Analysis of progress curves for a highly concentrated Michaelian enzyme in the presence or absence of product inhibition

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Methods are given for analysing the time course of an enzyme-catalysed reaction when the concentration of the enzyme itself is high, a situation which is often found *in vivo*. (1) The integrated form of the kinetic equation for a concentrated Michaelian enzyme in absence of product inhibition is given. Parameters are shown to be calculated easily using non-linear fitting procedures. (2) A general algorithm to analyse progress-curve data in more complex cases (i.e. when the analytical form of the integrated rate equation is not known or is exceedingly complex) is proposed. This algorithm may be used for any enzyme mechanism for which the differential form of the kinetic equation may be written analytically. We show that the method allows differentiation between the main types of product inhibition which may occur in the case of a highly concentrated Michaelian enzyme.

INTRODUCTION

Enzymes are generally much more concentrated in the cell than under conditions *in vitro*, at least for usual steady-state kinetic studies. Thus there is much current interest in the study of highly concentrated enzymes. High-enzyme-concentration effects are particularly important for understanding the regulatory and amplification properties of enzymes (Laurent & Kellershohn, 1984). However, under these conditions, initial velocities can hardly be measured, since depletion of substrate cannot be neglected when enzyme is highly concentrated, i.e. the reaction does not proceed to a negligible extent during the course of the assay. Thus, when possible, integrated rate equations must be preferably used in place of the corresponding differential velocity equations. Moreover, much more information is contained in a progress curve than merely the extrapolated rate at zero time.

Two reasons may explain why progress-curve data have been so little used in enzyme kinetics. First, it may be difficult, from a mathematical point of view, to obtain the integrated form of the differential rate equation corresponding to a particular enzyme mechanism. Secondly, when the integrated form can be written analytically, the solution is often rather complex, so that cumbersome procedures are required in order to analyse them. The integrated rate equations that Schwert (1969) derived for a number of complex mechanisms, illustrate this remark.

THEORY

1. Differential form of the kinetic equation for a concentrated Michaelian enzyme in the presence or absence of product inhibition

Consider a Michaelian enzyme that is inhibited by the product of the catalysed reaction, according to the following scheme:

Competitive, uncompetitive and non-competitive mechanisms of inhibition, as well as the absence of product inhibition, may be considered as special cases of this general mixed-type inhibition system.

By using the rapid-equilibrium hypothesis for the enzyme–product complexes EP and ESP, and the stationary-state hypothesis for the Michaelian complex ES, the velocity equation for this reaction scheme may be written as:

$$\frac{d(P)}{dt} = k_{+2}(ES) \quad (1)$$

with

$$(ES) = \frac{(E_T)_{app.} \cdot (S)}{K_{m, app.} + (S)} \quad (2)$$

where (P) is the concentration of free product, P, (S) is the concentration of free substrate, S, $(E_T)_{app.}$ and $K_{m, app.}$ are the apparent total enzyme concentration and Michaelis constant respectively. We have:

$$K_{m, app.} = \frac{\lambda}{\lambda'} K_m, \quad \text{with} \quad \lambda = 1 + \frac{(P)}{K_P}$$

$$\text{and} \quad \lambda' = 1 + \frac{(P)}{K'_P} \quad (3)$$

$$(E_T)_{app.} = (E_T)/\lambda' \quad (4)$$

K_P and K'_P are the dissociation constants for the EP and ESP complexes respectively.

At high enzyme concentration, i.e. when the enzyme concentration is not much lower than the substrate concentration, bound ligand may not be negligible compared with the total ligand. This means that enzyme–substrate and enzyme–product complexes may

not be neglected in the conservation equation for the substrate. This conservation equation may be written as:

$$(S) = (S_T) - (P) - \frac{\lambda - 1}{\lambda} (E_T) - \left(\lambda' - 1 + \frac{\lambda'}{\lambda} \right) (\overline{ES}) \quad (5)$$

where (S_T) is the total concentration of substrate (bound and free).

The stationary concentration of ES, i.e. $(\overline{ES})$, may be expressed as a function of the total substrate concentration by combining eqns. (2)–(5) and by solving for $(\overline{ES})$ the quadratic equation so far obtained. The result is:

$$(\overline{ES}) = \frac{\lambda K_m + \left(2 \frac{\lambda'}{\lambda} - 1 \right) (E_T) + \lambda' [(S_T) - (P)] - \sqrt{\left[\lambda K_m - (2\lambda' - 1) (E_T) + \lambda' [(S_T) - (P)] \right]^2 + 4\lambda \left(\lambda' - 1 + \frac{\lambda'}{\lambda} \right) K_m (E_T)}}{2\lambda' \left(\lambda' - 1 + \frac{\lambda'}{\lambda} \right)} \quad (6)$$

The differential form of the velocity equation corresponding to this mixed-type inhibition system is obtained by replacing $(\overline{ES})$, in eqn. (1) by its expression as a function of (S_T) (eqn. 6).

Some special cases must be considered.

$\lambda = \lambda' = 1$. This case corresponds to the absence of product inhibition. Eqn. (6) becomes identical with the expression given by Halfman & Marcus (1982) for the stationary concentration of ES, in the case of a highly concentrated Michaelian enzyme.

$\lambda' = 1$. Our reaction scheme reduces to a simple competitive product inhibition system for a highly concentrated Michaelian enzyme.

with

$$\begin{aligned} \Gamma_0 &= \frac{1 + \sigma\beta}{2(1 + \sigma'\beta)(\sigma\sigma'\beta^2 + 2\sigma'\beta + 1)} \\ \Gamma_1 &= 1 + \sigma\beta + \left[\frac{2(1 + \sigma'\beta)}{1 + \sigma\beta} - 1 \right] D + (1 + \sigma'\beta)(\alpha_0 - \beta) \\ \Gamma_2 &= [1 + \sigma\beta - (2\sigma'\beta + 1)D + (1 + \sigma'\beta)(\alpha_0 - \beta)]^2 \\ &\quad + 4D(\sigma\sigma'\beta^2 + 2\sigma'\beta + 1) \end{aligned}$$

The special cases $\lambda = 1$, $\lambda' = 1$ and $\lambda = \lambda'$ correspond to, respectively, $\sigma = 0$, $\sigma' = 0$ and $\sigma = \sigma'$.

2. Integrated rate equation for a highly concentrated Michaelian enzyme in the absence of product inhibition: fitting procedure to the integrated equation

In the special circumstance where there is no product inhibition, the differential form of the kinetic equation for a highly concentrated Michaelian enzyme [eqns. (1) and (6), with $\lambda = \lambda' = 1$], is simply:

$$\frac{d(P)}{dt} = \frac{k_2}{2} [K_m + (E_T) + (S_T) - (P)] - \sqrt{\{[K_m - (E_T) + (S_T) - (P)]^2 + 4K_m(E_T)\}} \quad (8)$$

This equation can be integrated analytically, as shown in Appendix 1. The result is:

$$\begin{aligned} V_M t &= \frac{1}{2} [(P) + \sqrt{\{[K_m - (E_T) + (S_T)]^2 + 4K_m(E_T)\}} - \sqrt{\{[K_m - (E_T) + (S_T) - (P)]^2 + 4K_m(E_T)\}}] \\ &\quad + [K_m + (E_T)] \ln \left[\frac{K_m + (E_T) - (S_T) - \sqrt{\{[K_m - (E_T) + (S_T)]^2 + 4K_m(E_T)\}}}{K_m + (E_T) - (S_T) + (P) - \sqrt{\{[K_m - (E_T) + (S_T) - (P)]^2 + 4K_m(E_T)\}}} \right] \\ &\quad + (E_T) \ln \left[\frac{K_m - (E_T) + (S_T) - (P) + \sqrt{\{[K_m - (E_T) + (S_T) - (P)]^2 + 4K_m(E_T)\}}}{K_m - (E_T) + (S_T) + \sqrt{\{[K_m - (E_T) + (S_T)]^2 + 4K_m(E_T)\}}} \right] \end{aligned} \quad (9)$$

$\lambda = 1$. In this case, product inhibition is uncompetitive.

$$\begin{aligned} \theta &= \frac{1}{2D} [\beta + \sqrt{\{(1 - D + \alpha_0)^2 + 4D\}} - \sqrt{\{(1 - D + \alpha_0 - \beta)^2 + 4D\}}] \\ &\quad + \left(1 + \frac{1}{D} \right) \log \left[\frac{1 + D - \alpha_0 - \sqrt{\{(1 - D + \alpha_0)^2 + 4D\}}}{1 + D - \alpha_0 + \beta - \sqrt{\{(1 - D + \alpha_0 - \beta)^2 + 4D\}}} \right] + \log \left[\frac{1 - D + \alpha_0 - \beta + \sqrt{\{(1 - D + \alpha_0 - \beta)^2 + 4D\}}}{1 - D + \alpha_0 + \sqrt{\{(1 - D + \alpha_0)^2 + 4D\}}} \right] \end{aligned} \quad (10)$$

$\lambda = \lambda' > 1$. This corresponds to a highly concentrated Michaelian enzyme that is non-competitively inhibited by the product of the catalysed reaction.

The velocity equation may be expressed conveniently as a function of dimensionless parameters. Let us define:

$$\begin{aligned} \alpha_0 &= \frac{(S_T)}{K_m}; & \beta &= \frac{(P)}{K_m}; & D &= \frac{(E_T)}{K_m}; \\ \theta &= k_{+2} t; & \sigma &= \frac{K_m}{K_p}; & \sigma' &= \frac{K_m}{K'_p} \end{aligned}$$

We have then:

$$v = \frac{d\beta}{d\theta} = \Gamma_0(\Gamma_1 - \sqrt{\Gamma_2}) \quad (7)$$

Conversely, eqn. (9) may be expressed as a function of the dimensionless parameters which were previously defined:

We verify that, in the case of very diluted enzymes $[(E_T) \ll (S_T)]$, eqns. (9) and (10) are identical with the classical form of the integrated Michaelis–Menten equation in absence of product inhibition. Fig. 1 shows the time courses predicted by eqn. (9) and the way in which they are affected by the value of the enzyme concentration.

In order to fit eqn. (9), a method similar to that described by Fernley (1974) or the method of Nimmo & Atkins (1976) may be used. To calculate $(P_{cal.})$ given t by means of the Newton–Raphson process, eqn. (9) is put in the form:

$$H = V_M t - f[K_m, (E_T), (S_T), (P)]$$

where $f[K_m, (E_T), (S_T), (P)]$ represents the right-side term of eqn. (9). For the non-linear regression analysis, an

Fig. 1. Influence of the enzyme concentration on a progress curve obtained for a concentrated enzyme in the absence of product inhibition

The curves were calculated from eqn. (9) with the following set of parameters values (arbitrary units): $(S_T) = 1$, $K_m = 1$ and $(E_T) = 0.001$ (dotted line, curve 1), 0.1 (curve 2), 1 (curve 3), 2 (curve 4) and 10 (curve 5). Curve 1 corresponds to the classical kinetic conditions of enzyme concentration [i.e. $(E_T) \ll K_m$].

algebraic expression for $[\partial H / \partial (P)]$ is necessary. We can show that this derivative is simply expressed as:

$$\frac{\partial H}{\partial (P)} = \frac{1}{2[(P) - (S_T)]} [K_m + (S_T) - (P) + (E_T) + \sqrt{\{[K_m + (S_T) - (P) - (E_T)]^2 + 4K_m(E_T)\}}] \quad (11)$$

Alternatively, the inclusive parameters $[K_m, V_M, (E_T)]$ may be determined by using the numerical method described in Appendix 2. In this case, progress-curves data are analysed by using the differential rate equation [eqn. (8)] and the knowledge of the integrated form of the velocity equation [eqn. (9)] is not necessary (see below).

3. Effect of product inhibition on progress curves for a highly concentrated Michaelian enzyme

When an enzymic reaction is to be monitored over an extended period, product inhibition cannot be simply ignored, as it is in the case of most of the initial-rate studies. Fig. 2 shows the effect of product inhibition on progress curves for a highly concentrated Michaelian enzyme. These curves are drawn comparatively for several mechanisms of product inhibition. For a given concentration of total substrate at zero time, (S_T) , we observe that the concentration of free product at the end of the reaction, $(P)_\infty$, is not identical according to the mechanism of product inhibition. In absence of product inhibition or when product inhibition is purely uncompetitive, $(P)_\infty$ is simply equal to the initial substrate concentration (S_T) . On the other hand, for competitive, non-competitive or mixed product inhibition, $(P)_\infty$ is less than (S_T) . This property is typical of a high-enzyme-concentration effect.

In fact, when free enzyme is able to form a complex with the reaction product, this complex is present in solution at the end of the reaction, its concentration being dependent on the thermodynamic equilibrium. However, when the enzyme is not highly concentrated [i.e.

Fig. 2. Effect of product inhibition on progress curves for a highly concentrated Michaelian enzyme

The curves correspond to distinct mechanisms of product inhibition. They were calculated for the following set of parameters values (arbitrary units): $(E_T) = 10$, $(S_T) = 10$, and $K_m = 5$. Curve 1, absence of product inhibition; curve 2, uncompetitive product inhibition ($K_P = 10$); curve 3, competitive product inhibition ($K_P = 10$); curve 4, non-competitive product inhibition ($K_P = K'_P = 10$); curve 5, mixed-type product inhibition ($K_P = 10$ and $K'_P = 2.5$).

$(E_T) \ll (S_T)$], the concentration of the enzyme-product complex is negligible compared with $(P)_\infty$, so that we have $(P)_\infty = (S_T)$, whatever the mechanism of product inhibition may be. On the other hand, at high enzyme concentration, $(P)_\infty$ is diminished compared with (S_T) from the amount of enzyme-product complex formed, $(EP)_\infty$.

For the schemes that we consider, we have:

$$(EP)_\infty =$$

$$\frac{(S_T) + (E_T) + K_P - \sqrt{\{(S_T) + (E_T) + K_P\}^2 - 4(E_T)(S_T)}}}{2} \quad (12)$$

[and $(P)_\infty \neq (S_T)$ when $(E_T) \ll (S_T)$]

Purely uncompetitive product-inhibition mechanism is the only mechanism for which the product is unable to form a complex with free enzyme. Thus, in all other cases of product inhibition, $(P)_\infty$ must be less than (S_T) when the enzyme is highly concentrated.

METHODS

When there is no product inhibition, as in Fig. 1, progress curves are simulated by using the integrated form of the corresponding differential rate equation [eqn. (9)]. However, in order to eliminate the trivial effect of enzyme concentration on the maximum velocity of the reaction, the abscissa of Fig. 1 corresponds to $\theta = V_M t$ instead of t . Then the curves specifically show the influence of enzyme concentration on the corresponding rate equation.

Although it should be possible to write, at least for the simplest cases, the integrated form of the kinetic equation for a highly concentrated enzyme in the presence of product inhibition, the solutions are exceedingly complex (results not shown). A laborious method of generating all

Fig. 3. Analysis of progress-curves for a concentrated Michaelian enzyme when $(S_T) = (P)_\infty$

(i) Simulation of data: data were simulated either from model II (uncompetitive product inhibition, *a*) or from model I (absence of product inhibition, *b*). The following set of parameters values was used: $(E_T) = 20$, $(S_T) = 10$, $K_m = 20$ and $1/K'_P = 0.2$ (*a*) or 0 (*b*). (ii) Analysis of data: in both cases, K_m and k_{cat} are assumed to be known. Obviously $(P)_\infty = (S_T)$. Therefore only models I and II have to be differentiated, the procedure described in the Methods section being used. (*a*) Fitting to model I (curve 1): $(E_T) = 8036$, $\Sigma^2 = 0.16$, lack of fit; fitting to model II (curve 2): $(E_T) = 19.78$, $K'_P = 5.0$, $\Sigma^2 = 0.003$. (*b*) Fitting to model I: $(E_T) = 20.12$, $\Sigma^2 = 0.0039$; fitting to model II: $(E_T) = 20.67$, $K'_P = 270$ (to be compared with $K_m = 20$), $\Sigma^2 = 0.0038$.

the corresponding equations and the algebraic calculation of the partial derivatives which are involved in the course of the fitting procedure, would be necessary and this would be very tedious. Thus it would be an unrealistic method for routine use and it is therefore profitable to consider another practical alternative.

The algorithm that we develop in Appendix 2 allows analysis of progress-curves data using the differential form of the rate equation. These equations are derived in the preceding section for the schemes that we consider. In all cases, we suppose that: (i) the studied enzyme is a concentrated Michaelian enzyme [$(E_T) > \text{or } \approx (S_T)$], and (ii) the reaction is far from equilibrium. The effects of several mechanisms of product inhibition on progress curves are shown in Fig. 2. Each curve is calculated by numerical integration of the corresponding differential rate equation.

In order to characterize the occurrence of product inhibition and to determine the type of inhibition using progress curves, experimental data are simulated and each set of data is fitted in turn to the equation corresponding to several models.

Simulation of experimental data

About 40–100 pairs of data (product, time) are calculated to simulate one progress curve. Generally the values of t are equispaced ($\Delta\theta = 0.5$ for Fig. 3 and curve 1 in Fig. 4 and $\Delta\theta = 1$ for curve 2 in Fig. 4). In the case of Fig. 5, data are distributed according to two distinct time scales: 50 points with $\Delta\theta = 0.1$ and 50 points with $\Delta\theta = 0.4$. Such a simulation mimics kinetic records obtained by using a data-acquisition system (for spectrophotometric recording, for instance).

Experimental data are simulated by adding, to each of the perfect values of the product concentration, one of a series of normally distributed pseudo-random numbers of mean zero and standard deviation 0.02 (Figs. 3 and 4) or 0.015 (Fig. 5) with respect to (S_T) . The variance of the experimental product concentration is therefore assumed to be constant.

Analysis of data

According to the theoretical section, $(P)_\infty$ is compared with the initial substrate concentration, (S_T) , allowing a first discrimination between some of the product-inhibition mechanisms (or the absence of product inhibition).

Data are then fitted to the remaining models to be differentiated. The following rules are used for the discrimination.

(i) When fitting to a sophisticated model suppresses the bias observed for a simpler model, the latter one is eliminated. From the statistical point of view, this corresponds to the minimization of the sum of squares of the deviations. In this way, model I for data in Fig. 3(*a*), model IV for data in Fig. 4 and models III and IV for data in Fig. 5 are eliminated.

(ii) The simplest model is retained when both of the following conditions are verified: (*a*) the sophisticated model does not improve the quality of the fit; (*b*) the value of the additional inhibition constant for the sophisticated model is so high that the corresponding mechanism of inhibition may be considered as non-significant. In this way, model II for data in Fig. 3(*b*) and model V for data in Fig. 4 are eliminated.

RESULTS

According to the previous section, the concentration of free product at the end of the reaction, $(P)_\infty$ (i.e. the plateau value of the progress curve), must first be compared with the initial substrate concentration, (S_T) .

(A) When $(P)_\infty = (S_T)$

Two possibilities may occur: (i) there is no product inhibition (model I); (ii) the reaction product is an uncompetitive inhibitor (model II).

Although there is no theoretical limit to the number of parameters to be determined using our algorithm, it would be hopeless to try to calculate up to four parameters [k_{cat} , K_m , K'_P and (E_T)] from the analysis of a single run. This limitation does not reflect a limitation inherent in our procedure. We must simply remember

Fig. 4. Analysis of progress curves for a concentrated Michaelian enzyme in the presence of product inhibition $[(P)_{\infty} \neq (S_T)]$: first example

(i) Simulation of data: data were simulated from model III (as described in the Methods section) by using the following set of parameters values (arbitrary units): $K_m = 20$, $(E_T) = 10$, and $K_P = 10$. Nine sets of experimental pairs of values $[(P), k_{cat} \cdot t]$ were calculated for distinct initial substrate concentrations ranging from $(S_T) = 2$ to $(S_T) = 200$. (b) Represents two of these nine sets of points corresponding to $(S_T) = 50$ (curve 1) and $(S_T) = 25$ (curve 2) respectively. (ii) Analysis of data: obviously, for each curve, $(P)_{\infty}$ is less than (S_T) , indicating that models I and II fail to be applied in these cases. According to the theoretical section, the secondary plot $(EP)_{\infty} = f(S_T)$ was drawn (a) and fitting to eqn. (12) gives the following set of adjusted parameters: $(E_T) = 10.09$ and $K_P = 10.0$. Then each set of data was analysed in turn on the remaining models to be tested (models III–V), as described in the Methods section. The following results were obtained for data in curve 1: fitting to model III: $\Sigma^2 = 0.176$, $K_m = 20.23$; fitting to model IV: $\Sigma^2 = 0.376$, lack of fit; fitting to model V: $\Sigma^2 = 0.178$, $K_m = 18.95$, $K_P = 143$ (to be compared with $K_P = 10.0$). The same results were obtained for curve 2 and the other sets of results not shown.

that no statistical analysis can extract information that is not present in the data, i.e. we cannot discriminate between two models when the expected differences are less than experimental errors. If no indication is provided independently on the occurrence of product inhibition or on the value of some parameters, we might expect that both models I and II enable accurate fitting of the data (with two distinct sets of parameters values). We would

Fig. 5. Analysis of progress-curves for a concentrated Michaelian enzyme in the presence of product inhibition $[(P)_{\infty} \neq (S_T)]$: second example

(i) Simulation of data: data were simulated from model V (mixed-type inhibition product) as described in the Methods section, with the following set of parameters values (arbitrary units): $K_m = 2$, $(E_T) = 50$, $K_P = 100$ and $K'_P = 2$. Lower and upper abscissae correspond to curves 2 and 1 respectively. Ten set of experimental pairs of values $[(P), k_{cat} \cdot t]$ were calculated for distinct initial substrate concentrations ranging from $(S_T) = 2$ to $(S_T) = 1200$. One of these sets of data corresponding to $(S_T) = 10$ is represented in the Figure. (ii) Analysis of data: the type of inhibition was supposed to be unknown. The first part of the analysis [plot $(EP)_{\infty} = f(S_T)$, results not shown] is similar to that described in Fig. 4. The following parameters values were obtained: $(E_T) = 49.73$ and $K_P = 10.0$. Then, each set of data was analysed in turn on models III–V: fitting to model IV (broken line): $K_m = 123.3$, $\Sigma^2 = 0.12$, lack of fit; fitting to model III: $K_m = 120.6$, $\Sigma^2 = 0.115$, lack of fit; fitting to model V (continuous line): $K_m = 1.79$, $K'_P = 2$, $\Sigma^2 = 0.018$. The same results were obtained for the other sets of results not shown. Obviously if product inhibition really occurs, its mechanism corresponds to the mixed-type model.

therefore have no means to discriminate between the two possibilities.

However, if we suppose that two parameters have been determined independently, our algorithm is efficient in order to distinguish between models I and II and to determine accurately the value of the two remaining parameters. This is illustrated in Fig. 3, where k_{cat} and K_m are assumed to be known.

When experimental data are simulated from model II (uncompetitive product inhibition), fitting to the equation corresponding to model I (absence of product inhibition) leads to a significant lack of fit. The bias disappears only when data are fitted to the equation corresponding to model II and the calculated values for the parameters D (or E_T) and K'_P are very close to the real values (Fig. 3a).

Contrarily, when experimental data are simulated from model I, fitting to the equation corresponding to model II does not improve the quality of the fit (Fig. 3b). Moreover, the calculated values for K'_P is so large in this case that it is easy to recognize that product inhibition is not significant.

For all sets of data that were analysed in this way, our method was successful in discriminating between the two

models. Of course, if the mechanism is assumed to be known (absence of product inhibition or uncompetitive inhibition), one can expect to determine more than two parameter values from the analysis of a single run. However, the convergence is not ensured in this case and it is strongly dependent on the precision of the initial provisional estimates given to the parameters to be determined.

(B) When $(P)_{\infty} < (S_T)$

Product inhibition occurs and the type of inhibition is either: (i) competitive (model III), (ii) non-competitive (model IV) or (iii) mixed (model V).

In the particular case where the type of product inhibition and some of the parameters values are already known, data may be analysed directly with our algorithm and the values of the remaining parameters may be calculated from a single run.

Generally, up to five parameters [$k_{cat.}$, (E_T) , K_m , K_P , K'_P] have to be determined. Of course, they cannot be obtained from the analysis of a single progress curve. One may proceed advantageously by performing several experiments at different initial substrate concentrations, (S_T) . For the sake of simplicity, we shall further consider that the $k_{cat.}$ value is already known. In fact, this parameter can be easily measured from an independent determination of the maximal initial velocity of the reaction for a given enzyme concentration. Moreover, if one of the progress curves has been obtained under the conditions $(S_T) \gg (E_T)$, K_m , $k_{cat.}$ may be estimated from the measurement of the initial slope of the corresponding progress curve [which is equal to $k_{cat.}(E_T)$]. When these conditions cannot be approached experimentally, the initial slope of the progress curve obtained for the highest experimental (S_T) concentration, allows us to calculate an underestimated value for $k_{cat.}$. Thus, by the use of our algorithm, three parameters have to be determined, but constraints are imposed on the minimal value of $k_{cat.}$ in the course of the fitting procedure. Thus convergence may be yet obtained unambiguously in this case.

According to eqn. (12), the plateau value obtained for each progress curve is independent of the values of the $k_{cat.}$, K_m and K'_P parameters. Therefore (E_T) and K_P values may be initially calculated by plotting $(EP)_{\infty}$ as a function of (S_T) and fitting eqn. (12) by least-squares criteria to experimental data.

Although eqn. (12) is non-linear in both (E_T) and K_P , good provisional estimates of these parameters are obtained from measurement of the initial slope and the plateau value in this secondary plot {they are equal to $[E_T/K_P + (E_T)]$ and (E_T) respectively}.

Once (E_T) and K_P have been determined, our algorithm may be used to calculate K_m and K'_P parameters from the analysis of any of the progress curve. (In order to use our two-step method, a minimal effort and a minimum of computer space are needed. However, for users having access to large calculators and to routines for solving non-linear simultaneous equations, it may be advantageous to improve our algorithm in order to analyse all the sets of data simultaneously, rather than to fit to each set in turn. For this, a procedure adapted from that described by Nimmo & Atkins (1976) should be implemented). Models III, IV and V are easily differentiated by using a procedure similar to that described above in the case of models I and II. Figs. 4 and 5 give some examples of such a differentiation.

DISCUSSION

In the present paper we propose a method to analyse progress curves for a highly concentrated Michaelian enzyme in the presence or absence of product inhibition. The algorithm of analysis combines both numerical integration of the differential rate equation and non-linear fitting procedures. Thus it does not require one to know the analytical form of the corresponding integrated rate equation. This point is important in the case where highly concentrated enzymes are studied, since the solutions of the integration (when they exist) are always exceedingly complex, except in the simplest case of a Michaelian enzyme in absence of product inhibition.

Nimmo & Atkins (1976) concluded that progress curves are unlikely to be of much use in determining the parameters of an enzymic reaction whose product is a competitive inhibitor. This conclusion is not justified in the case of a highly concentrated Michaelian enzyme. Of course, the analysis of progress curves must be carried out cautiously and sensibly. A thorough analysis of a progress curve will always yield more information than an estimate of the initial rate from the same data. The thoroughness of the analysis must avoid the expectation of extracting more information than that really present in the data. One must not forget that it is often possible for an incorrect equation to describe an experimental progress curve closely. Under these conditions, we show that it is possible, from the analysis of a small number of progress curves, to characterize the occurrence of product inhibition, and to determine the type of inhibition in the case of a highly concentrated Michaelian enzyme. However, this is only a particular use of our method. Of course, the study of the time course of a reaction is not exclusive from initial-rate studies. Yet we may argue that it is often much easier to obtain experimental progress curves than to measure initial velocities in the case of a highly concentrated enzyme, since depletion of substrate can hardly be neglected in this case. When possible, in the early stages of an enzyme-kinetic study, the usual initial-rate measurements may sometimes be useful to discover what mechanism is obeyed. It may be useful to introduce product effects in a controlled way. However, once these preliminaries have been completed, the reaction may be monitored over an extended period to obtain accurate values of the kinetic parameters from a small number of experiments. Our algorithm may be used directly at this stage.

Although we have considered only the relatively simple case of a concentrated Michaelian enzyme with product inhibition, the method discussed can be extended to other important cases in enzyme kinetics. Our algorithm may be used for any enzyme mechanism for which the differential form of the kinetic equation may be written analytically. In the framework of the study of highly concentrated enzymes, this is the case, for instance, for a reversible reaction which is catalysed by a Michaelian enzyme. On the other hand, such an equation cannot be written for a two-substrate reaction unless the concentration of one of the substrates may be considered as a constant. For such a concentrated enzyme, it may be convenient to find conditions where the multi-substrate reaction can be treated as a one-substrate reaction. Segels (1975) discussed an example of such a situation in the case of fructose-bisphosphate aldolase.

Although, in some cases, the kinetic behaviour of

highly concentrated enzymes may be studied *in vitro*, as, for example, the inhibition of fructose 1,6-bisphosphatase by AMP (Halfman & Marcus, 1982), our treatment is especially concerned with situations *in vivo*. Under these conditions, the product of an enzyme-catalysed reaction is typically consumed by the next reaction in the sequence. Thus progress curves may be used for studies *in vivo* only when one of the reaction products is not further metabolized. This is often the case for enzymes that catalyse the formation of several products. For instance, coenzyme oxidation or reduction may be measured directly, as in the case of the study of highly concentrated citrate synthase (Weitzman, 1973) or lactate dehydrogenase (Weitzman, 1973; Sawuka & Suzuki, 1970). Aspartate transcarbamylase is another example to which our treatment is applicable. In this case, enzyme activity is assayed by separating *N*-substituted derivatives of aspartate which lack the positive charge. Thus product formation may be measured in a system such as permeabilized cells which also contain other enzymes of the metabolic pathway (Penverne & Hervé, 1983). Another way to avoid the consumption of the product by the next reaction is to use synthetic substrates that are converted into non-metabolizable products. Such a method was used some time ago for the study *in vivo* of β -galactosidase (Monod *et al.*, 1951). Conversely, some cofactor may be omitted in the assay medium to prevent the next reactions from proceeding. For instance,

phosphofructokinase is assayed *in situ* in the absence of inorganic phosphate, so that further glycolytic steps are blocked and thus do not interfere in the study of the phosphofructokinase reaction (Reeves & Sols, 1973).

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REFERENCES

- Fernley, H. N. (1974) *Eur. J. Biochem.* **43**, 377–378
 Halfman, C. J. & Marcus, F. (1982) *Biochem. J.* **203**, 339–342
 Laurent, M. & Kellershohn, N. (1984) *J. Mol. Biol.* **174**, 543–555
 Monod, J., Cohen-Bazire, G. & Cohn, M. (1951) *Biochim. Biophys. Acta* **7**, 585
 Nimmo, I. A. & Atkins, G. L. (1976) *Biochem. J.* **157**, 489–492
 Penverne, B. & Hervé, G. (1983) *Arch. Biochem. Biophys.* **225**, 562–575
 Reeves, R. E. & Sols, A. (1973) *Biochem. Biophys. Res. Commun.* **50**, 459–466
 Sawuka, R. V. & Suzuki, I. (1970) *Biochem. Biophys. Res. Commun.* **40**, 1096–1101
 Schwert, G. W. (1969) *J. Biol. Chem.* **244**, 1278–1284
 Segels, I. H. (1975) *Enzyme Kinetics*, pp. 63–64, John Wiley and Sons, Chichester
 Weitzman, P. D. J. (1973) *FEBS Lett.* **32**, 247–250

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APPENDIX 1

INTEGRATED RATE EQUATION FOR A HIGHLY CONCENTRATED MICHAELIAN ENZYME IN THE ABSENCE OF PRODUCT INHIBITION

The differential form of the Michaelis–Menten equation for a concentrated enzyme, in absence of product inhibition (eqn. 6), may be rewritten as:

$$\int \frac{k_{+2}}{2} dt = \int \frac{du}{\sqrt{[u^2 + 4K_m(E_T)] - [u + 2(E_T)]}} \quad (A1)$$

with

$$u = K_m - (E_T) + (S_T) - (P).$$

Let us define:

$$v = \text{Arg sinh} \left[\frac{u}{2\sqrt{[K_m(E_T)]}} \right]$$

i.e.

$$du = 2\sqrt{[K_m(E_T)]} \cosh v \, dv$$

We have then:

$$\int \frac{k_{+2}}{2} dt = \int \frac{\cosh v \, dv}{\sqrt{[\sinh^2 v + 1] - \sinh v - \sqrt{[(E_T)/K_m]}}} \quad (A2)$$

Since $\sinh^2 v + 1 = \cosh^2 v$, we can write:

$$\frac{k_{+2}}{2} \int dt = \int \frac{\cosh v \, dv}{\cosh v - \sinh v - D^\dagger} \quad (A3)$$

with

$$D^\dagger = \sqrt{[(E_T)/K_m]}$$

Introducing exponential terms into eqn. (A3), we obtain:

$$k_{+2} \int dt = \int \frac{e^v}{e^{-v} - D^\dagger} dv + \int \frac{e^{-v}}{e^{-v} - D^\dagger} dv \quad (A4)$$

The right-side terms of eqn. (A4) are integrated as, respectively:

$$\int \frac{e^v}{e^{-v} - D^\dagger} dv = -\frac{1}{D^{\dagger 2}} \ln(1 - D^\dagger z) - \frac{z}{D^\dagger} + K_{+1}$$

and

$$\int \frac{e^{-v}}{e^{-v} - D^\dagger} dv = \ln z - \ln(1 - D^\dagger z) + K_{+2}$$

with $z = e^v$.

Thus eqn. (A4) is solved as:

$$k_{+2} t = -\frac{z}{D^\dagger} - \left(1 + \frac{1}{D^{\dagger 2}}\right) \ln(1 - D^\dagger z) + \ln z + K \quad (A5)$$

Coming back to the original variables, we have:

$$z = \frac{1}{\sqrt{[K_m(E_T)]}} [u + \sqrt{u^2 + 4K_m(E_T)}]$$

The condition $(P) = 0$ when $t = 0$ gives:

$$K = \frac{z_0}{D^\dagger} + \left(1 + \frac{1}{D^\dagger z_0}\right) \ln(1 - D^\dagger z_0) - \ln z_0$$

with

$$z_0 = \frac{1}{\sqrt{[K_m(E_T)]}} [u_0 + \sqrt{u_0^2 + 4K_m(E_T)}]$$

and

$$u_0 = K_m - (E_T) + (S_T).$$

Finally, the integrated form of the kinetic equation for a highly concentrated Michaelian enzyme is:

$$t = \frac{1}{k_{+2}} \left[\frac{z_0 - z}{D^\dagger} + \left(1 + \frac{1}{D^\dagger z_0}\right) \ln \left(\frac{1 - D^\dagger z_0}{1 - D^\dagger z} \right) + \ln \frac{z}{z_0} \right] \quad (A6)$$

Eqn. (9) of the main paper is obtained by replacing, in eqn. (A6), z and z_0 by their expression as a function of the original variables $[K_m, (E_T), (S_T)$ and $(P)]$.

APPENDIX 2

ALGORITHM FOR THE ANALYSIS OF PROGRESS CURVES USING THE DIFFERENTIAL FORM OF THE KINETIC EQUATION

Consider the following explicit differential equation:

$$\frac{d\beta}{d\theta} = f(\beta, \rho_k)$$

where ρ_k ($k = 1, 2, \dots, m$) represents the non-linear parameters to be determined. Suppose that N pairs of experimental values $[(\theta, \beta_{\text{exp}})_i, i = 1, 2, \dots, N]$ have been obtained.

The ρ_k parameters can be determined by using a method that combines both numerical integration of the differential equation and an iterative fitting procedure by least-squares criteria to experimental data.

Numerical integration of the differential equation

Numerical integrations were carried out by using the non-linear explicit algorithm developed by Kubicek & Visnak (1974). This algorithm is particularly efficient for the integration of stiff differential equations for which conventional methods, such as explicit Runge-Kutta methods, are not sufficiently stable (Dahlquist, 1963) and require excessive computation time (Garfinkel *et al.*, 1977).

The procedure of Kubicek & Visnak (1974) is a one-step method (it does not need to use an iterated corrector formula) in which an automatic step-size control is

Iterative fitting procedure

Our procedure is adapted from that described by Cleland (1967). It is based on Taylor's theorem. The partial derivatives $(\partial\beta/\partial\rho_k)$ must therefore be calculated. Since the integrated equation $[\beta = g(\theta, \rho_k)]$ is supposed unknown, these partial derivatives must be calculated numerically. The main steps of the fitting procedure are summarized below.

The least-squares criteria needs to minimize the following expression:

$$\sum_{i=1}^N [w_i(\beta_{\text{exp}} - \beta_{\text{cal}})_i^2] \quad (B1)$$

where $(\beta_{\text{exp}})_i$ and $(\beta_{\text{cal}})_i$ are the experimental and calculated values of $(\beta)_i$, respectively, for $\theta = \theta_i$; the weighting factor for each point, w_i , is the reciprocal of its variance. In our programs, we choose $w_i = 1$, i.e. the variance of β is assumed to be constant.

By using Taylor's theorem, we can write:

$$\beta_i \approx \beta_{i0} + \sum_{k=1}^m \left[(\rho_{1k} - \rho_{0k}) \left(\frac{\partial\beta}{\partial\rho_k} \right)_{i, \rho_{0k}} \right]$$

where ρ_{0k} are provisional estimates of the ρ_k parameters, ρ_{1k} are improved values of the ρ_k parameters, and β_{i0} is an approached value of β_i .

Expression B1 will be minimized by solving the set of linear equations:

$$\sum_{i=1}^N w_i \left(\frac{\partial\beta}{\partial\rho_k} \right)_{i, \rho_{0k}} \left[(\beta_{\text{exp}})_i - (\beta_0)_i - \sum_{k=1}^m (\rho_{1k} - \rho_{0k}) \left(\frac{\partial\beta}{\partial\rho_k} \right)_{i, \rho_{0k}} \right] = 0 \quad (B2)$$

Let us define:

$$\left. \begin{aligned} P_k &= \sum_{i=1}^N w_i \left(\frac{\partial\beta}{\partial\rho_k} \right)_{i, \rho_{0k}} \\ u_{k1} &= \sum_{i=1}^N w_i \left(\frac{\partial\beta}{\partial\rho_k} \right) \left(\frac{\partial\beta}{\partial\rho_1} \right) \\ q_k &= (\rho_{1k} - \rho_{0k}) \end{aligned} \right\} \begin{aligned} &[(\beta_{\text{exp}})_i - (\beta_0)_i] = \sum_{l=1}^m g_l u_{kl} \\ &\text{with } k, l = 1, 2, \dots, m \end{aligned}$$

implemented. For the computational process, the Jacobian element

$$\frac{\partial}{\partial\beta} \left(\frac{d\beta}{d\theta} \right)$$

must be evaluated. Since the differential equation is explicit, this expression may be derived algebraically.

System B2 may be rewritten under the following matricial form:

$$\|P\| = \|U\| \times \|Q\| \quad (B3)$$

where $\|P\|$ and $\|Q\|$ are unicolun vectors (with m elements) and $\|U\|$ is a $m \times m$ symmetrical matrix.

System B3 is solved as:

$$\|Q\| = \|U\|^{-1} \times \|P\| \quad (B4)$$

where $\|U\|^{-1}$ is the reciprocal of matrix $\|U\|$.

Scheme B1. Flowchart diagram

'KV subroutine' means numerical integration performed by using the Kubicek & Visnak's (1974) algorithm.

The improved estimates (ρ_{1k}) of the parameters ρ_k are obtained from eqn. (B4):

$$\rho_{1k} = \rho_{0k} + q_k$$

The process is repeated iteratively, until we have:

$$\sqrt{\left[\sum_{k=1}^m \left(\frac{\rho_{r,k} - \rho_{r-1,k}}{\rho_{r,k}} \right) \right]} < a \quad (\text{B5})$$

where the subscripts r and $r-1$ correspond to two successive cycles of iteration and a is an arbitrary threshold value.

When condition (B5) is satisfied, the best-fit estimates of the parameters ρ_k have been reached.

The standard error (s.e.) on the determination of the value of each parameter ρ_k , is given by:

$$\text{s.e.}(\rho_k) = \sqrt{[\Sigma^2 v_{kk}]} \quad (\text{B6})$$

where Σ^2 is the sum of squares of deviations:

$$\Sigma^2 = \frac{\sum_{i=1}^N (\beta_{\text{exp}} - \beta_{\text{cal}(r)})^2}{N - m} \quad (\text{B7})$$

where N is the number of pairs of experimental values $(\theta, \beta_{\text{exp}})_i$, m is the number of parameters ρ_k which have been calculated, $\beta_{\text{cal}(r)}$ is the calculated β -value obtained

after the last cycle of iteration, and v_{kk} are the diagonal elements of the matrix $\|U\|^{-1}$.

Numerical calculation of the partial derivatives $(\partial\beta/\partial\rho_k)$

By using Kubicek & Visnak's (1974) subroutine, the differential equation

$$\frac{d\beta}{d\theta} = f(\beta, \rho_k)$$

is integrated numerically for the following set of parameters:

$$\begin{aligned}\rho_k &= \rho_{0k} + \Delta\rho_k \\ \rho_1 &= \rho_{01}, 1 = 1, 2, \dots, m \\ l &\neq k\end{aligned}$$

Therefore N pairs of values $(\theta, \beta_{\text{cal}}^+)_i$ are obtained.

Similarly, the differential equation is integrated numerically for $\rho_k = \rho_{0k} - \Delta\rho_k$, while the other parameters ρ_1 are unchanged ($\rho_1 = \rho_{01}, 1 \neq k$). The values of β which are thus obtained for the set of θ_i values, are named $(\beta_{\text{cal}}^-)_i$.

The approximated numerical value of the derivative of β with respect to the ρ_k parameter so far considered, is, for each θ_i value:

$$\left(\frac{\partial\beta}{\partial\rho_k}\right)_i \approx \frac{(\beta_{\text{cal}}^+)_i - (\beta_{\text{cal}}^-)_i}{2\Delta\rho_k} \quad (\text{B8})$$

The procedure is repeated for each of the ρ_k parameters to be determined.

The flowchart of our procedure of calculation is given in Scheme B1. For each cycle of iteration, $(2m+1)$ processes of numerical integration of the differential equation must be performed. Since the convergence is

obtained after r cycles of iteration, the procedure of numerical integration is reinitiated in order to compute the $(\beta_{\text{cal}})_{i, (r)}$ values corresponding to the set of adjusted parameters $\rho_{k, (r)}$.

Our algorithm may be used to analyse any explicit first-order differential equation. In the particular case of the enzymic rate equation that we consider (eqns. 1 and 6), up to four parameters ρ_k have to be determined [K_m , K_p , K'_p and (E_T)]. When the velocity equation is expressed as a function of dimensionless parameters (eqn. 7), only three parameters ρ_k have to be considered (σ , σ' and D). When only one parameter ρ is unknown, the regression analysis does not require matricial calculations, since we have simply in this case:

$$\rho_1 - \rho_0 = \frac{\sum_{i=1}^N \left(\frac{\partial\beta}{\partial\rho}\right)_{\rho_0} (\beta_{\text{exp}} - \beta_{\text{cal}})_i}{\sum_{i=1}^N \left(\frac{\partial\beta}{\partial\rho}\right)_{\rho_0}^2}$$

A program written in BASIC for use with a Hewlett-Packard 9816 computer is available from the authors on request.

REFERENCES

- Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 1-32
- Dahlquist, G. (1963) *Bull. Inf. Sci. Tech. Commis. Energ. At. Fr.* **3**, 27-43
- Garfinkel, D., Marbach, C. B. & Shapiro, N. Z. (1977) *Annu. Rev. Biophys. Bioeng.* **6**, 525-542
- Kubicek, M. & Visnak, K. (1974) *Chem. Eng. Commun.* **1**, 291-296