0 mM); 0.0995 mM (0.0 mM); 0.0966 mM (50.0 mM); 0.0480 mM (0.0 mM); 0.0487 mM (24.0 mM); 0.0477 mM (50.0 mM); 0.0247 mM (0.0 mM); 0.0247 mM (50.0 mM); 0.146 mM (0.0 mM). For each curve, data were collected at enzyme concentrations of 13.8 and 5.2 ng/ml. Progress curve data were fitted to the integrated form of Eqn. 29, using a weighting factor of $1/A_0$. Steady-state rate data were fitted to the equation for competitive inhibition (NAD as inhibitor) or non-competitive inhibition (lactate as inhibitor), assuming equal variances of rates. The meanings of the kinetics constants are K_a, Michaelis constant for NADH; K_{ip}, $K_{i(intercept)}$ for lactate; and K_{iq}, $K_{i(slope)}$ for NAD. K_p^* is an inhibition constant for lactate and is defined by Eqn. 29. It could be obtained from the slope of a plot of $1/K_{iq}$ against lactate concentration.

Kinetic constants	Method of analysis	
	Progress curves	Steady-state rates
V (units/mg)	1140 ± 3	1176 ± 3
K_a (μM)	11.17 ± 0.11	9.19 ± 1.20
K_{ip} (mM)	420 ± 16	486 ± 151
K_p^* (mM)	28.5 ± 1.7	n.d.
K_{iq} (μM)	116 ± 4.1	90 ± 13

n.d., not determined.

the collection of data both early and late in the progress curve, without recourse to lengthy incubations. In addition, it allowed demonstration of the fact that no enzyme inactivation occurred during the assay as judged by the fact that, which different concentrations of enzyme, superimposable curves (Fig. 2) were obtained when product formed was plotted against the variable, time multiplied by enzyme added [22]. Before analysis, the data were normalised to identical enzyme concentrations by adjusting the time axis is proportion to the amount of enzyme added. V, K_a, K_{ip} and K_{iq} were also determined from steady-state rate measurements. The results obtained by the two methods (Table III) again illustrate the good agreement of the values of the kinetic parameters as obtained by the two procedures. Fig. 2 shows that the theoretical curve fits well to the experimental data.

Progress curves with unknown origins

The reliability of Eqn. 27 as a means of estimating the origin of a progress curve was assessed using simulated data. A theoretical progress curve of 70 points, covering 19—95% of the reaction, was calculated. The values of the kinetic constants were chosen to be similar to those obtained for lactate dehydrogenase, while the initial substrate concentration was 0.03 mM. A simulated data set was generated by adding to each data point, a random error drawn from a normal distribution with a mean of zero and a standard deviation of 0.00015 mM (0.5% of A_0). 18 sets of simulated data were generated in this manner. Each set of data was fitted to the integrated form of Eqn. 27, and to a polynomial in time. For the polynomial fitting, a stepwise procedure was used, including a higher power of time in each step until no significant reduction in the residual sum of squares was achieved. This analysis resulted in fits to a

TABLE IV

COMPARISON OF METHODS FOR DETERMINING THE ORIGIN AND INITIAL SLOPE OF PROGRESS CURVES

18 sets of simulated progress curve data were generated as described in the text and fitted to Eqn. 27 and to a polynomial in time. The intercept is equal to R_4 when data are fitted to Eqn. 27, or to the constant term when the data are fitted to a polynomial in time. The slope is calculated from Eqn. 28 when data are fitted to Eqn. 27, or is equal to the coefficient of t when the data are fitted to a polynomial in time. The 18 values of intercept and slope for each method of fitting were used to calculate the mean and standard deviation.

Method of fitting	Intercept $\times 10^3$ (mM)	Slope $\times 10^4$ (mM/s)
Polynomial in time	−0.491 ± 0.171	0.204 ± 0.005
Eqn. 27	−0.053 ± 0.287	0.189 ± 0.010
Expected values	0.000	0.188

quadratic equation for 11 sets of data, to a cubic equation for six sets, and to a quartic equation for one set of data. The results (Table IV) indicated that fitting of the simulated data to Eqn. 27 gave values for the slope and intercept which did not differ significantly from the expected values. On the other hand, fitting the same data to a polynomial in time gave values which differed significantly from the expected values. The sets of data which were fitted to a quadratic equation gave results which were only slightly worse than those fitted to higher order polynomials.

Discussion

It has been realised for many years that progress curve analysis offers a valuable technique for studying the kinetics of enzyme-catalysed reactions. Indeed the foundations of enzyme kinetics are built on the study of progress curves [23—25]. However, relatively little use has been made of the technique. It would appear that this was due both to concern about time-dependent changes in activity that the enzyme may undergo over the course of the reaction, and to the complexity of the algebra involved in deriving the necessary equations for non-linear regression.

Selwyn [22] has provided a simple test for detecting time-dependent changes in enzymic activity and the present work demonstrates procedures by which the algebra may be greatly simplified for irreversible systems. The algebra is less formidable when the system has, or behaves as if it has, only one substrate. Although it could appear that the requirement for irreversible one-substrate systems might limit the usefulness of the procedure, in practice this is not usually the case. For instance, in a Bi Bi reaction, provided one substrate is present in considerable excess, its concentration remains constant for all practical purposes and the system behaves as a Uni Bi. Raising the concentration of one substrate will also tend to make a system irreversible, Alternatively, removal or recycling of one of the products by chemical or enzymic means should prove to be a useful method of forcing a reversible two-substrate system into behaving as a one-substrate irreversible system.

When fitting experimental data to a theoretical model it is important to

choose appropriate weighting factors to be used in the analysis. However, it appears that no systematic study has been made of the random error in progress curve data. While the problem of the weighting of such data has been examined briefly in this laboratory, no definitive conclusions have been reached. The data presented here were analysed with a variety of weighting factors, but the values obtained for the kinetic parameters were not greatly affected by the weighting factor used. For instance, the data of the progress curves obtained with potato acid phosphatase were analysed using weighting factors of 1 (i.e. equal weighting), $1/A_0$, $(1/A_0)^2$, $1/A_t$, $1/z$ and $1/P_t$. Values for V varied from 102 to 126 units per mg while the values of K_a varied from 1.76 to 2.30 mM, and those for K_p varied from 0.250 to 0.265 mM. To a large extent, the insensitivity of the fitted parameters to the weighting factor used attests to the small magnitude of the errors in the data. A weighting factor of $1/A_0$ was used for the analysis of the progress curve data in Tables I, II and III.

The results obtained with prephenate dehydratase showed good agreement between steady-state rate measurements and progress curve analysis (Table I). Moreover, it was possible to determine the value for the inhibition constant for phenylpyruvate which could not be determined by conventional product inhibition experiments.

To verify that the technique gives values for kinetic parameters which are comparable with published values, studies were undertaken with potato acid phosphatase. Although the experiments were performed under slightly different conditions, the agreement between the values obtained and those of Hsu et al. [20] is good (Table II). Attempts were made to fit the data to more complex models which included product inhibition terms for *p*-nitrophenol, but these were not successful as in all cases the fitting procedure failed to converge. However, this is not particularly surprising as the inhibition constants for *p*-nitrophenol are of the order of 100 mM and the maximum concentration that *p*-nitrophenol reached in any experiment was less than 3 mM. These findings draw attention to the necessity of having product present in concentrations comparable to its inhibition constant(s) in order to ensure convergence and precise estimation of values for the kinetic parameters.

Consideration was then given to the study of a dehydrogenase reaction which could be made to behave as a Uni Bi reaction by the addition of high, but non-inhibitory, concentrations of one substrate. A further requirement was that either both products acted as strong inhibitors, or it was experimentally feasible to add to the system inhibitory concentrations of the weaker inhibitor. Lactate dehydrogenase met the second of these requirements, and progress curve analysis gave kinetic constants which agreed well with those obtained from steady-state rate measurements. A similar analysis was also attempted with liver alcohol dehydrogenase, but the application of Selwyn's test [22] indicated that the enzyme underwent a time-dependent decrease in activity under a wide variety of experimental conditions.

The technique described in the present report is based on the ability to analytically integrate rate equations and to write explicit functions for the partial derivatives. While this work was in progress, our attention was drawn to the work of Chandler et al. [26] who have developed a computer program which relies on numerical methods to obtain solutions for these equations. This latter

approach may be applied to systems which cannot be integrated analytically, but suffers from the disadvantage that it requires considerably more computer time.

The studies reported in this communication have been confined to enzymes whose kinetic mechanisms had been established. This choice was made because it was considered essential, in the first instance, to demonstrate that accurate values for kinetic parameters could be obtained from analysis of progress curve data. With the achievement of this aim, progress curve studies can be regarded as an adjunct to steady-state rate measurements in confirming reaction mechanisms and yielding values for kinetic constants. Further, it seems that the technique would facilitate studies of the effects of variables such as temperature, pH and ionic strength on the values for kinetic constants. Ultimately, it is hoped to use the procedure as a means of elucidating the kinetic mechanism of an enzyme for which this information is not available, and work is being undertaken in this area.

Appendix

Proof of Eqn. 9

Given a rate equation of the form of Eqn. 5, this may be integrated and rearranged to give Eqn. 7. Differentiating with respect to z, we get

$$\frac{d[F(z)]}{dz} = \frac{d[\int_0^t f(z) \cdot dz]}{dz} - \frac{d[V \cdot t]}{dz}$$

since

$$\frac{d[\int_0^t f(z) \cdot dz]}{dz} = f(z)$$

and

$$\frac{d[V \cdot t]}{dz} = 0$$

and, by definition

$$\frac{d[F(z)]}{dz} = F'(z)$$

then

$$F'(z) = f(z)$$

Proof of Eqn. 11

Given a rate equation of the form of Eqn. 5, this may be integrated to give Eqn. 6. Differentiating with respect to V, we get

$$\frac{d[V \cdot t]}{dV} = \frac{d[\int_0^t f(z) \cdot dz]}{dV}$$

from which

$$t = \frac{\mathrm{d}[\int_0^t f(z) \cdot \mathrm{d}z]}{\mathrm{d}z} \cdot \frac{\mathrm{d}z}{\mathrm{d}V}$$

since

$$\frac{\mathrm{d}[\int_0^t f(z) \cdot \mathrm{d}z]}{\mathrm{d}z} = f(z) = \Delta$$

then

$$\frac{\mathrm{d}z}{\mathrm{d}V} = \frac{t}{\Delta}$$

Proof of Eqn. 12

Given a rate equation of the form of Eqn. 10, integration gives

$$f_\theta(\theta) \cdot \int_0^t [f_z(z)] \cdot \mathrm{d}z + \int_0^t [g_z(z)] \cdot \mathrm{d}z = V \cdot t$$

Differentiating with respect to θ, we get

$$f_\theta(\theta) \cdot \frac{\mathrm{d}[\int_0^t [f_z(z)] \cdot \mathrm{d}z]}{\mathrm{d}\theta} + \frac{\mathrm{d}[f_\theta(\theta)]}{\mathrm{d}\theta} \cdot \int_0^t [f_z(z)] \cdot \mathrm{d}z + \frac{\mathrm{d}[\int_0^t [g_z(z)] \cdot \mathrm{d}z]}{\mathrm{d}\theta} = \frac{\mathrm{d}[V \cdot t]}{\mathrm{d}\theta}$$

Hence

$$f_\theta(\theta) \cdot \frac{\mathrm{d}[\int_0^t [f_z(z)] \cdot \mathrm{d}z]}{\mathrm{d}z} \cdot \frac{\mathrm{d}z}{\mathrm{d}\theta} + \frac{\mathrm{d}[f_\theta(\theta)]}{\mathrm{d}\theta} \cdot \int_0^t [f_z(z)] \cdot \mathrm{d}z + \frac{\mathrm{d}[\int_0^t [g_z(z)] \cdot \mathrm{d}z]}{\mathrm{d}z} \cdot \frac{\mathrm{d}z}{\mathrm{d}\theta} = 0$$

Rearranging, we get

$$\frac{\mathrm{d}z}{\mathrm{d}\theta} \cdot [f_\theta(\theta) \cdot f_z(z) + g_z(z)] = \frac{-\mathrm{d}[f_\theta(\theta)]}{\mathrm{d}\theta} \cdot \int_0^t [f_z(z)] \cdot \mathrm{d}z$$

From Eqn. 10

$$f_\theta(\theta) \cdot f_z(z) + g_z(z) = \Delta$$

Thus

$$\frac{\mathrm{d}z}{\mathrm{d}\theta} = -\left[\frac{\mathrm{d}[f_\theta(\theta)]}{\mathrm{d}\theta} \cdot \int_0^t [f_z(z)] \cdot \mathrm{d}z\right] / \Delta$$

References

1 Bates, D.J. and Frieden, C. (1973) J. Biol. Chem. 248, 7878—7884
2 Bates, D.J. and Frieden, C. (1973) J. Biol. Chem. 248, 7885—7890
3 Schonheyder, F. (1952) Biochem. J. 50, 378—384
4 Schwert, G.W. (1969) J. Biol. Chem. 244, 1278—1284
5 Klesov, A.A. and Berezin, I.V. (1972) Biokhimiya (Engl. edn.) 37, 141—151
6 Bizzozero, S.A., Kaiser, A.W. and Dutter, H. (1973) Eur. J. Biochem. 33, 292—300
7 Atkins, G.L. and Nimmo, I.A. (1973) Biochem. J. 135, 779—784
8 Segel, I.H. (1975) in Enzyme Kinetics, John Wiley and Sons, Inc., New York
9 Darvey, I.G., Shrager, R. and Kohn, L.D. (1975) J. Biol. Chem. 250, 4696—4701
10 Fernley, H.N. (1974) Eur. J. Biochem. 43, 377—378
11 Nimmo, I.A. and Atkins, G.L. (1974) Biochem. J. 141, 913—914
12 Johansen, G. and Lumry, R. (1961) C. R. Trav. Lab. Carlsberg 32, 185—214
13 Wilkinson, G.N. (1961) Biochem. J. 80, 324—332
14 Cleland, W.W. (1967) Adv. Enzymol. 29, 1—32
15 McCracken, D.D. and Dorn, W.S. (1964) in Numerical Methods and FORTRAN Programming, John Wiley and Sons, Inc., New York
16 Cornish-Bowden, A. (1975) Biochem. J. 149, 305—312
17 Gething, M.J.H. (1973) Ph.D. Thesis, University of Melbourne, Melbourne
18 Gibson, F. (1968) Biochem. Prep. 12, 94—97
19 Dudziński, P.K. and Morrison, J.F. (1976) Prep. Biochem. 6, 113—122
20 Hsu, R.Y., Cleland, W.W. and Anderson, L. (1966) Biochemistry 5, 799—807
21 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 104—137
22 Selwyn, M.J. (1965) Biochim. Biophys. Acta 105, 193—195
23 Brown, A.J. (1902) J. Chem. Soc. Trans. 373—388
24 Henri, V. (1903) in Lois Générales de l'Action des Diastases, Hermann, Paris
25 Michaelis, L. and Menten, M.L. (1913) Biochem. Z. 49, 333—369
26 Chandler, J.P., Hill, D.E. and Spivey, H.O. (1972) Comput. Biomed. Res. 5, 515—534