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The Reliability of Michaelis Constants and Maximum Velocities Estimated by Using the Integrated Michaelis–Menten Equation

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1. Experimental progress curves were simulated for a reaction obeying Michaelis–Menten kinetics. 2. K_m and V were estimated (*a*) by fitting the integrated Michaelis–Menten equation to the progress curves, and (*b*) from the initial slopes of the curves (i.e. from initial velocities). 3. The integrated equation could not be fitted successfully by a non-linear method, so it was transformed and fitted by a linear method. 4. Provided that the initial substrate concentration was greater than K_m and the data were precise enough, the integrated equation gave parameter estimates which were unbiased and as reliable as those derived from initial velocities although based on fewer experiments. 5. The integrated equation could be used for progress curves of unknown origin.

The Michaelis–Menten parameters of an enzyme-catalysed reaction (K_m and V) are usually found by measuring initial rates of reaction at several different substrate concentrations and then fitting the Michaelis–Menten rate equation to the data. Alternatively, the progress curve of the reaction at a single substrate concentration can be determined, and the integrated form of the equation fitted to the data (Laidler, 1958; Dixon & Webb, 1964).

The latter approach has the advantage that, at least for the simplest type of reaction, K_m and V can be estimated in a single experiment in which the concentration of product is continuously monitored. In contrast, when initial velocities alone are measured, at least three experiments are required, and up to ten are often performed. (Of course, more complicated types of reaction demand additional experiments whichever method is used.) Since it is often quite easy to monitor a reaction continuously and therefore to obtain a large number of points on the progress curve (e.g. by using a pH-stat device for an esterase, or a recording spectrophotometer for a dehydrogenase), it is at first sight surprising that the integrated equation has only occasionally been adopted, even though its theory has been well documented (Laidler, 1958; Booman & Niemann, 1956; Elmore *et al.*, 1963). One possible explanation is that the reaction does not conform to the integrated equation; because, for example, the enzyme or substrate is unstable, there is an appreciable non-enzymic reaction, or the product is also an inhibitor. A second possibility is that the origin of the progress curve is unknown; and a third is that although the whole curve is known, the values of the parameters derived from it are imprecise.

In the present paper we examine the second and third of these possibilities. We compare, by analysing simulated data for the simplest type of enzyme-catalysed reaction, the reliabilities of the parameters derived from initial velocities and from a progress curve. We assume, first, that the origin of the progress curve is known (for example, in a spectrophotometric assay the absorbance at zero time can generally be determined). We then make the alternative assumption that the origin and early part of the curve are not known but have to be estimated from the later part of the curve. This occurs in a continuous titration when the pH of the reagent added to start the assay is not exactly equal to the pH of the assay, so that initially the addition of titrant is either increased or delayed.

We found that the integrated equation could not be fitted by a well-tried non-linear method, but that a method which is strictly speaking invalid could be used instead. At certain substrate concentrations the latter method gave parameter estimates which were as reliable as those derived from initial velocities. In addition we found that it did not matter if the origin of the progress curve had also to be estimated.

Finally, we show how our results can be extended to certain more complex types of reaction by considering the situation that arises when the product is a competitive inhibitor with respect to the substrate.

Methods and Results

Data simulation

In the simplest type of enzyme-catalysed reaction, a single substrate is converted irreversibly into a product that does not inhibit the enzyme; the rate equation is:

$$\frac{\mathrm{d}p}{\mathrm{d}t}=\frac{V\cdot(s_0-p)}{K_m+(s_0-p)} \tag{1}$$

where p is the concentration of product at time t ($p = 0$ at $t = 0$); and s_0 is the initial concentration of substrate. Integration of eqn. (1) gives the equation describing the progress curve:

$$Vt = p - [K_m \cdot \ln(1 - p/s_0)] \tag{2}$$

A 'perfect' (i.e. error-free) progress curve was derived by setting $K_m = V = 1$ and s_0 to 0.5, 0.7, 1.0, 1.4 or 2.0, and then using eqn. (2) to calculate 15 roughly equally spaced values of t from values of p in the range $0.08s_0$–$0.80s_0$. 'Experimental' (i.e. error-containing) progress curves were simulated by adding to each perfect value of p one of a series of normally distributed pseudo-random numbers of mean zero and standard deviation either 0.005 or 0.01 (the series was generated by Edinburgh Regional Computing Centre program Random). The standard deviation of p was therefore independent of the magnitude of p, as is likely in practice. The value of 0.01 is large, but was chosen to provide a rigorous test of the methods used to analyse the data. At each standard deviation and substrate concentration 40 different progress curves were simulated (one example is given in Fig. 2).

Progress curve of known origin

Integrated equation. A theoretically valid way of estimating K_m and V is to choose a pair of values for these parameters, integrate eqn. (1) numerically to calculate the values of p corresponding to the observed values of t, and then search for the pair of values that minimizes the sum of the squares of the differences between the observed and calculated values of p. We did this, by using the Kutta–Merson procedure for numerical integration (Mayers, 1962), and the rapid-descent method of Davidon and Fletcher & Powell as exploited by Atkins (1971) for finding the minimum sum of squares of differences. However, the minimum finally reached was not unique but depended on the starting values of K_m and V, presumably because the minimum is ill-defined.

Other ways of estimating K_m and V depend on some form of least-squares linear regression based on eqn. (2): for example, the multiple regression of t on p and $\ln(1 - p/s_0)$, or the simple regression of

$$p/t \text{ on } \frac{1}{t} \cdot \ln(1 - p/s_0).$$

Neither of these regressions is theoretically valid, because t is really an independent variable and p a dependent one; however, both regressions must give unique answers. Fisher & Nimmo (1972) found that, despite its invalidity, the simple regression gave in a similar situation answers which were not biased and were more precise than those given by the multiple regression. Therefore we have used the regression of p/t on

$$\frac{1}{t} \cdot \ln(1 - p/s_0)$$

to estimate K_m and V.

The regression was first calculated with the points being weighted equally. Then, for comparison, it was calculated iteratively, with each point being given a weighting factor of:

$$\frac{t^2}{\left[1 + \dfrac{K_m}{s_0 - p}\right]^2}$$

until K_m converged to a constant value. This weighting factor was used because, as shown in Fig. 1, an error of Δp moves the point P to Q which is, by using Taylor's Theorem (Courant, 1937), to a first approximation of distance:

$$\frac{\Delta p}{t}\left[1 + \frac{K_m}{s_0 - p}\right]$$

above the line.

The results are in Table 1. For either regression all the mean values of K_m and V were less than the theoretical ones of unity, but improved markedly with increasing s_0. The mean K_m always differed more

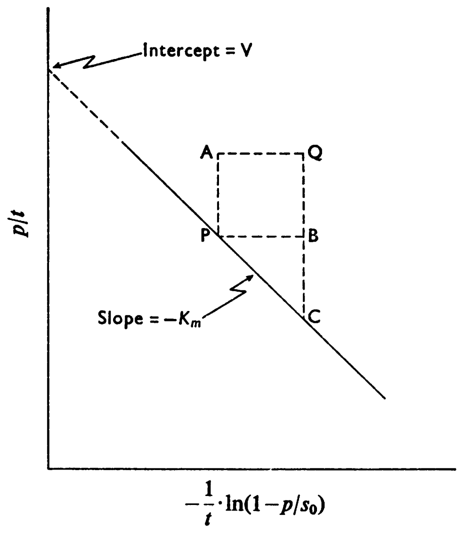

Fig. 1. *Effect of an error in p of* Δp *on the plot of* p/t *against* $-\frac{1}{t} \cdot \ln(1 - p/s_0)$

The point P moves to Q, such that:

$\text{QB} = \Delta p/t$

$\text{BC} = K_m \cdot \text{PB} \simeq \dfrac{K_m}{t}\left[\dfrac{\Delta p}{s_0 - p}\right]$ (by Taylor's Theorem)

$\text{QC} \simeq \dfrac{\Delta p}{t}\left(1 + \dfrac{K_m}{s_0 - p}\right)$

Table 1. *Mean estimates of K_m and V for progress curves of known origin*

Values are means ±S.D. ($n = 40$).

	Integrated unweighted		Integrated weighted		Initial velocities	
s_0	K_m	V	K_m	V	K_m	V
S.D. = 0.005						
0.5	0.53±0.27	0.65±0.20	0.60±0.13	0.70±0.10		
0.7	0.76±0.19	0.84±0.13	0.83±0.14	0.83±0.10		
1.0	0.85±0.17	0.91±0.10	0.91±0.08	0.95±0.05	0.97 ± 0.07	0.99 ± 0.03*
1.4	0.96±0.14*	0.98±0.07*	0.99±0.06*	1.00±0.03*		
2.0	0.98±0.07	0.99±0.03	0.99±0.04*	1.00±0.02*		
S.D. = 0.01						
0.5	0.05±0.24†	0.30±0.18	0.09±0.21†	0.31±0.15		
0.7	0.37±0.27	0.58±0.18	0.47±0.13	0.63±0.09		
1.0	0.54±0.27	0.74±0.16	0.69±0.12	0.82±0.07	0.96 ± 0.13*	0.99 ± 0.05*
1.4	0.84±0.15	0.92±0.08	0.87±0.10	0.93±0.05		
2.0	0.92±0.15	0.97±0.07	0.96±0.09*	0.98±0.04*		

* $P > 0.04$ that there is no difference from theoretical value of 1.00, calculated from interval estimate of median (Campbell, 1967).

† Many of the values were negative.

from its theoretical value than did the corresponding mean V, and also had the larger standard error. In general, the weighted regression gave the better answers.

Rate equation. Initial velocities were equated to the first-order term of a cubic fitted to the whole progress curve and constrained to pass through the origin. (The reason for choosing a cubic is explained in the next section.) The mean values of K_m and V, computed from initial velocities by the method of Wilkinson (1961), are in Table 1. They are about as reliable as the answers given by the weighted form of the integrated equation when $s_0 = 1.4$. But the latter method is to be preferred because it requires one-fifth the number of experiments to achieve a given reliability.

Progress curve of unknown origin

The origin of the progress curve has to be found when, for example, in the continuous titration of an esterase the pH chosen for the assay is slightly greater than that of the reagent added to start the reaction. There is a rapid addition of extra alkali, after which the total amount of alkali added is always greater than the equivalent amount of product formed (see Fig. 2). The progress curve can only be analysed by subtracting this extra alkali (p_0) from the total amount added (p).

We have assumed that the first part of the curve was undetermined, but that it had its true shape by the time 8% of the substrate had been consumed.

Integrated equation. Two methods for finding p_0 were tested. In the first method, a polynomial was fitted to the values of t and p, p_0 was equated to its zero-order term and K_m and V were estimated from the linear regression of $(p-p_0)/t$ on

$$\frac{1}{t}\cdot\ln(1-(p-p_0)/s_0).$$

The optimum order for the polynomial was established by fitting in turn a quadratic, a cubic and a quartic to the progress curves for $s_0 = 1.0$ and estimating K_m and V by using the unweighted form of the linear regression. Table 2 shows that the cubic gave the best answers, so only it was used at the other values of s_0, and for deriving initial velocities (see above). The mean values of K_m and V calculated from the low-error curves by using the cubic and the weighted form of the linear regression are in Table 3; those calculated from the high-error curves and those given by the unweighted form of the regression were less reliable and have not been included. As for the curves of known origin (Table 1), the means were significantly less than their true values when s_0 was under 1.4, and their standard errors decreased as s_0 increased. There was also little quantitative difference between the means for the curves of known and of unknown origin, which indicates that the cubic polynomial gave an unbiased estimate of the origin.

The second method for finding p_0 was a modified Fibonacci search (Atkins, 1973) used in conjunction with both the unweighted and the weighted forms of the linear regression. The mean estimates were slightly

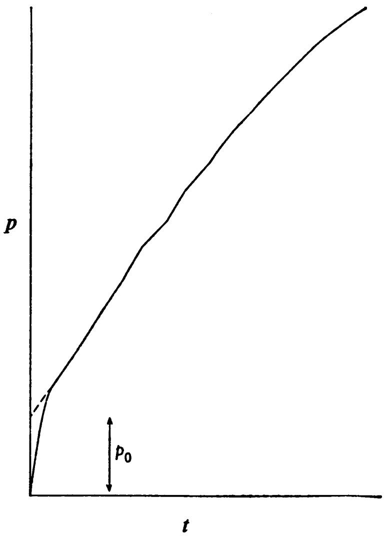

Fig. 2. *Progress curve of unknown origin*

The amount of extra alkali added is p_0, so the origin is really $(0, p_0)$. For this curve $K_m = V = 1$, $s_0 = 2.0$ and the S.D. of $p = 0.005$.

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lower than those in Table 3, and have not been tabulated.

Rate equation. Initial velocities were calculated from the first-order term of the cubic, and K_m and V were again computed from them by Wilkinson's (1961) method. The means are in Table 3; they are less precise than those given by the integrated equation when s_0 was 1.4 or 2.0.

Effect of data spacing

At the lower initial substrate concentrations all the integrated methods underestimated K_m and V. This is explained qualitatively by Fig. 3, which shows the effect of a fixed error in p on the plot of p/t against

$$-\frac{1}{t}\cdot\ln(1-p/s_0).$$

It can be seen that if p is underestimated a point which should have fallen on the line in fact lies below its true position and to the left of it, whereas if p is overestimated, the point lies above and to the right of its true position. As a result, low values of the abscissa are correlated with values of the ordinate that are erroneously low, and high values of the abscissa with erroneously high values of the ordinate. It follows that the least-squares regression through points containing error tends to be rotated counter-clockwise and therefore to have a decreased slope (i.e. K_m) and intercept (i.e. V). This tendency is more marked at the low substrate concentration than at the high one.

Table 2. *Effect of the order of the polynomial on the estimates of K_m and V*

Values are means±S.D. ($n = 40$), and are for $s_0 = 1.0$ and unweighted linear regression only.

S.D.	K_m	V
Quadratic		
0.005	0.93±0.09	0.96±0.06
0.01	0.61±0.19	0.77±0.12
Cubic		
0.005	0.96±0.22*	0.99±0.15*
0.01	0.71±0.31	0.83±0.21
Quartic		
0.005	0.94±0.38*	0.98±0.26*
0.01	0.57±0.45	0.76±0.30

* $P > 0.04$ that there is no difference from theoretical value of 1.00, calculated from interval estimate of median (Campbell, 1967).

An attempt was made to improve the estimates of K_m and V at $s_0 = 0.5$ by using all the data to find p_0 but only the larger values of p ($p \geqslant 0.157$) in the subsequent linear regression. However, the estimates were substantially the same. On the other hand, using only smaller values of p for the regression gave negative values of K_m.

Application to other types of reaction

Although these results are for the simplest type of reaction, they can be applied to more complex ones. For example, if the reaction product is a competitive inhibitor of constant K_i, eqn. (2) becomes:

$$\frac{p}{t}=\frac{1}{1-(K_m/K_i)}\cdot V+\frac{K_m+(K_m/K_i)\cdot s_0}{1-(K_m/K_i)}\cdot\frac{\ln(1-p/s_0)}{t} \qquad (3)$$

[Note that this equation is correct, whereas in Laidler (1958) eqn. (29) and Fig. 28 are wrong.]

The apparent value of K_m [K', the coefficient of $\ln(1-p/s_0)/t$] is linearly related to s_0, and both K_m and K_i can be calculated from the slope and intercept of the plot of K' against s_0. The variances of this slope and intercept can be found from the residual variance about the plot, that is, from the variance of K', which can in turn be found in, for example, Table 3. Thus the errors to be expected in K_m, K_i and V can all be estimated.

Discussion

The simplest form of the integrated Michaelis–Menten equation can be used only if its rate equation holds throughout the whole reaction. Among the

Table 3. *Mean estimates of K_m and V for progress curves of unknown origin*

Values are means ±S.D. (n as indicated).

	Integrated weighted ($n = 40$)		Initial velocities ($n = 39$)	
s_0	K_m	V	K_m	V
0.5	0.60±0.13	0.70±0.11		
0.7	0.84±0.17	0.89±0.13		
1.0	0.94±0.14	0.97±0.10*	0.99	1.01
1.4	1.02±0.09*	1.01±0.06*	±	±
2.0	1.00±0.06*	1.00±0.03*	0.18*	0.08*

* $P > 0.04$ that there is no difference from theoretical value of 1.00, calculated from interval estimate of median (Campbell, 1967).

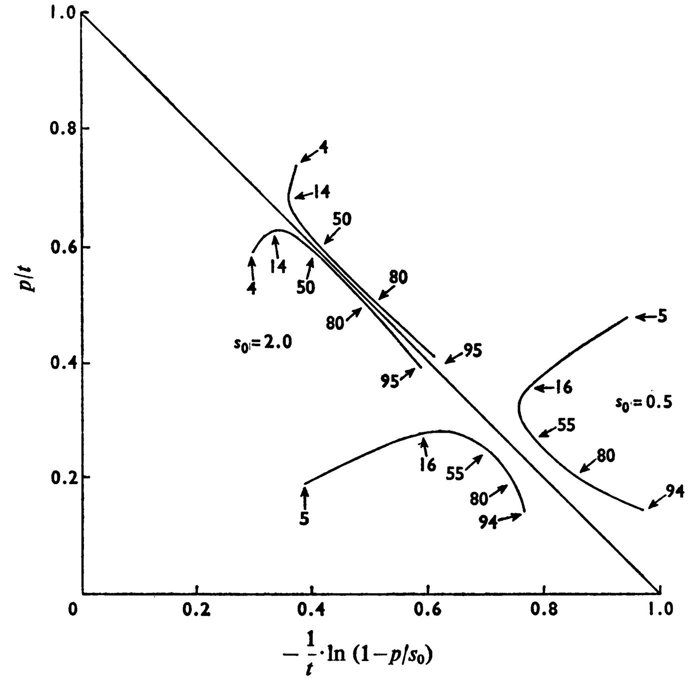

Fig. 3. *Effect of error on the plot of p/t against* $-\frac{1}{t}\cdot ln(1-p/s_0)$

$K_m = V = 1$, $s_0 = 0.5$ and 2.0 and the error in $p = \pm 0.01$. Points containing this error lie on the curves above (p overestimated) and below (p underestimated) the straight line and not on it. The percentage conversion of substrate into product is shown at five points on each curve.

factors preventing its use are the irreversible inactivation during the reaction of the enzyme [see Selwyn (1965) for a test of this], a departure from steady state in the concentration of enzyme–substrate complex, the reversibility of the reaction, or the presence or formation of an inhibitor (Laidler, 1958). However, the rate equation can be modified to take some of these factors into account: for instance, it is possible in practice to allow for the reversibility of the reaction (Alberty & Koerber, 1957), or for the presence of a non-competitive inhibitor which is a reaction product (Schwert, 1969). Progress curves can also be used to quantify irreversible two-substrate reactions (Schwert, 1969).

Thus some form of integrated equation is in principle applicable to several different types of reaction. Our results show that the simplest form of the equation had to be fitted to the data by a theoretically invalid method. However, this method gave unbiased estimates of the parameters, provided that the data were sufficiently precise and the initial concentration of substrate was greater than K_m. In these conditions, the estimates were as good as those derived from initial velocities, and were based on fewer experiments. On the other hand, when s_0 was not greater than K_m, the estimates were too low. These conclusions can be extended to more complex types of reaction. For example, when the reaction product is a competitive inhibitor, the proviso that the coefficient of the

$$\frac{1}{t}\cdot\ln(1-p/s_0)$$

term (that is, the apparent value of K_m) be roughly twice the initial concentration of substrate is met if the true value of K_m is also twice this concentration.

Our results also show that weighting the regression (albeit with factors which are approximate) improved the precision of the estimates of the parameters, and that the origin of the progress curve could be calculated with but little loss in precision. It is not possible by using the linear regression to estimate the standard deviations of the parameters derived in an individual experiment. However, in any regression which should be solved by non-linear methods (like the one considered here, or one fitting a rectangular hyperbola) the calculated standard deviations are only approximate (Bliss & James, 1966) and must be interpreted with caution. Nevertheless, our results do indicate what values might be expected for these standard deviations. Thus when $s_0 = 1.4$ and the standard deviation of $p = 0.005$, the standard deviation of the residuals about the curve relating p to t must average 0.005, or roughly 0.5% of the maximum observed value of p. Table 3 shows that in these conditions the coefficients of variation of K_m and V are respectively 9% and 6%.

These calculations only apply when the experimental error is random and not systematic. It is conceivable that factors like slow changes in pH or temperature, or instrumental drift, could introduce systematic errors. We have not attempted to assess quantitatively the effect on the parameter estimates of such errors. However, their presence would probably result in a curvilinear plot of p/t against

$$-\frac{1}{t}\cdot\ln(1-p/s_0),$$

and could thus be detected.

Therefore we have concluded that, in the absence of systematic error, there is no reason why K_m and V should not be determined from progress curves. Conformity of a reaction to Michaelis–Menten kinetics is better tested by using initial velocities, but when its parameters are being estimated as a routine, the progress curve could be the method of choice. Full details of the computer program, written in IMP, are available from the authors on request.

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