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Use of Progress Curves to Investigate Product Inhibition in Enzyme-Catalysed Reactions

APPLICATION TO THE SOLUBLE MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE

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1. Several methods of analysing progress curves of enzyme-catalysed reactions are discussed briefly in relation to their usefulness in a situation where a reaction product has a K_i much lower than the K_m for the substrate 2. A comparison is made of different methods of estimating initial rates in this situation. 3. The use of a computer curve-fitting routine capable of handling functions of more than one variable for the extraction of kinetic parameters from progress curves is described. 4. This method and that of fitting time as a polynomial in product concentration are applied to progress curves for the soluble mitochondrial adenosine triphosphatase and the results are compared with values obtained by more conventional methods.

Numerical analysis of progress curves of enzyme reactions should not only provide better estimates of the initial rates than can be obtained visually but also provide information about inhibition by the products of the reaction. The use of initial velocities, introduced by Michaelis & Menten (1913), avoids the problem of changing conditions and time-dependent inactivation of the enzyme during the assay but ignores the information in the rest of the progress curve. However, by making a suitable choice of buffer etc. it is usually possible to maintain conditions constant within acceptable limits and it is simple but essential, if progress curves are to be analysed, to test for inactivation of the enzyme during the assay (Selwyn, 1965).

Analysis of progress curves is particularly valuable when one or more of the products has a low K_i, for the decrease in rate is then very marked and visual estimation of the initial rate is most unreliable (see Table 1). However, in this case the two most commonly used methods for analysing progress curves are also unsatisfactory.

In one method, which is based on a rearrangement of the integrated rate equation, the data are plotted as x/t against $(1/t)\ln[s/(s-x)]$, where t is time, x is the product concentration and s is the initial substrate concentration. This was originally used by Walker & Schmidt (1944), but later Foster & Niemann (1953) showed that this plot could be used where the products of the reaction are competitive inhibitors. In this plot the initial point of the progress curve is lost (both co-ordinates are undefined when $x = t = 0$) but where the products are competitive inhibitors this point has to be found for each progress curve. This point can be obtained from the intersection of the plotted functions of the progress curve and a line of slope s drawn through the origin. However, when K_i is smaller than K_m the lines from the progress curve have a positive slope and the intersection with the line of slope s is ill defined. Under these conditions re-plots of intercepts at various substrate concentrations have been used (Bendetskii, 1968) but these require the use of substrate concentrations in the range of K_i for the estimation of this parameter. This may have two disadvantages, namely that the reaction may be difficult to follow at low substrate concentrations and also that another set of progress curves at substrate concentrations around K_m will still be required to obtain K_m and $V_{max.}$.

Klesov & Berezin (1972) rearranged the integrated rate equation by using an approach similar to that used by Guggenheim (1926) to obtain rate constants of first-order reactions. Although this method has advantages over that of Foster and Niemann (1953) in some cases, when K_i is much smaller than K_m, it is subject to the same objections as those mentioned above and we found that it was of little practical use in this case.

The other method often used for numerical estimation of initial rates is to fit a polynomial in time to the progress curve

$$x = a + bt + ct^2 \ldots \quad (1)$$

The coefficient a is a measure of the contamination of the substrate with the products of the reaction. (Under some circumstances, such as when large concentrations of enzyme are used, a could be a measure of an initial burst of reaction or, if of negative value, can indicate a lag period.) When a is

sufficiently close to zero the coefficient b may be taken to be the initial steady-state rate of the reaction. Polynomials can also be used in an empirical fashion as interpolation formulae to give the velocity dx/dt at various values of x and this information can be applied to the rate equation, left in its differential form, to obtain K_i as well as K_m and $V_{max.}$.

When K_i is much smaller than K_m, fitting polynomials in t to the progress curves is not satisfactory and we show that fitting polynomials in x

$$t = a + bx + cx^2 + dx^3 \ldots . \quad (2)$$

gives a better fit with a lower-order polynomial.

It is sometimes possible to increase the usefulness of polynomial fitting by relating the empirical polynomial to a polynomial expansion of the function describing the curve being fitted. An example of this, when applied to the progress curves of enzyme reactions, is the method of Alberty & Koerber (1957). However, it will be shown that even when the polynomial gives a good fit to the data the estimate of K_i from the coefficients of the higher-order terms is not reliable.

A method for dealing with particular types of integrated rate equation has been given by Schwert (1969). This technique depends on comparison of pairs of curves at different initial substrate concentrations to yield simplified equations from which some of the kinetic constants can be obtained and substituted into the original equation for the evaluation of the remaining constants. This method will doubtless be extended to other types of integrated rate equation; for example it can be easily applied to eqn. (1).

Materials and Methods

Soluble mitochondrial ATPase* from ox heart (Pullman *et al.*, 1960) was prepared by the method of Selwyn (1967) modified in the following way. The 40–50% precipitate from the first $(NH_4)_2SO_4$ stage was redissolved in 20mM-Tris–HCl–30% (v/v) glycerol, pH7.0, and desalted on a column (25cm × 2cm) of Sephadex G-25 equilibrated with the same buffer. The enzyme solution was then applied to a column (15cm × 1cm) of DEAE-cellulose DE22 and was eluted with a gradient (100ml) of 30% glycerol containing 20–120mM-Tris–HCl buffer. The fractions containing ATPase were pooled, diluted 6-fold with 30% glycerol, readjusted to pH7.0 and applied to another column (5cm × 1cm) of DEAE-cellulose also equilibrated in the buffer of 20mM-Tris–HCl–30% glycerol, pH7.0. The enzyme was removed rapidly from this column by application of 200mM-Tris–HCl buffer, pH7.0, and a 37–52%-$(NH_4)_2SO_4$ precipitation was done. The precipitate from this step was redissolved in 50% (v/v) glycerol–20mM-Tris–HCl, pH7.8, and stored at −20°C until required. The specific activity of these preparations was typically 50–80μmol of P_i/min per mg of protein (under the assay conditions specified below) and they had a half-life of approx. 30days when stored at −20°C.

In the assay system used the progress of the reaction is followed by measurement of the pH change it produces. This was done by feeding the recorder output of a Radiometer PHM 52 instrument through a voltage-divider network to a 10mV recorder; this gave a full-scale deflexion on the recorder that corresponded to a pH change of 0.1 unit. Assays were done at 30°C in Hepes buffer adjusted to pH7.6 with KOH, 50mM-KCl and at equimolar concentrations of ATP and $MgCl_2$. The concentration of Hepes buffer was varied to obtain convenient rates but was usually 2.5mM. An ATP concentration of 2.5mM was used in the assays done during the purification procedure and in that used to determine the specific activity. The total assay volume was 5ml. As the buffer capacity of the assay mixture varies with ATP concentration it was necessary to calibrate for each assay with a suitable volume of a standard HCl solution (normally 30μl of 20mM), the pH then being adjusted to 7.6 with KOH. This was done before the reaction was started since the Tris buffer added with the enzyme makes a negligible addition to the overall buffering capacity.

The ratio H^+/P_i was determined in a calibration experiment by measurement of the amount of phosphate produced by the reaction and the corresponding pH change. P_i was measured by the method of Fiske & SubbaRow (1926). The value found for P_i/H^+ at pH7.6 was 1.03.

The low value of K_i for ADP makes it essential that the ATP used contains no ADP. This was achieved chromatographically on a column (15cm × 3cm) of DEAE-Sephadex A-25. The ATP was applied as a 50mM solution in 20mM-NaCl–1mM-Tris–HCl buffer, pH8.2, and eluted with a gradient that started with this composition and increased to 300mM-NaCl. The third of the 260nm-absorbing peaks from the column was assumed to be ATP and these fractions were checked for ADP by using the coupled phosphoenolpyruvate, lactate dehydrogenase and NADH system (Pullman *et al.*, 1960). Total adenine nucleotide concentration was estimated from E_{260} measurement. Only those fractions from the ATP peak that contained no detectable ADP (limits of detection estimated to be about 0.1% of the ATP concentration in these fractions) were kept. These were stored frozen until needed.

Hepes buffer was obtained from Stuart Kinney and Co., London W1A 4ES, U.K. ATP, NADH,

* Abbreviations: ATPase, adenosine triphosphatase; ITPase, inosine triphosphatase; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K.

The computer programs used in this work were based on the following subroutines.

(i) F4CFORPL (source, International Computers Ltd.). This routine will, when given a set of data points (x_k, y_k, w_k), successively provide approximating polynomials $Q_N(x)$ $(N = 1, 2, 3$ etc.) such that the expression

$$S_n = \Sigma\, w_j[y_j - Q_N(x_j)]^2$$

is minimized.

A fuller description of this routine can be found in the ICL 1900 Fortran Scientific Subroutines Manual, pp. 23–26.

(ii) NCLF (source, P. D. Bolton, University of New South Wales). This routine can be used with functions of more than one variable and obtains best-fit values for parameters by the n-dimensional Newton's method. This routine also allows the programmer to specify that the signs of the parameters shall not change from those of the approximate values initially provided; this facility was used. These programs were run on the ICL 1905E computer of the University of East Anglia.

Results and Discussion

The integrated rate equation for a reaction where the products are competitive inhibitors was given by Huang & Niemann (1951):

$$t = \frac{1}{V_{max.}}\left[x\left(1 - \frac{K_m}{K_i}\right) + K_m\left(1 + \frac{s}{K_i}\right)\ln\frac{s}{s-x}\right] \quad (3)$$

where s is the initial substrate concentration, x is the product concentration at time t and K_i is the inhibitor constant for the product if only one product inhibits or, in general, $1/\Sigma(1/K_n)$ where n products inhibit. This equation may be converted into a polynomial by a method based on that which Alberty & Koerber (1957) used on a similar, but not identical, equation. First, the logarithmic term is expressed as a power series in x/s giving:

$$t = \frac{1}{V_{max.}}\left[x\left(1+\frac{K_m}{s}\right)+\frac{K_m x^2}{2s}\left(\frac{1}{s}+\frac{1}{K_i}\right)+\frac{K_m x^3}{3s^2}\left(\frac{1}{s}+\frac{1}{K_i}\right)....\right] \quad (4)$$

This can then be expressed as a series in t by the use of the approximation $x/s = V_{max.}t/(s + K_m)$, which of course only holds for small values of x/s. This gives:

$$\frac{x}{s} = \frac{V_{max.}t}{s+K_m} - \left(\frac{K_m}{s+K_m}\right)\left(1+\frac{s}{K_i}\right)\left(\frac{V^2_{max.}t^2}{2(s+K_m)^2}+\frac{V^3_{max.}t^3}{3(s+K_m)^3}....\right) \quad (5)$$

For small values of x/s eqns. (4) and (5) may be sufficiently good approximations when taken to three or four terms to allow them to be equated with the best-fit polynomial through the data points of the same order. For x/s equal to 0.25 (with $s = 400\,\mu$M, $K_i = 16\,\mu$M, $K_m = 400\,\mu$M) eqn. (4) for instance is only 5% in error if terms in x^4 and higher are ignored. For lower values of x/s and for less unfavourable ratios of K_m/K_i the error is less.

When polynomial fitting was tried on a set of progress curves for ATPase the estimate of initial rate obtained by fitting t as a polynomial in x was very different from that obtained by fitting x to a polynomial in t and the estimates obtained graphically by drawing tangents at zero time were also different.

In order to test these methods of estimating initial rates they were applied in a situation in which the correct answer was known; simulated progress curves were generated by using eqn. (3) and values of $K_m = 400\,\mu$M, $K_i = 16\,\mu$M, $V_{max.} = 2.6\,\mu$M/s. For graphical estimates these curves were drawn by the graph plotter of the computer and several members of the biochemistry section were asked to draw initial slopes to them (Table 1). For the polynomial-fitting procedures 20 values of x were chosen randomly in the range $0 < x/s < 0.25$ and the corresponding t values calculated. This method of selecting x values was used to ensure that the points were not at equal spacings of t or x, a situation that was also avoided with the experimental progress curves since equal spacing of the variables may cause problems with the polynomial-fitting routines. The theoretical data sets were then fitted to t as a polynomial in x and to x as a polynomial in t by using subroutine F4CFORPL. The results obtained from this procedure are summarized in Table 1. This simulation is unrealistic to the extent that the curves are error-free; this is very unlikely to be true in practice. However, a check of this at one substrate concentration (1 mM), with 40 theoretical progress curves to which normally distributed errors had been added, showed that the average initial rates obtained (although in no case exactly correct) did display the same pattern as for the error-free curves.

Table 1. *Comparison of estimates of initial rates obtained by using different methoas*

Progress curves were constructed by using eqn. 1 in the text and values of $K_m = 400\mu M$, $K_i = 16\mu M$, $V_{max.} = 2.6\mu M/s$. Curves for the visual estimate were drawn on the computer graph plotter and five members of the biochemistry department were asked to draw initial rates to these. Polynomial-fitting estimates were obtained by using the subroutine F4CFORPL (see the text). Initial rate is defined as $\frac{(dx)}{(dt)_{x=0}}$.

	Initial rate (μM/s)				
Initial substrate concn. (μM) ...	1000	400	200	140	100
Calculated	1.86	1.30	0.867	0.674	0.520
Visual estimate					
Mean	1.97	1·26	0.82	0.652	0.515
S.D.	±0.238	± 0.1	±0.035	±0.019	± 0.013
Spread	1.5–2.5	1.0–1.6	0.7–0.9	0.6–0.7	0.5–0.55
3rd order in x*	1.86	1.30	0.867	0.675	0.521
4th order in x	1.86	1.30	0.867	0.674	0.520
3rd order in t	1.07	0.890	0.688	0.573	0.465
7th order in t	1.60	1.21	0.843	0.665	0.517
11th order in t	1.75	1.27	0.863	0.673	0.520

* Indicates that the curve was fitted to $t = a + bx + cx^2 + dx^3$ where t is time and x is product concentration.

Table 1 shows clearly that when K_i is considerably less than K_m visual estimates of initial rates are most unreliable and also that in this case it is better to fit t as a polynomial in x than to fit x as a polynomial in t, since the former achieves the correct initial rate with a polynomial of fewer terms than the latter. For real progress curves the fit of a polynomial in x shows no significant improvement judged by the sum of the squares of the residuals criterion after a third-order polynomial and a polynomial in t no significant improvement after a fourth-order polynomial. Fig. 1 shows third-order fitted curves in x and t and the points through which they were fitted.

Fig. 2 is a Lineweaver–Burk plot using initial rates estimated by fitting t to a polynomial in x. The value for K_m of 400μM lies between two other published values of 290μM (Selwyn, 1967) and 520μM (Akimenko *et al.*, 1972).

From the coefficients of x^2 of the fitted polynomials it should be possible to find K_i, since these coefficients should be approximations for the coefficient of x^2 in eqn. (4). This could be done by multiplying the coefficient by s and plotting this against $1/s$, which should give a line of intercept $-1/K_i$. When this was done with the coefficients from third-order polynomials fitted to the error-free theoretical curves the

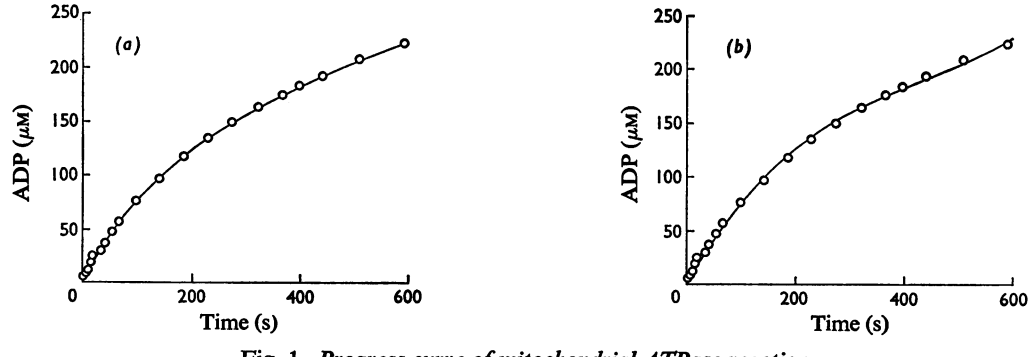

Fig. 1. *Progress curve of mitochondrial ATPase reaction*

The lines are calculated from best-fit third-order polynomials fitted to the same 20points. (*a*) Polynomial in product concentration. (*b*) Polynomial in time. Conditions were as stated in the Materials and Methods section, the initial ATP concentration being 1mM.

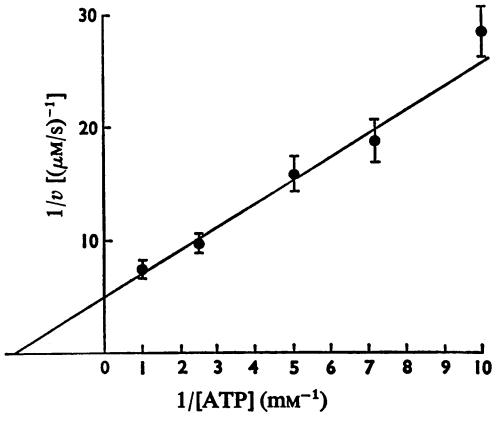

Fig. 2. *Lineweaver–Burk plot of initial-rate estimates obtained by fitting third-order polynomials in product concentration to the progress curves for ATPase*

Plotted values are the mean of six determinations except for that for the lowest substrate concentration, which is the mean of four. The vertical bars indicate the standard errors.

correct value for K_i was obtained, but unfortunately the coefficients of x^2 from the experimental curves were of insufficient accuracy for this.

To overcome this difficulty a more direct approach was adopted for the extraction of values of K_m, K_i and $V_{max.}$ from the progress curves. This was to consider a number of progress curves at one time, in which case the initial substrate concentration as well as the product concentration becomes a variable. With t in eqn. (4) thus considered as a function of two variables the subroutine NLCF can be used to obtain the best-fitting values of K_m, K_i and $V_{max.}$.

In this procedure the value obtained for K_i is of course more dependent on the later points than are the values of K_m and $V_{max.}$ and so it is more sensitive to error on the later points. This makes it important to attach appropriate statistical weights to the data points. An analysis of the times taken to reach fixed product concentrations on five sets of five progress curves showed that the standard deviation on the time to reach a given product concentration was roughly proportional to the mean time. The usual weighting is $1/\sigma^2$ and on this basis a statistical weight of $1/t^2$ was attached to the data points for the curve-fitting procedure. The procedure required a minimum of four progress curves at substrate concentrations between 100 and 1000μM and the values obtained were $K_m = 440\mu$M and $K_i = 16\mu$M. The standard errors on these values as calculated by the curve-fitting procedure were about 10% of the values of the parameters.

The K_m value is in reasonable agreement with that obtained by the polynomial-fitting procedure. The purified ATPase also catalyses the hydrolysis of ITP; since IDP (and also P_i) is not a competitive inhibitor the progress curves have an appreciable linear region and accurate calculation of initial rates can be made graphically. ADP is a competitive inhibitor of the ITPase activity of the enzyme and the K_i for ADP measured in this system is 22μM (Fig. 3). The close agreement with the value obtained from analysis of the progress curves for ATP hydrolysis is taken to be good evidence for the reliability of that method. Not only does the analysis of progress curves for ATP hydrolysis provide a value for the K_i in a more physiological system, but also, although the method of analysis of the inhibition of ITPase activity was simpler it required 25 assays compared with 5 for the ATPase system.

The soluble ATPase from mitochondria presents a rather extreme example of the case where the K_i for one of the products is smaller than the K_m for the substrate; a K_m/K_i ratio of 25 is not a situation one expects to meet frequently. However, the curve-fitting procedure we have had to adopt is obviously applicable in other cases where the integrated rate-equation

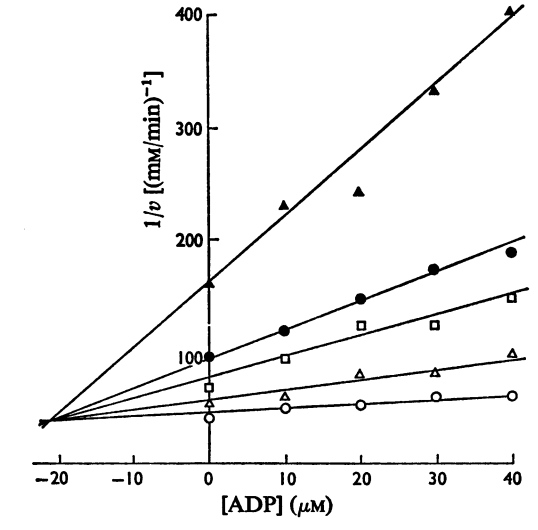

Fig. 3. *Dixon plot to determine the K_i for ADP acting as a competitive inhibitor of the hydrolysis of ITP by the mitochondrial ATPase*

Equimolar concentrations of ITP and $MgCl_2$ were used. Other conditions were as stated in the Materials and Methods section. ○, 5mM-ITP; △, 2.5mM-ITP; □, 1.5mM-ITP; ●, 1mM-ITP; ▲, 0.5mM-ITP.

is known. In those cases where the equation is not known it is worth considering that fitting t as a polynomial in x should give better estimates of the initial rate than fitting x as a polynomial in t.

It is worth noting that in normal statistical practice t is considered to be error free (in comparison with x). We have used an empirical method to estimate the weighting for the points when fitting a polynomial in x, since this assumes that x is error free. If the weighting of x values is known it is possible to derive a theoretical expression for transforming the weights. However, as has been noted previously (Selwyn, 1965) the value of x is a function of the product of time and enzyme concentration. Errors in the amount of enzyme added are equivalent to errors in the time-scale and may well be a major source of error.

Computer routines such as NLCF, that can produce least-squares fits to functions with more than one variable and several parameters, are of course already used in many fields of science and their use in enzyme kinetics may be expected to increase. These routines enable one to obtain values for parameters in theoretically derived equations that might otherwise be considered too complex to be of practical use.

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