

Short Communications

Analysis of Progress Curves in Enzyme Kinetics

By A. J. CORNISH-BOWDEN

Department of Biochemistry, University of Birmingham, P.O. Box 363,
 Birmingham B15 2TT, U.K.

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Since Michaelis & Menten (1913) demonstrated that many of the problems encountered by earlier workers in enzyme kinetics could be avoided by measuring initial rates and using kinetic equations in their differential forms, most biochemists have been reluctant to use integrated rate equations. However, confining analysis to the initial linear part of a progress curve wastes much of the available information, and also wastes materials and time, because more experiments are needed to obtain estimates of the kinetic parameters. Balcom & Fitch (1970) have proposed a method of analysing progress curves in which the kinetic equations are used in their familiar differential forms, by estimating the velocities at different times in the experiment from the differences between adjacent points on the curve. For a set of observations of substrate concentrations, $s_0, s_1, s_2 \dots s_n$, at times $t_0, t_0 + \Delta t, t_0 + 2\Delta t \dots t_0 + n\Delta t$, they estimate velocities as:

$$v_i = \frac{s_{i-1} - s_{i+1}}{2\Delta t} \quad (1)$$

for values of i from 1 to $n-1$. From this set of velocities and substrate concentrations they obtain estimates of the kinetic parameters by means of the computer programs of Cleland (1963).

The method of Balcom & Fitch (1970) would, if valid, be an extremely valuable one, because it would permit the highly developed techniques for analysing rate equations to be applied to progress curves. This would permit much more information to be obtained from each experiment, and it would remove the subjective element inherent in most estimates of initial rates. Unfortunately the method is not valid, because most of the observations are given very little weight in the analysis. It has long been recognized (Roseveare, 1931) that methods similar to that of Balcom & Fitch (1970) are unsuitable for estimating the slopes of straight-line plots, because all points except the first two and last two cancel out in the calculations. The analysis is less simple for enzyme kinetic equations, but the conclusions to be drawn are similar. Intuitively, one would expect that an error in any point other than the first two or the last two would produce equal and opposite errors in two velocity estimates, which would approximately cancel

one another out in the estimates of the kinetic parameters. More detailed examination supports this expectation.

Balcom & Fitch (1970) discussed their method in connexion with a mechanism involving a significant back reaction and product inhibition, and proposed that it could be used generally for complex rate equations. However, any general method ought to be valid for the simplest cases, and the Michaelis-Menten equation is taken here as an example:

$$v_i = \frac{V \cdot s_i}{K_m + s_i} + \epsilon_i \quad (2)$$

For a set of velocities v_i at substrate concentrations s_i , the best-fit values of V and K_m are those that minimize the sum of squares of residuals ϵ_i , assuming that the velocities are normally and independently distributed with uniform variance. The requirements for normality and uniform variance are not important in the present context, because similar conclusions would result from any reasonable distribution that might be assumed. The requirement for independence is much more important: if the velocities are defined by eqn. (1), then, if the values of s_i are independently distributed with uniform variance, the values of v_i are not independent; for each i the correlation coefficient between v_i and v_{i+2} is -0.5 , a value quite sufficiently different from zero to invalidate the analysis. Now it might nonetheless be argued that minimization of the sum of squares would still lead to reasonable estimates of the kinetic parameters. It is therefore desirable to demonstrate that this is not the case.

For eqn. (2), the sum of squares SS is defined by:

$$SS = \sum \epsilon_i^2 = \sum_{i=1}^n \left(v_i - \frac{V \cdot s_i}{K_m + s_i} \right)^2 \quad (3)$$

Partial differentiation with respect to V and K_m gives:

$$\frac{\partial SS}{\partial V} = -2 \sum_{i=1}^n \frac{v_i \cdot s_i}{K_m + s_i} + 2V \sum_{i=1}^n \frac{s_i^2}{(K_m + s_i)^2} \quad (4)$$

$$\frac{\partial SS}{\partial K_m} = 2V \sum_{i=1}^n \frac{v_i \cdot s_i}{(K_m + s_i)^2} - 2V^2 \sum_{i=1}^n \frac{s_i^2}{(K_m + s_i)^3} \quad (5)$$

\hat{V} and \hat{K}_m may be defined as the values of V and K_m such that SS is a minimum. Then, after setting both derivatives to zero, \hat{V} may be eliminated to give the following expression for \hat{K}_m :

$$\sum_{i=1}^n \frac{v_i \cdot s_i}{\hat{K}_m + s_i} \cdot \sum_{i=1}^n \frac{s_i^2}{(\hat{K}_m + s_i)^3} = \sum_{i=1}^n \frac{v_i \cdot s_i}{(\hat{K}_m + s_i)^2} \cdot \sum_{i=1}^n \frac{s_i^2}{(\hat{K}_m + s_i)^2} \quad (6)$$

This equation cannot be solved analytically for \hat{K}_m , whence the need for iterative procedures, such as that of Wilkinson (1961), but it can be used to examine the influence of the various observations on \hat{K}_m . If the velocities are calculated by the method of Balcom & Fitch (1970), substitution of eqn. (1) into eqn. (6) gives:

$$\sum_{i=1}^{n-1} \frac{s_i (s_{i-1} - s_{i+1})}{\hat{K}_m + s_i} \cdot \sum_{i=1}^{n-1} \frac{s_i^2}{(\hat{K}_m + s_i)^3} = \sum_{i=1}^{n-1} \frac{s_i (s_{i-1} - s_{i+1})}{(\hat{K}_m + s_i)^2} \cdot \sum_{i=1}^{n-1} \frac{s_i^2}{(\hat{K}_m + s_i)^2} \quad (7)$$

The first summation on the left-hand side of eqn. (7) can be expanded as:

$$\sum_{i=1}^{n-1} \frac{s_i (s_{i-1} - s_{i+1})}{\hat{K}_m + s_i} = \frac{s_1 (s_0 - s_2)}{\hat{K}_m + s_1} + \frac{s_2 (s_1 - s_3)}{\hat{K}_m + s_2} + \frac{s_3 (s_2 - s_4)}{\hat{K}_m + s_3} + \dots \quad (8)$$

It is convenient to consider the odd and the even terms of the expansion separately; the sum of odd terms, \sum_{odd} , is given by:

$$\sum_{odd} = \frac{s_1 (s_0 - s_2)}{\hat{K}_m + s_1} + \frac{s_3 (s_2 - s_4)}{\hat{K}_m + s_3} + \dots + \frac{s_{n-1} (s_{n-2} - s_n)}{\hat{K}_m + s_{n-1}} \quad (9)$$

$$= \frac{s_0 \cdot s_1}{\hat{K}_m + s_1} + s_2 \left[\frac{s_3}{\hat{K}_m + s_3} - \frac{s_1}{\hat{K}_m + s_1} \right] + \dots \\ \dots + s_{n-2} \left[\frac{s_{n-1}}{\hat{K}_m + s_{n-1}} - \frac{s_{n-3}}{\hat{K}_m + s_{n-3}} \right] - \frac{s_{n-1} \cdot s_n}{\hat{K}_m + s_{n-1}} \quad (10)$$

The ratio of the first to the second terms in eqn. (10) is:

$$-s_0 \cdot s_1 (\hat{K}_m + s_3) / [\hat{K}_m \cdot s_2 (s_1 - s_3)]$$

Now, for closely spaced observations, the difference between successive observations must be small, so that this ratio will normally be numerically very large. The actual value will, of course, be different in different experiments, but a typical run might consist of about forty s_i values from $2\hat{K}_m$ to $0.5\hat{K}_m$, which would give a value of about -60 for this ratio. By the same argument, all of the intermediate terms in eqn. (10) are very small compared with the first and the

last. The even terms in eqn. (8) may be analysed similarly, so that the total value of the expression is determined very largely by s_0, s_1, s_{n-1} and s_n , with very little weight being given to the other observations.

By similar arguments it may be shown that the first summation on the right-hand side of eqn. (7) is also determined largely by the same four observations. It is not necessary to consider the other two summations in eqn. (7), since they do not involve the 'velocities'. Although in this method the 'velocities' and concentrations both derive from the same set of

observations, the errors in the 'velocities' are relatively far greater than the errors in the concentrations,

because the difference between two approximately equal values is much less well-defined than the values themselves.

It follows, therefore, that the expression that defines \hat{K}_m , namely eqn. (7), weights the first two and the last two observations very much more heavily than any of the others. The more observations there are, the closer they will be, and so the less will be the weight attached to the intermediate values. This result would be serious enough in any case, but it is made more so by the fact that it is common for the first observations in an enzyme kinetic run to be the least accurate, because of the time taken for instruments to settle down etc.

There is no reason to suppose that the method discussed in this communication would be any more

reliable when applied to more complex examples than the Michaelis–Menten equation. Indeed, the correlation between the velocity estimates arises from eqn. (1), which is independent of the kinetic model used. Fortunately there are alternatives to this method that do not suffer from the same objections: Elmore *et al.* (1963) have described a method in which progress curves are fitted by the method of orthogonal polynomials. The resulting polynomials may then be differentiated to obtain velocities at any substrate concentrations. This method is more laborious than that of Balcom & Fitch (1970), but not excessively so. It provides an excellent and objective method of estimating the initial slope of a progress curve; however, it is probably unwise to take more than one velocity from each curve for further analysis, because, although all of the information is used in calculating the polynomial, the resulting velocities are not statistically independent. In order to use the whole

curve it is better to use an equation that represents the whole curve, namely the appropriate integrated rate equation. Schwert (1969) has given the integrated forms of several of the important enzyme kinetic equations, and has shown how they may be applied. It is likely that his approach will prove to be more reliable than the one that has been discussed in the present communication.

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