68. *Computation of Enzyme Kinetic Parameters.*

By J. E. BANFIELD.

A new method involving a doubly integrated Michaelis–Menten equation is developed for either hand or computer operation to extract the kinetic parameters V and K_M.

FOR some time we have been concerned with the determination of precise values of the Michaelis parameters K_M and V for enzyme reactions, in particular for the hydrolysis of penicillin with penicillinase. Determination of the velocities at a number of substrate concentrations requires either the results of numerous different experiments or the abstraction of instantaneous velocities from a number of points, *e.g.*, on a curve. Both these methods can lead to considerable error. Elaborate statistical methods for treating such results have recently been described [1] and prompt us to record a complementary approach along different lines.

Whereas Elmore, Kingston, and Shields [1] use a more general procedure involving curve fitting to a set of orthogonal polynominals and calculate the velocities at particular points from the coefficients, we prefer a method of numerical integration which is particularly useful where large numbers of experimental points of reasonable accuracy are available, as from the use of an autotitrator. For a curve-fitting method it seems preferable to use the theoretical equation to the curve, *if known*, rather than an arbitrary form.

The dangers attendant on the application of our method, described below, to cases where strict Michaelis–Menten kinetics (or the form where K_M' and V', the apparent parameters, replace K_M and V in the equation, for compeditive and anti-compeditive inhibition, etc.) are not followed will be obvious.* Should product inhibition, or consecutive reactions of comparable rate, affect the kinetic form then the use of our method is inappropriate and should be replaced by, *e.g.*, methods involving determination of initial rates. To ensure that the rate equation of Michaelis–Menten type is applicable over the full course (excluding the pre-steady state) of the reaction our programme incorporates a built-in check in which each point calculated from the integrated rate equation is compared with each experimental value; systematic errors greater than experimental errors should signal rejection of the calculation. This provides a very sensitive test for applicability of the rate equation.

The integrated Michaelis–Menten equation (where $[S]$ and t refer, respectively, to the substrate concentration and time)

$$[S] + K_M \ln [S] = -Vt + \text{Constant}$$

or

$$[S] - [S_0] + K_M \ln ([S]/[S_0]) = -V(t - t_0)$$

can in principle be fitted to sets of three points to yield both parameters; in practice, sensitivity to experimental error makes use of all suitable combinations of points imperative, statistical weighting difficult, and computation impractically long. We early abandoned such an approach.

The minimum input of data to a computor must comprise two experimental quantities for each point. Whereas the aforementioned curve-fitting procedure might involve handling each such point as many as in $!n/[6!(n - 3)]$ times, numerical integration can involve handling each such point only twice and is a simple and rapid task. Thus was indicated the use of a doubly integrated form of the Michaelis–Menten equation the usual form of which is transcendental:

$$\int Vtds = -K_M[S] \ln [S] + K_M[S] + [S]K_M \ln [S_0] + [S]Vt_0 + [S][S_0] - [S^2]/2 + \text{Constant.}$$

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* This warning is added at the suggestion of a Referee, who quotes Booman and Niemann (*J. Amer. Chem. Soc.*, 1956, **78**, 3642) and Dixon and Webb (" The Enzymes," Longmans, London, 1958, p. 119).

[1] Elmore, Kingston, and Shields, *J.*, 1963, 2070.

In this form, $[S_0]$ and $[t_0]$ refer to a particular concentration and time. This equation can be simplified to a useable form giving the area (A) under the concentration–time curve from a given experimental point to infinite time for the particular case where $[S] = [S_0]$ as:

$$\int_0^{S_0} Vt\mathrm{d}s = K_\mathrm{M}[S_0] + [S_0]Vt_0 + 0{\cdot}5[S_0]^2;$$

i.e.,

$$A = \int_0^{S_0} (t - t_0)\mathrm{d}s = K_\mathrm{M}[S_0]/V + [S_0]^2/2V$$

or

$$\frac{2\int_0^{S_0} t\mathrm{d}s}{[S_0]} - 2t_0 = \frac{2K_\mathrm{M}}{V} + \frac{[S_0]}{V}$$

The left-hand part of the last equation is referred to below as the " area function; " it is computed for various values of $[S_0]$ and plotted against $[S_0]$, the subscript then being dropped; the slope (obtained by a least-squares calculation) is the reciprocal velocity and the negative intercept (on the $[S]$ axis) is twice K_M. A more precise value for K_M is obtainable when the accurate V value so obtained is used to construct a " time difference plot " [2] * of the once integrated rate equation or its slightly modified form:

$$x_1 - x + (t - t_1)V = -K_\mathrm{M} \ln [S] + \text{Constant},$$

where the left-hand side of the equation may be referred to by analogy [2] as the " volume difference function " and is plotted against ln $[S]$, giving K_M as the negative slope (x is a reading at time t; $[S] = x_\infty - x$; the subscript 1 refers to the first reading).

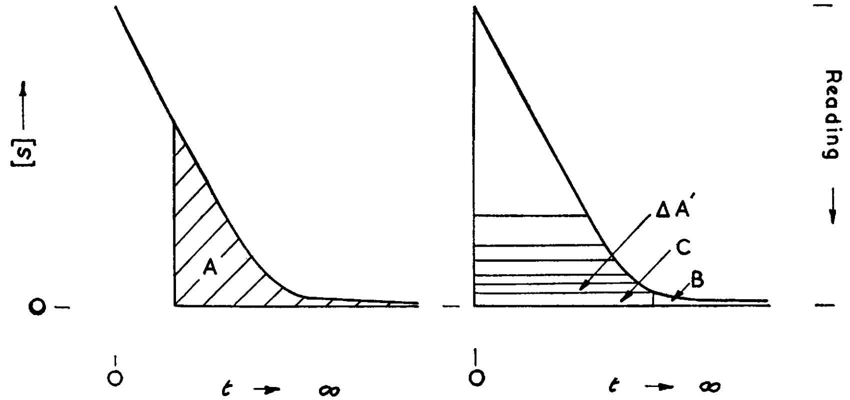

FIG. 1. A typical Michaelis–Menten plot showing areas referred to in the text.

The above procedure is applicable to calculation with a hand-machine. From approximate estimates of V and K_M we calculate the area (B) under the curve from the last experimental point to $t = \infty$ and add this to the area of (C). Graphical integration by the trapezoid rule can then continue from this point giving (B) + (C) + $\Sigma\Delta A'$ (see Fig. 1), and at intervals (equal for the hand-calculation to simplify least-squares calculations; at each experimental point not necessarily at equal intervals for the computer programme) the " area function " is calculated. Points at low substrate concentration are unduly

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* All rates and Michaelis constants of ref. 2 were recalculated by this procedure, giving more accurate values. Qualitative conclusions of ref. 2 were not affected with the exception that the Arrhenius plot for V now shows a slight smooth downward curvature, and thus the explanation for the deviation of the point at 47° is inappropriate. Unfortunately all results and calculations were lost in a fire which destroyed this Department in 1958; results of only one other run survived the fire and are given below.

[2] Banfield, *Experientia*, 1957, **13**, 403.

affected by inaccuracies in the infinity reading ([*S*] being in the denominator), causing the curves to bend characteristically as shown in Fig. 2; no difficulty is experienced in obtaining a good value of *V* from the linear part of such curves although the intercepts are rather more affected. From deviant points at low [*S*] a corrected value of the infinity reading can readily be obtained and the calculation repeated as necessary.

A more elaborate procedure is made feasible by a programme * recently written in " autocode " for the Ferranti " Sirius " computor.† The $\Delta A'$ areas are first calculated and stored for use in all later cycles. For estimated values of K_M and *V* the end correction ($B + C$) is calculated, the " area function " is calculated for each point, and its regression line against [*S*] is determined. The revised values for K_M and *V* are then used to redetermine ($B + C$), etc., and these are sensibly constant after the three cycles. In these operations the least-squares calculation ignores the five points at lowest [*S*] although the corresponding experimental readings are incorporated into the area function through the integration. Deviations from the line for the last five points are then used to calculate five corrections to the infinity point which is then adjusted according to their average. If this adjustment is trivial the calculation jumps one or more of the refinement stages in which the whole calculation to this point is repeated. (For runs at initial substrate concentrations not much greater than K_M, this adjustment is unnecessary and undesirable and a shorter modification of the programme or other methods should be used.) Printed-out values of the infinity value, *V*, and $-2K_M$ are given at each refinement stage. Provision is also made for the use of the accurate *V* value to determine K_M from the above equation for the " concentration difference function " and a least-squares procedure. Finally the " difference function " is compared with the least-square line and the difference between calculated and observed readings for each point is printed out.

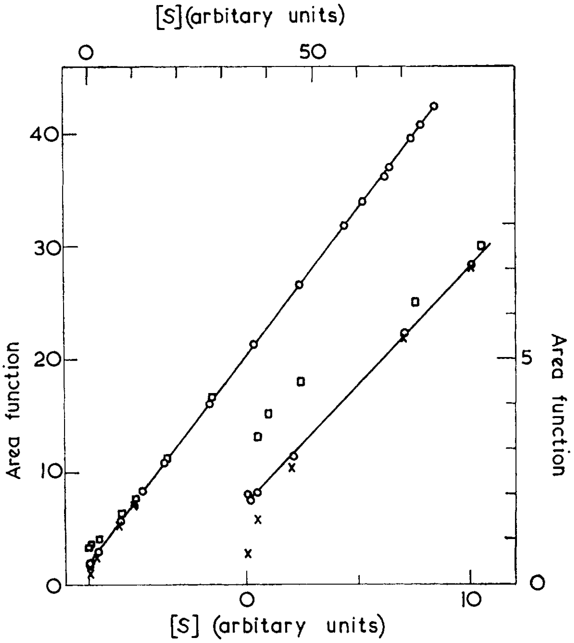

FIG. 2. Plot of " area function " against substrate concentration [S] for hydrolysis of penicillin with penicillinase at constant pH. Points ○ refer to a calculation made with a desk machine for a measured infinity reading of 77·08; points × and □ illustrate the errors introduced by taking wrong infinity readings of 76·90 and 77·50, respectively, for the calculation. Points at low [*S*] values are shown on expanded scales in the lower right inset.

An advantage of both hand and computor calculations is that a single deviant experimental point, being incorporated into the area function through an integration, has very little effect on the final result. It is, therefore, unnecessary to consider statistical criteria

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* It is hoped that copies may be available on application to the author.

† I am informed that " Atlas " will later accept " Sirius " autocode.

for rejection of such points (which in the author's opinion is a highly dubious procedure). On the other hand a trend, caused, *e.g.*, by a systematic experimental error for part of a run, is made very evident both in the area function plot and in the calculated differences.

RESULTS

Points calculated from the integrated Michaelis–Menten equation with $V = 1{\cdot}000$, $K_M = 1/2{\cdot}303$, and an infinity reading of 33·00 were used to test the programme after introduction of arbitrary errors into both " experimental " points and estimates * of V, K_M, and the infinity reading. Rapid convergence (1·003, 1·003, 1·003, 1·001, 1·001, 1·001, 1·001, 1·001, 1·001, 1·000, 1·000, 1·000) to the true V was obtained with a slightly slower convergence (−0·790, −0·779, −0·779, −0·831, −0·834, −0·834, −0·852, −0·854, −0·854, −0·860, −0·860, −0·860) to within 0·004 of the true K_M and to within 0·018 of the correct value of the infinity reading (32·940, 32·971, 32·982).

The experimental run comprised 70 points and it is impractical to give full details. The slope was invariant at 0·524 throughout the calculation, and negative intercepts had successive values of −3·598, −3·588, −3·588, −3·636, −3·637, −3·637 −3·648, −3·649, −3·649 with infinity readings 77·10 (input) 77·132, 77·139, 77·141. The calculated K_M of 1·825 from the negative intercept is to be compared with the value of 1·813 obtained from the " volume difference function "–log [S] plot; the difference is not significantly greater than the error in measurement of [S] (±0·01).

Differences, $[S]_{\text{calc.}} - [S]$, for all points of the run are rounded to the nearest 1/100 unit are as follows: 0·07, 0·13, 0·13, 0·07, 0·03, 0·01, −0·03, −0·05, −0·06, −0·03, −0·08, −0·08, −0·08, −0·07, −0·05, −0·07, 0·07, −0·06, −0·05, −0·07, −0·03, 0·08, 0·12, 0·12, 0·09, 0·04, 0·01, 0·00, −0·01, −0·02, −0·02, 0·03, −0·01, 0·01, 0·02, 0·03, 0·00, 0·02, 0·03, 0·02, 0·01, 0·02, −0·01, 0·01, 0·02, 0·01, 0·01, 0·02, 0·02, 0·03, 0·04, 0·05, 0·04, 0·04, 0·06, 0·06, 0·05, 0·12, 0·07, 0·05, −0·29, −0·18, −0·19, −0·12, −0·07, −0·06, −0·01, 0·02, 0·11, 0·11.

The largest individual error (0·3) represents an error of 11 sec. in a run of 40 min.

EXPERIMENTAL

General methods are described in ref. 2. Times were determined for null readings with a double-handed stopwatch (Camier and Cuss). The pH-stat system included a " Cambridge " bench-pattern pH meter used on the millivolt scale and modified to increase the sensitivity with a damping circuit and an external galvanometer (future work is planned with a specially built vibrating capacitor electrometer). The glass electrode used was kept in solutions of ionic strength of 0·02 when not in use, a second electrode being used for determination of pH so that the first electrode was not subjected to reference solutions. As this run is used solely to illustrate the computation procedure, absolute values are not given.

DISCUSSION

The above computational methods (hand or programme) have proved most useful in our work where reasonably precise data at many points of a kinetic run are available and the products of reaction are demonstrably without effect. It is hoped that results of this type, by enabling fine details of enzyme kinetics to be unravelled for suitable experimental cases, will lead to a deeper understanding of enzymic action.

However, it is emphasised that the method is inappropriate for use with data of low precision or where only a few experimental points are available. In some cases a more refined numerical integration (such as Simpson's rule [3]) might extend the scope of the method for runs where fewer points of high accuracy are available.

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* Lineweaver–Burk plots (Lineweaver and Burk, *J. Amer. Chem. Soc.*, 1934, **56**, 658) are satisfactory for obtaining these estimates for experimental runs.

[3] Courant, " Differential and Integral Calculus," Blackie, London, Vol. I, 2nd ed., 1954, p. 344.