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Some uses of extrapolation in kinetics

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Extrapolation procedures are shown to be useful for obtaining kinetic parameters from irreversible enzymic reactions in which there are two intermediates, under both single-turnover and steady-state conditions. Small excesses of one component are treated as if they were large excesses, which is convenient in practice. The method has also been applied to a non-enzymic reversible bimolecular reaction.

INTRODUCTION

The reactions encountered in biochemistry consist of unimolecular or bimolecular steps, and the rate of the latter depends on the product of the concentrations of two species. The kinetics are simplier if the concentration of one partner greatly exceeds that of the other. This principle applies both to non-enzymic and (rather differently) to enzymic reactions, and is of course familiar and widely used. Little attention, however, has been paid to the quantitative aspects of the condition that the concentration of one partner should greatly exceed that of the other. The point of the present paper is to show that only very modest differences in concentration may suffice if extrapolation procedures are used. This will often be a great practical convenience, and so the procedures should be useful in a wide variety of kinetic contexts. We consider an irreversible two-intermediate enzyme reaction under both single-turnover and steady-state conditions; the treatment is illustrative rather than exhaustive, but we have tried to provide enough examples to convince the reader of the potentialities of the procedure, and to show how the accuracy can be tested in a particular case. Extrapolation is, of course, a customary procedure in linear plots of initial-rate data, but we are here concerned with less familiar aspects. The simpler kinetics of non-enzymic reactions are treated first.

THEORY

Non-enzymic reactions

Irreversible second-order reactions. The reaction is written as:

$$A + B \rightarrow C$$

Lower-case italic letters denote the concentrations of the corresponding species, and the rate constant is k. The expression for a second-order reaction may be written (with $x = c/a$, the fractional extent of reaction) as:

$$-\ln(1-x) = k\cdot b\cdot t - k\cdot a\cdot t - \ln[1-(a/b)x] \quad (1)$$

which is to be compared with the usual first-order expression:

$$-\ln(1-x) = k_{obs.}\cdot t \quad (2)$$

The second and third terms on the right-hand side of eqn. (1) become small when a is small, and are zero when $a = 0$. The least-square line will be given by minimizing the difference between the second-order and first-order rate equations, i.e. minimizing:

$$\int_0^t \{k\cdot b\cdot t - k\cdot a\cdot t - \ln[1-(a/b)x] - k_{obs.}\cdot t\}^2\cdot dt$$

whence by differentiation with respect to $k_{obs.}$, followed by integration and solution for $k_{obs.}$:

$$k_{obs.} = k\cdot b - k\cdot a - 2\ln[1-(a/b)x]/t$$

When a/b is small $k_{obs.}$ is a linear function of a that equals $k\cdot b$ when $a = 0$.

Irreversible reactions: procedure. The first-order rate constant, $k_{obs.}$, is determined by a least-squares fit of the data to $\ln[a/(a-z)] = k_{obs.}\cdot t$, where z is the value of [C] at time t; this is done for two or more different initial concentrations of A. The measurements should be made to the same extent of reaction (e.g. 90%) in each experiment. Plot $k_{obs.}$ against a; linear extrapolation to $a = 0$ then gives a value for $k\cdot b$, where k is the second-order rate constant. If only two concentrations of A are used then it is obviously possible to utilize the fact that the ordinate intercept of a line through the pair of points x_1, y_1 and x_2, y_2 is given by $(x_2y_1 - x_1y_2)/(x_2 - x_1)$; in particular, if $x_1 = x_2/2$ then the intercept is $2y_1 - y_2$. The error introduced by the procedure is less than 1% if a/b is no greater than 0.4. Indeed, even with a pair of points in which a/b was 0.5 and 0.75 the extrapolated value was within 10% of the true value.

As an example, the reaction of 6β-bromopenicillanic acid (2 μM) with β-lactamase I (4 μM) ($a/b = 0.5$) had an apparent first-order rate constant, measured spectroscopically, of 2.25 min^{-1}, and the reaction of 3 μM-6β-bromopenicillanic acid with 4 μM-β-lactamase I ($a/b = 0.75$) had an apparent first-order rate constant of 1.58 min^{-1} (measured as above), and hence the extrapolated value is $[(0.75\times 2.25) - (0.5\times 1.58)]/(0.75 - 0.25)$, i.e. 3.6 min^{-1}, and so the second-order rate constant is 3.6/4, i.e. 0.9 $\mu M^{-1}\cdot min^{-1}$, in agreement with the reported value (from 19 measurements) of 0.78 $\mu M^{-1}\cdot min^{-1}$ (Knott-Hunziker *et al.*, 1980). A practical point, which is sometimes important, is that the absorption coefficient does not need to be known when the second-order rate constant is obtained by this procedure.

Reversible second-order reaction. For the reaction in Scheme 1 the time is given, in terms of the equilibrium concentration of C, z_e, as:

$$t = (1/k_{+1}\cdot q)\cdot\ln\{[z_e(M-z)]/[M(z_e-z)]\}$$

where:

$$q = \{[a+b+(k_{-1}/k_{+1})]^2 - 4a\cdot b\}^{\frac{1}{2}}$$

and $M = z_e + q$. Moreover, the concentration of C is given by:

$$z = M\cdot z_e\cdot[1-\exp(-k_{+1}\cdot q\cdot t)]/[M - z_e\cdot\exp(-k_{+1}\cdot q\cdot t)] \quad (3)$$

A	+	B	$\overset{k_{+1}}{\underset{k_{-1}}{\rightleftharpoons}}$	C	
a		b		0	concentration at time 0
$a-z$		$b-z$		z	concentration at time t

Scheme 1.

The fact that these are rather complicated equations to plot, and that they demand prior knowledge of the equilibrium constant to plot them, makes the pseudo-first-order method all the more attractive, and here too extrapolation is entirely satisfactory.

Reversible reactions: procedure. The procedure here is similar except that $k_{obs.}$ is plotted against b for several experiments; the slope gives the forward rate constant k_{+1} and the intercept gives the reverse rate constant k_{-1}. The errors are again low; e.g. for $a/b = 0.2$ and 0.4 and $k_{-1}/k_{+1} \cdot b = 0.2$ for reactions measured to 90% completion the errors in the slope and intercept were 1.4% and 0.4% respectively.

More complex reactions. The method of extrapolation is potentially even more advantageous when there is no analytical solution for the scheme containing the second-order reaction. Such is the case when the step C → D is added to the reversible reaction just considered; this scheme has been thoroughly explored, and the pseudo-first-order approximation has been tested (Summers *et al.*, 1987). Although the extrapolation method was not considered, the results given strongly suggest that it would be applicable.

Enzymic reactions

Interpretation of the first-order rate constant in single-turnover kinetics. The analytical solution for the 'two-intermediate' mechanism:

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} X \xrightarrow{k_{+2}} Y \xrightarrow{k_{+3}} E+P$$

under pseudo-first-order conditions of constant enzyme concentrations is:

$$y+p = k_{+1}' \cdot k_{+2} \cdot s \cdot \{[1-\exp(-\lambda_1 \cdot t)]/\lambda_1 - [1-\exp(-\lambda_2 \cdot t)]/\lambda_2\}/(\lambda_1-\lambda_2) \quad (4)$$

where $k_{+1}' = k_{+1} \cdot e$, $\lambda_1 \cdot \lambda_2 = k_{+1}' \cdot k_{+2}$ and $\lambda_1+\lambda_2 = k_{+1}' + k_{-1} + k_{+2}$. If $\lambda_1 \gg \lambda_2$ then:

$$y+p = s \cdot [-\exp(-\lambda_2 \cdot t)]$$

and

$$\lambda_2 = k_{+1}' \cdot k_{+2}/(k_{-1}+k_{+1}')$$

and if $k_{-1} \gg k_{+2}$ and $k_{-1}/k_{+1} = K_s$ then:

$$\lambda_2 = k_{+2} \cdot e/(K_s+e) \quad (5)$$

The theoretical value for $k_{obs.}$ was calculated by fitting the values of $y+p$ as a function of time to the semi-logarithmic linear form of first-order rate equation; the 'rapid equilibrium' assumption was used in the simulations, which were carried out with C. Frieden's KINSIM program (Barshop *et al.*, 1983). These are the usual assumptions made in treating stopped-flow measurements of such reactions, when the data are regarded as being well fitted by a single exponential.

When only p, rather than $y+p$, is being measured, and the data are fit by a single exponential, then if k_{+3} is much less than λ_2:

$$p = s \cdot [1-\exp(-k_{+3} \cdot t)]$$

Thus here $k_{obs.}$ does not depend on the concentration of enzyme, and gives k_{+3}. When $k_{+3} \gg k_{+2}$, on the other hand, y is small compared with p, and so the solution for p is the same as that already given for $y+p$, and $k_{obs.}$ varies with e. When the two rate constants are of the same order of magnitude the data will not fit a single exponential.

In practice one usually wants to use as small an excess of enzyme as is permissible. Hence this application of the extrapolation method has been explored quite thoroughly.

The kinetic scheme is that given above, and, unless otherwise stated, we assume that [Y]+[Z] is being measured, e.g. the first product in a hydrolysis catalysed by chymotrypsin. The reaction was modelled with the KINSIM program. Values are given in Table 1 for the $k_{obs.}$ obtained by the first-order least-squares line, which is up to 20% low, and for the extrapolated value, which is correct to within 1%. When two points for which [S]/[E] differed by a factor of 2-fold were used for extrapolation then the formula (2 × smaller [S]/[E] value) − (greater [S]/[E] value) was used, equivalent to linear extrapolation. When three points were used, rational extrapolation was found to be superior to polynomial extrapolation; the parameters in an expression of the form $y = (p_0+p_1 \cdot x)/(1+q_1 \cdot x)$ were found by solving the simultaneous equations, where p_0 is the desired value. It is indeed remarkable that it is so easy to get good values by extrapolation over what are, in effect, large distances.

Occasionally, only [P], rather than [Y]+[P], is measured. If $k_{+2} \gg k_{+3}$ there will be a lag in the progress

Table 1. Single-turnover kinetics

Values found by simulation with initial concentration of enzyme [E] = 100 (arbitrary units) and k_{+3} = 100, measured to 90% completion. First series, K_s = 10 and k_{+2} = 100; second series, K_s = 10 and k_{+2} = 10; third series, K_s = 100 and k_{+2} = 100; fourth series, K_s = 1 and k_{+2} = 100. Linear or rational extrapolation was used as described.

[S]/[E]	$k_{obs.}$ (s^{-1})	Concentration used	Extrapolation (s^{-1})	Error (%)	Series
0.2	89.7				1
0.4	87.8	0.2 and 0.4	91.6	0.7	
0.6	85.3	0.2, 0.6 and 1	91.2	0.3	
1	77	0.6, 0.8 and 1	91.8	1.2	
0.2	9.01				2
0.4	8.9	0.2 and 0.4	9.12	0.3	
0.6	8.73	0.2, 0.6 and 1	9.01	0.3	
1	8.27	0.6, 0.8 and 1	9.12	0.3	
0.2	47.9				3
0.4	45.6	0.2 and 0.4	50.1	0.2	
0.6	43.4	0.2, 0.6 and 1	50.2	0.3	
1	39.2				
0.2	98.9				4
0.4	98.6	0.2 and 0.4	99.3	0.3	
0.6	98.2	0.2, 0.6 and 1	99.1	0.1	
0.8	97.3	0.6, 0.8 and 1	99.1	0.1	
1	94.8				

curve, and so in the simulations the least-squares line in the plot of $\ln\{[S]_0/([S]_0-[P])\}$ against time was no longer constrained to pass through the origin. In one example, when measurements of [P] over 40% to 90% reaction were simulated, with $K_s = 100$, $k_{+2} = 100$, $k_{+3} = 10$, $[E]_0 = 100$ and $[S]_0 = 10$ and 20, or 10, 20 and 40, the extrapolated values of the first-order rate constant were 9.964 and 9.963 respectively, i.e. close to k_{+3}, as expected in this case (see above). However, when the values of k_{+2} and k_{+3} were interchanged, the extrapolated value of the first-order rate constant was 5.00. This is because measuring [P] is now virtually the same as measuring [Y]+[P], and so eqn. (5) applies.

'Steady-state' reactions. When the concentration of enzyme is not much less than that of the substrate the assumptions of steady-state kinetics cease to hold; thus the free and total substrate concentrations differ. Experiments in which $[S]_0$ does not greatly exceed $[E]_0$, i.e. the range $[E]_0/[S]_0 = 0.05$–0.5, are now considered. Apparent values of the parameters can be obtained and plotted against $[E]_0/[S]_0$, and the intercept obtained by extrapolation. In what follows, the values so obtained from simulations are compared with the theoretical values.

Use of half-time plots. Progress curves for the two-intermediate reaction were analysed by the method of Wharton & Szawelski (1982). Linear plots were obtained when k_{+2} was less than or equal to k_{+3}, but when k_{+2} was greater than k_{+3} there was a burst and points had to be taken after the burst had finished. We observed that a satisfactory straight line was no guarantee that the derived parameters would be correct, a point that was noted in many of the situations studied here. The results in Fig. 1 suggest that satisfactory values of $k_{cat.}$ and of $k_{cat.}/K_m$ can be obtained by linear extrapolation of points up to $[E]/[S] = 0.5$. As usual, these are the two parameters obtained directly, and K_m is obtained by division. However, these conclusions will depend on the mechanism, and the user of the method should test the accuracy of extrapolation in the particular circumstances.

When initial rates, rather than progress curves, are used to obtain values for the kinetic parameters, extrapolation methods may again be useful, but this has been less studied. Part of the difficulty lies in the fact that what is measured experimentally as the initial rate will depend on the mechanism and the values of the rate constants.

Slow-binding inhibition

The equation for the formation of product when there is an inhibitor present that binds slowly according to Scheme 2 is (Morrison & Walsh, 1988):

$$p = v_f \cdot t + (v_0 - v_f)[1 - \exp(-k \cdot t)]/k \qquad (6)$$

One of the assumptions that has to be made when this equation is being deduced is that the concentration of substrate has remained virtually unchanged. Thus the sole reason for the decrease in the rate of reaction has to be the binding of inhibitor coming to equilibrium. However, the detection of the transient is harder if the concentration of the substrate is too high, and so substrate depletion is often a cause for concern when applying eqn. (6) to experiments at appreciable concentrations of enzyme. Simulations to measure, and extrapolations to correct for, the effects of substrate depletion are now reported.

At the highest concentration of enzyme there was approx. 25% of the substrate consumed during the portion of the reaction analysed, and the parameters k, v_0 and v_f obtained by a least-squares fit to eqn. (6) were

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EI^*$$

Scheme 2.

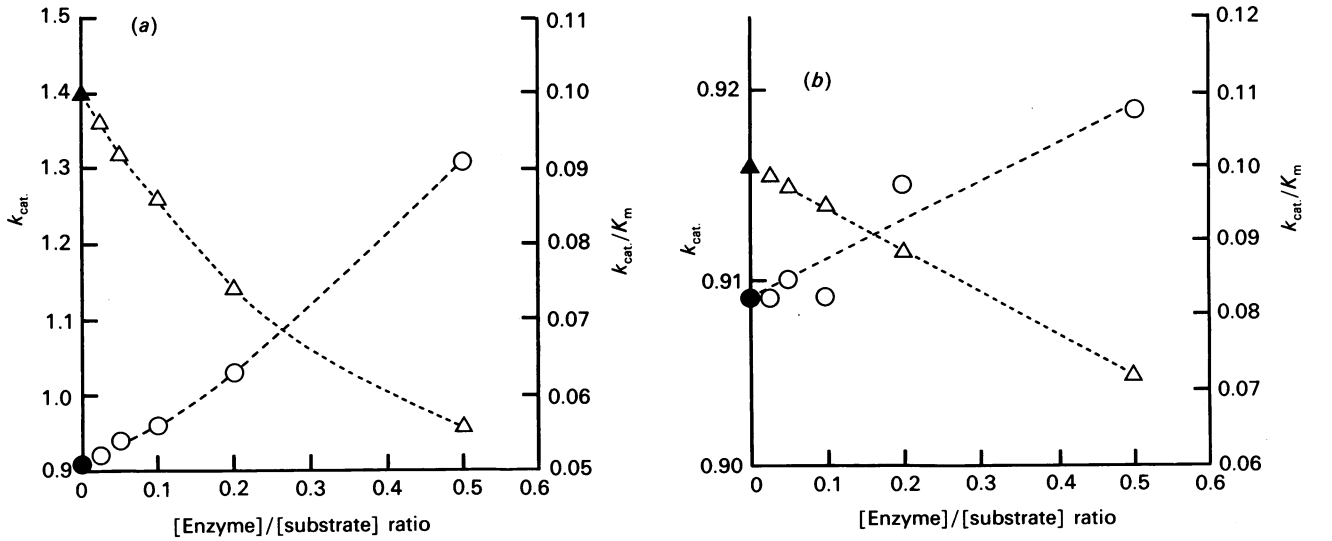

Fig. 1. Dependence of kinetic parameters obtained from half-time plots of progress curves on the concentration of enzyme

Progress curves for the two-intermediate mechanism were simulated, on the assumption that the conversion of the first intermediate into the second was being measured (arbitrary units): (*a*) [S] = 20, $K_s = 10$, $k_{+2} = 1.0$ and $k_{+3} = 10$; (*b*) [S] = 20, $K_s = 100$, $k_{+2} = 10$ and $k_{+3} = 1.0$. Kinetic parameters were obtained from the progress curves by the half-time method, and are plotted against the quotient [enzyme]/[substrate]. Symbols: ○ and ●, $k_{cat.}$; △ and ▲, $k_{cat.}/K_m$. ● and ▲ refer to the theoretical values used for the simulation.

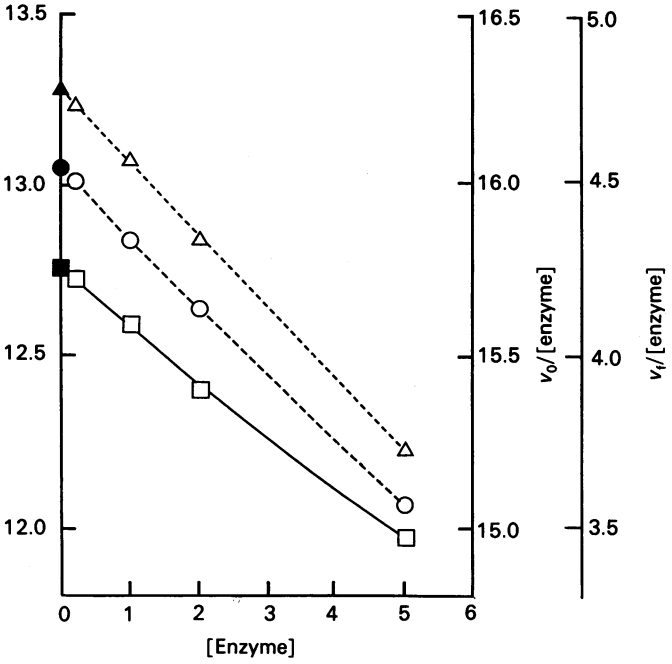

Fig. 2. Dependence of parameters v_0, v_f and k for slow-binding inhibition on the concentration of enzyme

Progress curves were simulated for slow-binding inhibition, [S] = 50, [I] = 500, K_i = 1700, k_{+2} = 60 and k_{-2} = 357, and then fitted by non-linear regression to eqn. (6) in the text. The parameters thus obtained are plotted against the enzyme concentration. Symbols: ○ and ●, rate constant k; △ and ▲, original rate, normalized to unit enzyme concentration, v_0/e; □ and ■, final rate, normalized to unit enzyme concentration, v_f/e. ●, ▲ and ■ refer to the theoretical values obtained as described by Crompton *et al.* (1988).

in error by about 7%, 7% and 28% respectively. Nevertheless, the plots of the parameters against the concentration of enzyme were approximately linear (Fig. 2), and the extrapolated values were within 1% of the true values. Thus extrapolation to zero concentration of enzyme can be recommended in the use of eqn. (6) to interpret slow-binding inhibition, and the same may well apply when eqn. (6) is used for hysteretic enzymes (Frieden, 1979).

CONCLUSIONS

The usual procedure for simplifying the kinetics of a simple irreversible bimolecular reaction is to use an excess of one component; it has been suggested that the excess may be as little as 10:1, but that 40:1 or even 100:1 is to be preferred (Bunnett, 1986). There are, however, practical drawbacks to the use of a large molar excess of one component; it may contain impurities, or hinder the measurement of the concentration of the other component, or it may alter the medium.

Extrapolation procedures could be used more widely now that their reliability, at least in some cases, has been demonstrated. One area of potential use is in the study of low-activity mutants of enzymes produced by site-directed mutagenesis. Here it may be necessary to use the enzyme at, say, one-fifth or one-tenth the concentration of the substrate in order for the rate to be high enough to be conveniently and accurately measurable. Thus it may well be advantageous to measure apparent values of the parameters and extrapolate, as described in the present paper. Other examples of the usefulness of extrapolation are in the determination of specificity constants (Crompton & Waley, 1986), in the kinetics of 'suicide substrates' (De Meester *et al.*, 1987) and in the determination of the permeability number for β-lactam antibiotics (Waley, 1988).

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