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The determination of specificity constants in enzyme-catalysed reactions

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A convenient and accurate procedure for determining the kinetic parameter $V_{max.}/K_m$ is described. This avoids the error in the usual method of taking the observed first-order rate constant of an enzymic reaction at low substrate concentration as $V_{max.}/K_m$. A series of reactions is used in which the initial concentation of substrate is below K_m (e.g. from 5% to 50% of K_m). Measurements are taken over the same extent of reaction (e.g. 70%) for each member of the series, and treated as if the kinetics were truly first-order. The reciprocal of the observed first-order rate constant is then plotted against the initial concentration of substrate: the reciprocal of the ordinate intercept is $V_{max.}/K_m$. The procedure, as well as being applicable to simple reactions, is shown to be valid when there is competitive inhibition by the product, or when the reaction is reversible, or when there is competitive or mixed inhibition. The hydrolysis of cephalosporin C by a β-lactamase from *Pseudomonas aeruginosa* is used to illustrate the method.

INTRODUCTION

Specificity is the hallmark of catalysis by enzymes, and so the determination of $k_{cat.}/K_m$, the specificity constant (Nomenclature Committee of the International Union of Biochemistry, 1982; Fersht, 1985), is a common task. This amounts, of course, to determining the $V_{max.}/K_m$ when the concentration of enzyme is (implicitly) introduced. Moreover, the effects of pH and of isotopic substitution are often described in terms of $V_{max.}/K_m$. On occasion, too, practical considerations such as the solubility of the substrate or a very high K_m mean that it is only $V_{max.}/K_m$ that can be reliably determined. The present paper makes two points. First, it describes a simple and convenient method for determining $V_{max.}/K_m$. The proposed method is an interesting, and sometimes advantageous, alternative to the use of initial rates. When the initial rate declines rapidly (owing to substrate depletion), it is hard to estimate accurately by 'pencil and paper' (tangent at the origin) methods. Instead, the use of progress curves is commonly advocated (Cornish-Bowden, 1975; Boeker, 1982), and the latest method utilizes non-linear regression (Duggleby, 1985). However, the simple procedure for determining $V_{max.}/K_m$ proposed here is satisfactory when the rate is declining and is also essentially a 'pencil and paper' method, but one that exploits the whole of the reaction. The second point is also important, in that a common procedure can lead to appreciable errors. This common procedure consists of treating reactions in which the initial concentration of substrate is appreciably below K_m as if they were truly first-order reactions. However, such semi-logarithmic progress-curve plots turn out to be much more widely used than might have been expected.

THEORY

Error in the usual procedure

We first consider the slope of the semi-logarithmic plot when the progress curve is treated as first-order. The slope is related to the parameter $V_{max.}/K_m$ by the treatment now presented. The progress curve for a simple enzymic reaction is given by:

$$t = x \cdot \frac{s_0}{V_{max.}} - \left(\frac{K_m}{V_{max.}}\right) \cdot \ln(1-x)$$

Here t is the time, s_0 is the initial concentration of substrate, s the concentration at time t, and $x = (s_0 - s)/s_0$ is the fractional extent of reaction. This may be rearranged to:

$$-\ln(1-x) = \left(\frac{V_{max.}}{K_m}\right) \cdot t - x \cdot \frac{s_0}{K_m} \quad (1)$$

In a first-order plot, instead of eqn. (1) we have:

$$-\ln(1-x) = C \cdot t$$

where C is a parameter whose value is now found. The line in such a first-order plot will usually by obtained by minimizing the absolute (unweighted) errors, i.e. the least-squares approximation obtained by minimizing:

$$S = \sum_i \left(\frac{V_{max.}}{K_m} \cdot t_i - x_1 \cdot \frac{s_0}{K_m} - C \cdot t_i\right)^2$$

with respect to C. Hence:

$$\frac{\partial S}{\partial C} = -2\sum_i \left[\left(\frac{V_{max.}}{K_m} - C\right) t_i - \frac{s_0}{K_m} \cdot x_i\right] \cdot (t_i) = 0$$

and thus:

$$\left(\frac{V_{max.}}{K_m} - C\right) \sum_i t_i^2 - \frac{s_0}{K_m} \sum_i x_i \cdot t_i = 0$$

and so:

$$C = \frac{V_{max.}}{K_m} - \frac{s_0}{K_m} \cdot \frac{\Sigma x_i \cdot t_i}{\Sigma t_i^2} \quad (2)$$

Values of C are given in Table 1 as a percentage of $V_{max.}/K_m$. The error brought about by taking C as $V_{max.}/K_m$ is thus seen to vary from 4.5% (top left entry) to about 26% (bottom right entry). An important point is that, experimentally, the plots will appear to be first-order, at least for most of the examples listed in

Table 1. Slopes of first-order plots

The values in the Table were derived by the use of eqn. (2) in the text, derived on the basis that a line was drawn in a first-order plot by minimizing the absolute errors. The row marked with an asterisk (*) shows the values obtained if the relative (rather than the absolute) errors are minimized. The slopes of semi-logarmithmic progress-curve plots are given as percentages of the true $V_{max.}/K_m$. The initial concentration of substrate is given as a percentage of K_m, and the extent of reaction is also given as a percentage.

Extent of reaction (%)	Initial concn. of substrate (% of K_m)...	Slope of first-order plot													
		5	10	15	20	25	30	35	40	45	50	55	60	65	70
15		95.5	91.4	87.7	84.2	81.0	78.1	75.3	72.7	70.3	68.1	66.0	64.0	62.2	60.4
30		95.8	92.0	88.5	85.2	82.2	79.3	76.7	74.2	71.9	69.7	67.7	65.7	63.9	62.2
60		96.6	93.3	90.3	87.5	84.8	82.3	80.0	77.8	75.6	73.6	71.8	70.0	68.2	66.6
75		97.0	94.1	91.5	88.9	86.5	84.3	82.1	80.0	78.1	76.2	74.4	72.7	71.1	69.6
75*		96.3	92.9	89.7	86.7	84.0	81.4	79.0	76.7	74.5	72.5	70.6	68.8	67.1	65.4
90		97.6	95.3	93.1	91.0	89.0	87.1	85.2	83.4	81.7	80.1	78.5	77.0	75.5	74.1

* Errors assumed proportional, not absolute.

Table 1. The example of Fig. 1, where both theoretical and experimental results are depicted, bears this out. The plots are linear, but if the slopes were taken as $V_{max.}/K_m$ the values would be about 15% low. Thus it is erroneous to conclude that a good first-order plot gives the correct value of $V_{max.}/K_m$. But such a plot is still useful, and we now discuss how to obtain $V_{max.}/K_m$ from C.

Use of 'first-order plots' to determine $V_{max.}/K_m$

Exceptionally, K_m may already be known, and then Table 1 can be used to find $V_{max.}/K_m$. Normally, K_m is not known, and the procedure now described should be used. When y is defined as $-\ln(1-x)$ and eqn. (1) is differentiated with respect to t, we have:

$$\frac{dy}{dt} = \frac{V_{max.}}{K_m} - \frac{s_0}{K_m} \cdot \frac{dx}{dt}$$

and, since $dx/dt = (1-x)dy/dt$, the slope of a semi-logarithmic progress-curve plot of y against t is given by:

$$\frac{dy}{dt} = \frac{V_{max.}}{s_0(1-x)+K_m} \qquad (3)$$

When the deviation from linearity is, in practice, negligible, the (constant) slope is $k_{obs.}$, the pseudo-first-order rate constant. And thus:

$$\frac{1}{k_{obs.}} = s_0 \cdot \frac{(1-\bar{x})}{V_{max.}} + \frac{K_m}{V_{max.}} \qquad (4)$$

Eqn. (4) gives the reciprocal of the slope of a semi-logarithmic progress-curve plot as a function of the kinetic parmeters, and of s_0 and $\bar{x}$, where $\bar{x}$ is now a mean value of x. Hence a plot of $1/k_{obs.}$ against s_0 for a series of reactions will enable the true $V_{max.}/K_m$ to be obtained (as the reciprocal of the ordinate intercept) as long as $\bar{x}$ is effectively constant for the series (see below). Thus, for a series of reactions, the measurements should be taken to the same final extent of reaction (e.g. 50%), so that $\bar{x}$ is indeed effectively constant. The practical point is that the convenience of first-order plots is retained, and that the extrapolation to $s_0 = 0$ gives a reliable, and precise, value of $V_{max.}/K_m$. Tests with stimulated data have shown that the procedure is adequately robust.

When we found that stimulated data gave a straight line when plotted according to eqn. (4), we realized that this meant that $\bar{x}$ had to be effectively constant (at some sort of mean value) for the series with varying s_0. This was checked as follows. The slope of the least-squares line through the origin when y is plotted against t is $\sum_i y_i \cdot t_i / \sum_i t_i^2$, and so, from eqn. (3):

$$\frac{\sum_i y_i \cdot t_i}{\sum_i t_i^2} = \frac{V_{max.}}{s_0(1-\bar{x})+K_m}$$

which may be solved for $\bar{x}$ to give:

$$\bar{x} = \frac{(s_0+K_m)\sum y_i \cdot t_i - V_{max.}\sum t_i^2}{s_0 \sum y_i \cdot t_i}$$

For example, $\bar{x}$ only varied from 0.233 to 0.229 for a series of reactions followed to 50% when s_0/K_m was varied from 0.05 to 50.

Competitive inhibition by the product

When the product of the reaction is a competitive inhibitor, with dissociation constant K_p, eqn. (4) is replaced by:

$$\frac{1}{k_{obs.}} = s_0 \left(\frac{x\left(\frac{K_m}{K_p}-1\right)+1}{V_{max.}} \right) + \frac{K_m}{V_{max.}} \qquad (5)$$

and thus the intercept of the plot of $1/k_{obs.}$ against s_0 is unaltered. This is a practical convenience: it is not necessary to find out if there is competitive product inhibition to find $V_{max.}/K_m$, as long as the inhibition is not so marked that a straight line can no longer be drawn through the points in the semi-logarithmic plot. It may be shown that $\bar{x}$ is now given by:

$$\bar{x} = \frac{(s_0+K_m)\sum y_i \cdot t_i - V_{max.}\sum t_i^2}{s_0\left(1-\frac{K_m}{K_p}\right)\sum y_i \cdot t_i}$$

Competitive and mixed inhibition

If there is either purely competitive or mixed (Cornish-Bowden, 1979, pp. 74–82) inhibition, the value of the kinetic parameter found by the extrapolation method described will be:

$$\frac{K_m}{V_{max.}}\left(1+\frac{i}{K_i}\right)$$

where i is the concentration of inhibitor, and K_i the dissociation constant for the equilibrium:

$$E+I \rightleftharpoons EI$$

Thus, even if there is mixed inhibition, the competitive part is directly obtained. It is convenient when studying inhibitors to use a relatively low concentration of substrate, so that the effect of the inhibitor is evident. This suggests that the present method may be a useful alternative to an earlier method for determining K_i in which progress curves were also employed (Waley, 1982).

Reversible reactions

If the reaction is reversible, with equilibrium constant $K_{eq.}$, then it may be shown that eqn. (4) is now replaced by:

$$\frac{1}{k_{obs.}}=\frac{K_m}{V_{max.}}\cdot F\left\{1+s_0\left[\frac{1}{K_m}+x\cdot F\left(\frac{1}{K'_m}-\frac{1}{K_m}\right)\right]\right\}$$

where K'_m is the Michaelis constant for the reverse direction, x is now $p/p_{eq.}$, $p_{eq.}$ being the concentration of product at equilibrium, and the 'effect of reversibility' factor F is:

$$F=\frac{1}{1+\frac{1}{K_{eq.}}}=\frac{p_{eq.}}{s_0}$$

As long as the usual plots of y against t are linear a value of C may be obtained and $1/C$ plotted against s_0 for a series of experiments followed to the same extent (x being effectively constant); the intercept is:

$$\frac{K_m}{V_{max.}}\cdot F$$

Use of initial rates to determine $V_{max.}/K_m$

When the initial rate declines markedly early in the course of the reaction, either measurements have to be restricted to the first (say) 3% of reaction, or progress curves have to be used to estimate the initial rate. If a discontinuous assay is being employed and material is scarce, an estimate of the initial rate (v_0) can be obtained from only two measurements of the product (p) formed from the following ('upside-down') formula based on the work of Boeker (1982):

$$v_0=\frac{\frac{1}{t_1}-\frac{1}{t_2}}{\frac{1}{p_1}-\frac{1}{p_2}}$$

The wrong way to proceed, when $V_{max.}/K_m$ is the parameter of interest, is to estimate $V_{max.}$ and K_m separately. This is because the relative error in $V_{max.}/K_m$ will be the sum of those of $V_{max.}$ and K_m, and the relative error in K_m is commonly the largest of the three. The right way is to determine $V_{max.}/K_m$ directly, for instance from a plot of s_0/v_0 against s_0 (Hanes plot), which is statistically preferable to the reciprocal plot (Cornish-Bowden, 1979, pp. 26–28). It may be noted that the 'first-order' method described above utilizes a plot akin to the Hanes plot, s_0/v_0 being replaced by $1/k_{obs.}$.

Instability of enzyme

The first-order plot has to be used with care if the enzyme is unstable. If all forms (i.e. bound and free) of the enzyme lose activity exponentially with rate constant k_i, then $V_{max.}/K_m$ can be found (to within 3%) if $k_i < 0.1 \times V_{max.}/K_m$ if the reaction is followed to 50%

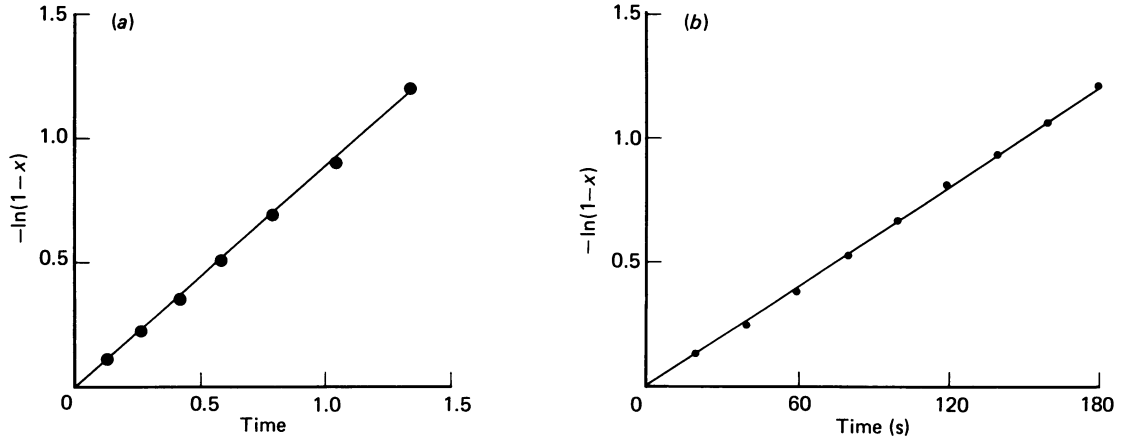

Fig. 1. Progress curves treated as first-order plots

(*a*) (Theoretical) plot of $-\ln(1-x)$, where x is the fractional extent of reaction, against time, when $s_0/K_m = 0.2$, for the first 70% of reaction. The points were calculated from the integrated Michaelis–Menten equation. (*b*) as (*a*) for the hydrolysis of cephalosporin C (10.6 μM) catalysed by a β-lactamase from *Pseudomonas aeruginosa* (0.64 nM); $s_0/K_m = 0.25$. The reaction was carried out in 100 mM-NaCl/20 mM-Mops buffer, pH 7, at 30 °C; A_{260} was measured. The measurements shown correspond to the range 0–70% reaction.

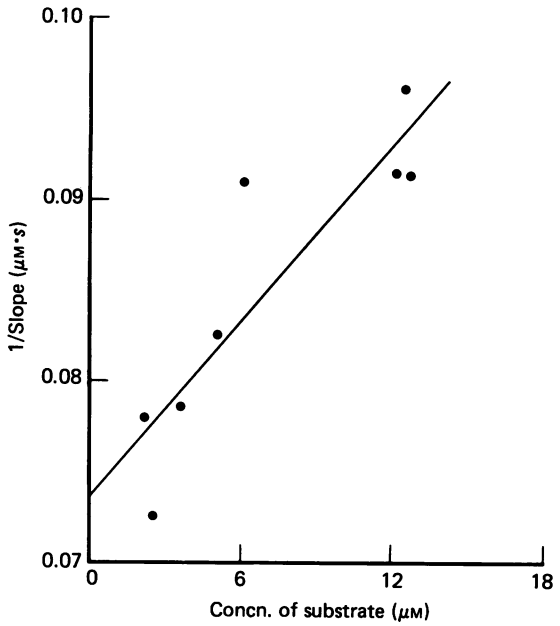

Fig. 2. Variation of reciprocal of first-order rate constant with concentration of substrate

The first-order rate constant was determined from experiments as in Fig. 1(*b*); the concentration of substrate varied over the range 2–10 μM (0.05–0.25 K_m). The plot shows the linear dependence of the reciprocal of the first-order rate constant on the concentration of substrate [eqn. (3) in the text]; the ordinate intercept gives $K_m/V_{max.}$ in general, here $K_m/k_{cat.}$ as the rates were expressed on the basis of unit enzyme concentration.

completion; about 10% of the enzyme will be inactivated at this stage. Naturally, initial rates are safer here, and, if only the first 5% of othe reaction is followed and k_i is equal to $V_{max.}/K_m$, only negligible (0.02%) errors are introduced.

MATERIALS AND METHODS

The β-lactamase was from the constitutive mutant of *Pseudomonas aeruginosa* 1822S/H (Flett *et al.*, 1976; Berks *et al.*, 1982), and was purified by affinity chromatography (Cartwright & Waley, 1984); cephalosporin C was a gift from Glaxo Group (Greenford, Middx., U.K.) and was recrystallized (Newton & Abraham, 1956). The enzymic reaction was carried out in 100 mM-NaCl/20 mM-Mops/NaOH buffer, pH 7, at 30 °C; A_{260} was measured in a Cary 219 spectrophotometer.

RESULTS

Linear first-order plots were obtained (Fig. 1*b*); eight experiments were carried out, in which the concentration of substrate varied from 5% to 25% of K_m. The slopes gave $k_{obs.}$. Then $1/k_{obs.}$ was plotted against the initial concentration of substrate (Fig. 2). From the intercept $k_{cat.}/K_m$ was obtained as $807 \pm 40\ \mu M^{-1} \cdot min^{-1}$.

CONCLUSIONS

Although $V_{max.}/K_m$ is written as a quotient, i.e. a derived quantity, it is preferable, both conceptually and experimentally, to regard $V_{max.}/K_m$ as a primary kinetic parameter and to determine it directly, a view that has been emphasized by Northrop (1983). When the initial concentration of substrate is below 5% of K_m, then the customary procedure of taking the observed first-order rate constant as $V_{max.}/K_m$ is not greatly in error, but at higher concentrations of substrate the errors are appreciable (Table 1). Nevertheless, the first-order constant is easy to measure accurately, and so the procedure of using it to obtain the true $V_{max.}/K_m$ should be generally useful. For instance, it is better to determine K_i from the apparent $K_m/V_{max.}$ than from the apparent K_m because the former can be obtained more accurately. Perhaps the most likely limitation of the procedure is instability of the enzyme, and some guidance was given above. First-order rate constants can, of course, be obtained graphically, or by a non-parametric method (Nimmo & Atkins, 1979), or by direct non-linear regression (Bicknell & Waley, 1985).

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