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Half-time analysis of the integrated Michaelis equation

Simulation and use of the half-time plot and its direct linear variant in the analysis of some α-chymotrypsin-, papain- and fumarase-catalysed reactions

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Substitution of half-time parameters in the integrated form of the Michaelis–Menten equation for any enzyme-catalysed reaction yields an equation that gives a linear relationship between the half-time of the reaction and the substrate concentration at that point of the reaction. The logarithmic term of the integrated equation becomes a constant as a result of the substitution, which means that the use of the half-time plot of the equation requires calculation only of half-time and substrate-concentration values at various stages of the reaction. The half-time method is both simple and exact, being analogous to an $[S_0]/v_i$ against $[S_0]$ plot. A direct linear form of the half-time plot has been devised that allows very simple estimation of Michaelis parameters and/or initial velocities from progress-curve data. This method involves no approximation and is statistically valid. Simulation studies have shown that linear-regression analysis of half-time plots provides unbiased estimates of the Michaelis parameters. Simulation of the effect of error in estimation of the product concentration at infinite time $[P_\infty]$ reveals that this is always a cause for concern, such errors being magnified approximately an order of magnitude in the estimate of the Michaelis constant. Both the half-time plot and the direct linear form have been applied to the analysis of a variety of experimental data. The method has been shown to produce excellent results provided certain simple rules are followed regarding criteria of experimental design. A set of rules has been formulated that, if followed, allows progress-curve data to be acquired and analysed in a reliable fashion. It is apparent that the use of modern spectrophotometers in carefully designed experiments allows the collection of data characterized by low noise and accurate $[P_\infty]$ estimates. $[P_\infty]$ values have been found, in the present work, to be precise to within $\pm 0.2\%$ and noise levels have always been below 0.1% (signal-to-noise ratio $\simeq 1000$). As a result of the considerations above, it is concluded that there is little to be feared with regard to the analysis of enzyme kinetics using complete progress curves, despite the generally lukewarm recommendations to be found in the literature. The saving in time, materials and experimental effort amply justify analysis of enzyme kinetics by progress-curve methods. Half-time plots linear to $\geqslant 90\%$ of reaction have been obtained for some α-chymotrypsin-, papain- and fumarase-catalysed reactions.

Analysis of enzyme-kinetic mechanisms has, up to the present time, relied almost entirely upon estimation of initial-velocity values, followed by appropriate graphical and numerical manipulation (see, e.g., Laidler & Bunting, 1973; Cornish-Bowden, 1979; Wharton & Eisenthal, 1981). The measurement of initial velocities is time-consuming, tedious, sometimes expensive and sometimes difficult, owing to premature curvature. The great advantage of this approach is, however, the relatively unambiguous analysis and interpretation of the data that is possible. Thus the inherent reliability of the method strongly compensates for the generally unsophisticated nature of the experimental approach.

Empirical equations having the same parametric form as the integrated form of the Michaelis–Menten equation (see eqn. 1 below) have been available for

Abbreviation used: Moc-Gly-ONp, methoxycarbonylglycine *p*-nitrophenyl ester.

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the analysis of progress curves since times which predate the discrete formulation of enzyme-kinetic mechanisms (Henri, 1903; Abderhalden & Michaelis, 1907; Philoche, 1908).

The integrated Michaelis–Menten equation has the general form:

$$V_{app.} \cdot t = [P] + K_m(app.) \cdot \ln\left(\frac{[P_\infty]}{[P_\infty]-[P]}\right) \quad (1)$$

in which [P] is the product concentration at time t, $[P_\infty]$ is the value of [P] at equilibrium (infinite time) and $V_{app.}$ and K_m(app.) have meanings which depend upon the kinetic mechanism of the reaction but have their usual simple meanings for a Michaelian system. Eqn. (1) may be expressed in terms of substrate concentrations as in eqn. (1a):

$$V_{app.} \cdot t = ([S_0]-[S]) + K_m(app.) \cdot \ln\left(\frac{[S_0]}{[S]}\right) \quad (1a)$$

where [S] is the substrate concentration at time t and $[S_0]$ the initial substrate concentration.

A number of papers have appeared in the literature since the early days of the empiricist approach which describe methods based upon the use of eqn. (1) for the analysis of progress-curve data. Perhaps the best known is that of Walker & Schmidt (1944), which makes use of a plot of $[P]/t$ against

$$\ln\left(\frac{[P_\infty]}{[P_\infty]-[P]}\right)/t.$$

This and similar methods based upon linear transformations of eqn. (1) require a considerable amount of computation, which detracts from the intrinsic appeal of the analysis of data-rich progress curves.

Methods have more recently been proposed (Lee & Wilson, 1971; Yun & Suelter, 1977) which involve relatively simple arithmetical procedures, but these are approximate unless correction factors are applied and rely upon the double-reciprocal plot, which as is well-known, introduces statistical bias unless subject to weighted regression analysis (Dowd & Riggs, 1965; Wharton & Eisenthal, 1981).

The effect of errors in $[P_\infty]$ has led to comment on several occasions (e.g. Fleischer, 1953; Klyosov & Berezin, 1972; Newman *et al.*, 1974; Cornish-Bowden, 1975), since such errors can have a seriously deleterious effect upon the parameter estimates. Although this represents a serious theoretical problem for all methods, with the partial exception of that of Klyosov & Berezin (1972), it should be possible with modern instrumentation to circumvent this problem in practice.

Many of the papers that have appeared in the literature whose subject has been the analysis of progress-curve data have relied either upon computer simulation of data or on isolated experimental examples chosen (presumably) to enhance the reputation of the method in question. Very rarely have the previously published methods been applied in the course of systematic investigations of enzyme-kinetic mechanisms by the original authors or by others. This suggests that there is some fundamental dislike or mistrust of progress-curve-analysis techniques among enzymologists. One of the purposes of the present paper is to attempt to dispel such disquiet by the presentation of a set of rules which have allowed the reliable application of progress-curve analysis by means of the relatively simple half-time method.

Recent theoretical studies of the validity of the integrated rate equation have shown that it applies in a wide range of circumstances, including some relaxation of the steady-state assumption, provided $[S_0] > 100[E_0]$ (Stayton & Fromm, 1979; Bartha, 1980).

Cornish-Bowden (1975) has presented an ingenious variant of the direct linear plot for the analysis of progress curves. He has made the point that this method, which makes use of an approximation of the logarithmic term of eqn. (1) (see also Yun & Suelter, 1977), supplies excellent estimates of initial-velocity values in circumstances where premature curvature in assay traces makes initial-velocity estimation very difficult. Although excellent for initial-velocity estimation, this method yields Michaelis parameters that may be subject to large errors depending upon the validity of the approximation to the logarithmic term (which is most accurate at low [P]). Thus the method cannot be recommended, at least in its approximate form, for the determination of Michaelis parameters from whole progress curves. Application of the half-time method to this type of plot has allowed the formulation of a simple but exact form suitable both for the measurement of initial velocities and Michaelis parameters.

It is well known that equations having the parametric form of eqn. (1) apply to a wide range of kinetic mechanisms (Schwert, 1969; Laidler & Bunting, 1973; Bates & Frieden, 1973*a*,*b*; Duggelby & Morrison, 1977, 1978); some of these cases and some deviations from the form of eqn. (1) are considered in terms of the half-time method in the present paper.

The widespread availability of relatively inexpensive microprocessor systems readily amenable to interfacing with spectrophotometers greatly enhances the desirability of analysing enzyme-kinetic data in terms of complete progress curves.

Theory

Derivation of the half-time equation

The time taken for an enzyme-catalysed reaction to proceed to half-completion is obtained by sub-

stitution of the relation $[S] = [S_0]/2$ when $t = t_{\frac{1}{2}}$ into eqn. (1a), which gives eqn. (2), the half-time equation.

$$V_{app.} \cdot t_{\frac{1}{2}} = K_m(app.)\ln 2 + [S_0]/2 \quad (2)$$

The substrate concentration at which the reaction is initiated is known as the *initial* substrate concentration, in contrast with the local substrate concentration at any time t. Thus a plot of $t_{\frac{1}{2}}$ against local $[S_0]$ yields a straight line of slope $\frac{1}{2}V_{app.}$ and intercept $\ln 2K_m(app.)/V_{app.}$. The half-time plot is constructed by taking local $[S_0]$ values at various stages of the reaction and calculating $t_{\frac{1}{2}}$ values at each of these points (see the Materials and methods section).

The substitution procedure shown above is a specific case of a more general procedure in which any fraction of reaction may be substituted as in eqn. 3.

$$V_{app.} \cdot t_n = K_m(app.)\ln n + [S_0]/n \quad (3)$$

In the present work we use the half-time form, since this method is widely used in chemical kinetics (see, e.g., Pilling, 1975; Hammes, 1978) and it is conceptually the simplest form. We have, however, no reason to suppose that the choice of half-reaction has any intrinsic merit other than ease of calculation as compared with any other fraction (up to perhaps $\frac{1}{3}$, beyond which the fraction of reaction would become rather small to measure accurately).

At low substrate concentration, when *local* $[S_0] \ll K_m(app.)$, the reaction is first-order and the half-time is constant, whereas at high substrate concentration, where local $[S_0] \gg K_m(app.)$, the half-time is directly proportional to the substrate concentration.

The procedure outlined above applies to all enzyme-catalysed reactions that are characterized by equations having the same parametric form as eqn. (1), i.e. product inhibition, reversibility etc. (Schwert, 1969; Laidler & Bunting, 1973; Bates & Frieden, 1973*a*,*b*).

Half-time plots of reversible reactions in which a significant concentration of substrate remains at equilibrium are constructed by using as the abscissa the apparent substrate concentration, which is defined as $[S] - [S_{eq.}]$, where $[S]$ is the *local* substrate concentration at time t and $[S_{eq.}]$ is the substrate concentration at equilibrium.

The kinetic parameters of reversible enzyme-catalysed reactions are obtained from the slopes and intercepts of secondary plots of the intercept values obtained from half-time plots of the primary data, the slopes of the primary plots and the Haldane relationship. The secondary plotting procedure is performed essentially as described by Yun & Suelter (1977).

The half-time plot bears considerable analogy to the half-reciprocal plot of initial-velocity data in the form $[S_0]/v_i$ against $[S_0]$. This is easily seen, since the slope and intercept parameters for both of these plots have the same form.

The direct linear form of the half-time plot

Following Cornish-Bowden (1975), eqn. (1) may be recast in parameter space as eqn. (4):

$$V_{app.} = ([S_0] - [S])/t + K_m(app.)\ln([S_0]/[S])/t \quad (4)$$

Substitution of the half-time relations into eqn. (4) gives the half-time form eqn. (5):

$$V_{app.} = \frac{[S_0]}{2t_{\frac{1}{2}}} + \frac{\ln 2K_m(app.)}{t_{\frac{1}{2}}} \quad (5)$$

Thus if a direct linear plot (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden, 1975) is constructed with values of $-[S_0]/2$ on the abscissa and $[S_0]/2t_{\frac{1}{2}}$ (which represents the slope of the chord joining a point at $[S_0]$ to a point at $[S_0]/2$ on the progress curve) on the ordinate, the lines joining these points should (for perfect data) intersect at a common point, namely $V_{app.}, \ln 2K_m(app.)$ in the first quadrant of the plot. This form of plot may also be used to advantage for obtaining initial-velocity estimates from data characterized by short or non-existent linear regions as described by Cornish-Bowden (1975).

Materials and methods

Moc-Gly-ONp was synthesized as described by Werber & Shalitin (1973) and had m.p. 81°C (literature value 80–83°C). Dithiothreitol, *N*-benzoyl-L-tyrosine *p*-nitroanilide, *N*-transcinnamoylimidazole, L-malic acid and fumaric acid were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Indole, *p*-cresol, acetonitrile and dimethyl sulphoxide were obtained from BDH, Poole, Dorset, U.K. All of these materials were used as received. Buffer salts were from the usual commercial sources.

α-Chymotrypsin was obtained as the thrice-crystallized product of Sigma. Titration of the enzyme with *N*-transcinnamoylimidazole by the method of Schonbaum *et al.* (1961) revealed that the enzyme was 98.6% active. Papain was prepared by the method of Baines & Brocklehurst (1979) from completely soluble latex supplied by Powell and Schofield, Liverpool, U.K. The purified enzyme after activation at pH 6.5 for 5 min with 5 mM-dithiothreitol followed by passage through a column of Sephadex G-25 was found by titration with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) to have 0.3–0.45 reactive thiol groups per mol of enzyme protein.

Fumarase was partially purified from ox heart by

homogenization, adjustment to pH 5.2 with 1 M-acetate buffer, pH 4.0, and absorption on to calcium phosphate gel in a centrifuge tube at 4°C. The enzyme was removed at 4°C from the gel with 5% $(NH_4)_2SO_4$ in 0.1 M-phosphate buffer, pH 7.3, and separated from the gel by centrifugation. The supernatant was precipitated with $(NH_4)_2SO_4$ and redissolved after centrifugation in a minimum quantity of 0.1 M-phosphate buffer, pH 7.0. The enzyme was prepared by second-year undergraduate students during a practical class in this Department.

Stock solutions of Moc-Gly-ONp were prepared in acetonitrile which had previously been dried over a molecular sieve. L-Malic acid and fumaric acid were dissolved in water and adjusted to pH 8.0 with 2 M-NaOH before making up to volume with a small quantity of water.

Spectrophotometric assays

The criteria of rules 4 and 5 (see the Results and discussion section) were applied to the design of all enzyme assays; reaction progress was measured with a Pye–Unicam SP.1800 spectrophotometer. Under these conditions $[P_\infty]$ could be reliably determined to 0.1–0.2% of $[S_0]$, the 0–1.0 absorbance scale of the spectrophotometer being essentially drift-free over 30 min.

Assays were performed as follows: the appropriate quantity of buffer (0.1 M- or 0.3 M-phosphate, pH 7.0, for α-chymotrypsin and papain experiments, and 15 mM-phosphate, pH 8.0, for the fumarase experiments) was preincubated at 25°C and transferred to a cuvette thermostatically controlled at 25°C. Organic solvent, where required, was added, followed by enzyme (10–100 μl) and the mixture was allowed to equilibrate to 25°C (detected by a thermocouple probe inserted into the cell contents). The reactions were started by addition of substrate and monitored as described above.

Moc-Gly-ONp was shown to release 100 ± 1% of the expected quantity of *p*-nitrophenolate anion when hydrolysed in 100 mM-NaOH, an ε_{400} value of 18 000 litre·mol^{-1}·cm^{-1} for *p*-nitrophenolate being assumed. The aqueous rate (i.e. the spontaneous rate in the absence of enzyme) of hydrolysis of Moc-Gly-ONp in 0.1 M-phosphate buffer at pH 7.0 is significant, being characterized by a rate constant ($k_{obs.}$) of 1.68×10^{-4} s^{-1}.

Method for the construction of half-time plots

A four column table is made up from the progress curve comprising:

Time Absorbance $[S_0]$ local value $t_{\frac{1}{2}}$

The absorbance at time t is subtracted from that at infinite time $[A_\infty]$ and divided by the absorption coefficient, which is determined either from the A_∞ of the particular experiment or from independent measurements, to give the local $[S_0]$ value. To obtain the half-time, the absorbance value at the local $[S_0]$ is added to that for $[P_\infty]$ (i.e. A_∞) and divided by 2, followed by measurement of the half-time directly from the chart. The half-time is plotted on the ordinate and local $[S_0]$ on the abscissa; $V_{app.}$ and K_m(app.) being determined as outlined above. This procedure has been found in most cases to give good straight-line plots using 12–15 points covering 10–90% of the reaction.

The *direct linear* form of the half-time plot is constructed in a similar fashion, except that the local $[S_0]/2t_{\frac{1}{2}}$ values are determined from the slopes of the chords joining the local $[S_0]$ values to the appropriate $[S_0]/2$ values. Thus neither plot form requires more than the most rudimentary calculation.

Results and discussion

Simulation studies

The results of simulation studies of the half-time method are given in Tables 1 and 2. The methods used to generate and analyse the data are given in the legends to these Tables. It is clear from Table 1 that excellent estimates of $V_{app.}$ and K_m(app.) are

Table 1. *Unweighted linear-regression analysis of the half-time method using simulated progress-curve data*
Progress curves were simulated by calculation of 2000 points using eqn. (1) with $V_{app.} = 1.00$, K_m(app.) = 1.00 and the Newton–Raphson method at equal intervals of t from [P] = 0 to [P] = 90% $[S_0]$. Gaussian noise of S.D. = $0.005K_m$ was added to each value of [P]. Of such curves, 50 were generated for each value of $[S_0]/K_m$. For each of 20 [P] values between 0 and 70% $[S_0]$, $t_{\frac{1}{2}}$ values were calculated by using values of [P], from the 2000 available, closest to the values required to give the half-time. The values given in the Table represent the mean values for the 50 curves, which were subject to regression analysis for each value of $[S_0]/K_m$.

			95% Confidence limits				95% Confidence limits	
S_0/K_m	K_m (app.)	S.D.	Upper	Lower	$V_{app.}$	S.D.	Upper	Lower
0.50	1.136	0.425	0.926	1.206	1.107	0.335	0.936	1.166
1.00	1.014	0.102	0.969	1.046	1.010	0.067	0.978	1.032
1.50	1.012	0.061	0.989	1.039	1.007	0.034	0.997	1.022
2.00	0.989	0.042	0.973	1.004	0.995	0.020	0.988	1.001

Table 2. *Effect of errors in estimation of* $[P_\infty]$ *on the parameters obtained from simulated progress curves by unweighted linear-regression analysis using the half-time method*

Progress-curve generation and analysis was as described in Table 1, with the exception that random error was *not* added to the calculated progress-curve data.

$[S_0]/K_m$ ...	0.5		1.0		2.0		5.0	
Error in $[P_\infty]$ (%)	K_m (app.)	$V_{app.}$	K_m (app.)	$V_{app.}$	K_m (app.)	$V_{app.}$	K_m (app.)	$V_{app.}$
+2.0	1.513	1.357	1.294	1.158	1.199	1.074	1.147	1.028
+1.0	1.212	1.148	1.134	1.072	1.095	1.036	1.072	1.014
+0.5	1.089	1.061	1.067	1.037	1.043	1.016	1.037	1.008
−0.5	0.903	0.930	0.944	0.970	0.958	0.985	0.969	0.994
−1.0	0.831	0.880	0.892	0.942	0.921	0.971	0.930	0.986
−2.0	0.710	0.795	0.795	0.889	0.842	0.942	0.871	0.975

obtained when the initial $[S_0] \geqslant K_m$(app.), the mean values of the parameter estimates being well within the 95% confidence limits. If $[S_0] = 0.5K_m$(app.) the mean values are again within the 95% confidence limits, but differ considerably from the correct values. For best results the half-time method should be used in circumstances where it is possible to arrange that the initial $[S_0] \geqslant K_m$(app.). These results also show that the application of unweighted least-squares regression analysis to half-time plots gives unbiased estimates of the Michaelis parameters.

Several weighting strategies have been tested; that in which weights proportional to the square of the local $[S_0]$ values are applied produced a modest improvement. The benefit achieved by application of weighted regression analysis is, however, deemed insufficient to justify complication of the simple regression method.

Table 2 shows the results of simulations in which error has been introduced into the $[P_\infty]$ (i.e. $[S_0]$) value in the absence of random error in the progress curve.

α-Chymotrypsin-catalysed hydrolysis of Moc-Gly-ONp

It is well known that α-chymotrypsin is relatively 'well-behaved' in that reactions catalysed by this enzyme have been found to closely obey Michaelis–Menten kinetics (see, e.g., Bardsley *et al.*, 1980). It thus represents a good choice for the practical evaluation of a kinetic-analysis method under 'best case' circumstances. A number of α-chymotrypsin-catalysed reactions have been subject to half-time analysis of progress-curve data. In all cases very good half-time plots have resulted which have allowed accurate estimation of the Michaelis parameters using ruler and pencil. An example of such a reaction is shown as a half-time plot in Fig. 1(*a*) and in direct linear form in Fig. 1(*b*). The progress curve relevant to Figs. 1(*a*) and 1(*b*) was obtained in the presence of 10% acetonitrile. In the presence of 0.33% acetonitrile the Michaelis constant was too

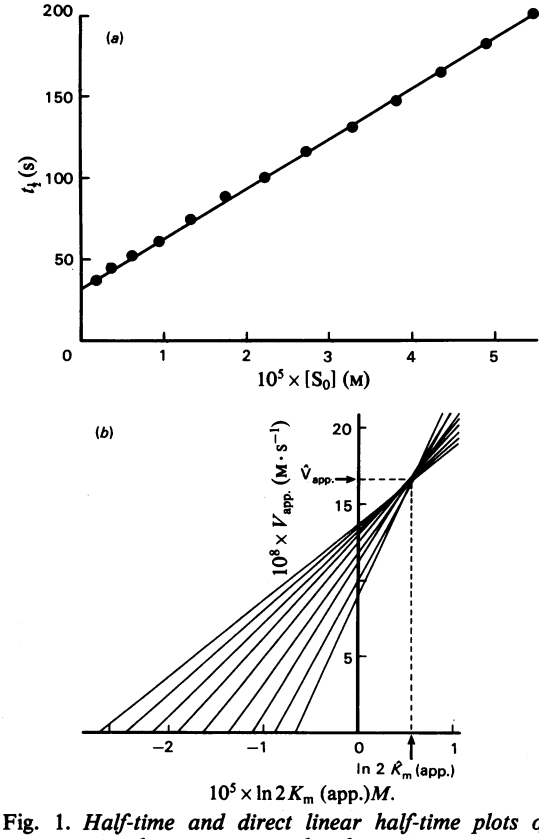

Fig. 1. *Half-time and direct linear half-time plots of α-chymotrypsin-catalysed reactions*

The relevant Michaelis parameters are given in Table 3. (*a*) Half-time plot of the α-chymotrypsin-catalysed hydrolysis of Moc-Gly-ONp. The reaction was monitored at 420nm with initial $[S_0] = 57\mu$M in 0.1M-phosphate buffer, pH6.98, containing 10% acetonitrile, 25°C. (*b*) Direct linear half-time plot of data shown in (*a*). The points on the abscissa are local $[S_0]/2$ values, the points on the ordinate scale represent values of $[S_0]/2t_{\frac{1}{2}}$ calculated (see the Theory section) using the same local $[S_0]$ as the point to which it is joined on the abscissa. The broken lines give the median estimates of V and $\ln 2K_m$. The intercepts of the nine lines define a 96.5% joint confidence limit for V and $\ln 2K_m$.

low to measure accurately, even when the 0–0.1 absorbance scale was used, since the reaction, as observed, was zero-order virtually to completion. The Michaelis parameters were estimated to be: $k_{cat.} = 0.10\,s^{-1}$; $K_m = 0.48\,\mu M$ from a poor-quality half-time plot.

The initial substrate concentration was varied from $57\,\mu M$ to $11.4\,\mu M$ (five runs), there being no discernible trend in the Michaelis parameters obtained from half-time plots under these conditions. It is thus apparent that the products of the reaction do not cause inhibition. The complete absence of curvature in half-time plots up to $\geqslant 90\%$ completion of the reaction indicates that the enzyme does not suffer loss of activity during progress-curve accumulation. The method of Selwyn (1965) has been used to confirm this. Reactions were performed at three enzyme concentrations at three chart speeds such that all curves should be superimposable in the absence of activity loss. All three curves could be exactly superimposed by small (~2s) shifts in the time axis, these being required since the starting times were slightly in error. The contribution of the aqueous rate was always $\leqslant 1\%$ of the total rate at the start of the reaction; a low K_m makes possible use of a low initial substrate concentration, which has a small associated aqueous contribution to the rate. Michaelis parameters relevant to Figs. 1(*a*) and 1(*b*) are given in Table 3.

Papain-catalysed hydrolysis of Moc-Gly-ONp

Initial experiments were conducted in 0.1 M-phosphate buffer, pH 7.0, in the presence of 3.3% acetonitrile at an initial substrate concentration of 3.42 mM. The half-time plots were characterized by marked curvature at high local $[S_0]$, but were approximately linear below 1.5 mM local $[S_0]$. The degree of curvature was reduced but not eliminated by increasing the enzyme concentration until further reduction in curvature was not observed. The component of curvature removed in this way arises from the aqueous component of the total rate which is considerable at an initial substrate concentration of 3.42 mM.

The residual curvature was found to be due to a pH change which occurred during the course of the reaction. Measurement of the pH at the start and finish of the reaction revealed that a change from 6.98 to 6.87 occurred. Increasing the buffer strength to 0.3 M reduced the pH change to 0.04 unit and resulted in almost complete elimination of curvature in the half-time plots. Attempts to employ phosphate buffer of concentration greater than 0.3 M resulted in precipitation of the substrate. We were rather surprised to find a pH change of this magnitude at this substrate concentration, but calculation predicts a pH change of 0.09 unit for 0.1 M-phosphate buffer assuming 1.5 protons are

Table 3. *Collected Michaelis parameters*

The Michaelis parameters are given to more than normal significance in order to facilitate detailed comparison between the various methods.

Reaction	Method of data analysis	Half-time plot	Direct linear form of half-time method	Atkins & Nimmo (1973)	Initial-velocity method*
α-Chymotrypsin–Moc-Gly-ONp	...	$6.15 \pm 0.04 \times 10^{-2}\,s^{-1}$†	$6.14 \times 10^{-2}\,s^{-1}$‡	$6.03 \pm 0.06 \times 10^{-2}\,s^{-1}$	—
	$k_{cat.,app.}$				
	K_m (app.)	$7.92 \pm 0.23 \times 10^{-6}$ M†	7.86×10^{-6} M‡	$7.45 \pm 0.39 \times 10^{-6}$ M	—
Papain–Moc-Gly-ONp	$k_{cat.,app.}$	$10.96 \pm 0.26\,s^{-1}$	$10.75\,s^{-1}$	$11.22 \pm 0.55\,s^{-1}$	$10.5 \pm 0.2\,s^{-1}$
	K_m (app.)	$8.93 \pm 0.51 \times 10^{-4}$ M	8.46×10^{-4} M	$8.96 \pm 1.34 \times 10^{-4}$ M	$5.4 \pm 0.2 \times 10^{-4}$ M
Fumarase (Fumarate ⇌ L-malate)	$V_{app.}$	$7.25 \pm 0.09 \times 10^{-6}$ M·s^{-1}	7.29×10^{-6} M·s^{-1}	$7.70 \pm 0.11 \times 10^{-6}$ M·s^{-1}	—
	K_m (app.)	$9.64 \pm 0.22 \times 10^{-4}$ M	9.75×10^{-4} M	$10.87 \pm 0.36 \times 10^{-4}$ M	—

* The initial-velocity experiments were performed under the same conditions as the progress-curve experiments (see the text); 39 initial velocities which were corrected for aqueous hydrolysis were measured at 13 $[S_0]$ values from $11.4\,\mu M$ to 3.4 mM. Regression analysis was performed by using the method of Wilkinson (1961).

† See Fig. 1(*a*).

‡ See Fig. 1(*b*).

released per molecule of substrate that is hydrolysed. This value of the pH change is in reasonable agreement with the observed value and illustrates the danger of preconceived notions in this regard. The reason why the reaction is so sensitive to pH change is that *p*-nitrophenol has a pK_a close to 7.0 and so the quantity of *p*-nitrophenolate, the light-absorbing species under the conditions of assay, will change maximally with change in pH. The decrease in absorption coefficient due to the pH change from pH 6.98 to pH 6.87 represents a 13% change. The Michaelis parameters of papain-catalsyed reactions are pH-independent near pH 7.0, so the pH change will not affect the values of these parameters. *p*-Nitroaniline has a pK_a of $\simeq 1.0$ and so *p*-nitroanilide substrates, which have become popular for simple enzyme assays, will not be affected in this way.

The Michaelis parameters are presented in Table 3 together with parameters determined from an extensive initial-velocity study. The initial-velocity studies were characterized by an aqueous rate component of about 50% of the total rate at 3.42 mM-substrate. Thus a large fraction of the rate had to be subtracted at high substrate concentration. The agreement between the methods is good if this is taken into account.

Fumarase-catalysed interconversion of L-*malate and fumarate*

A number of reports have appeared in the literature to the effect that the fumarase-catalysed interconversion of L-malate and fumarate deviates from simple reversible Michaelis–Menten kinetics, particularly at high substrate concentration (Alberty & Bock, 1953; Alberty *et al.*, 1954; Alberty & Koerber, 1957; Bardsley *et al.*, 1980). For this reason many of the kinetic studies of fumarase have been restricted to measurements at low substrate concentrations (see, e.g., Darvey *et al.*, 1975). Despite the above observations, we have found that half-time plots of progress-curve data in both directions are linear to 90% of approach to equilibrium even when the initial $[S_0]$ values are greater than the apparent Michaelis constants. The fumarase reaction was studied at five initial substrate concentrations in each direction (from 1.5 mM to 12 mM for L-malate and from 0.3 mM to 3.0 mM for fumarate). Secondary plots of the intercepts of these primary half-time plots against the initial L-malate or fumarate concentrations were found to be accurately linear. Table 4 reveals that the agreement with the parameters obtained from initial-velocity studies is good. It is noteworthy that the results from the half-time studies in each direction are in very good agreement. Our results are thus consistent with the observations of Andersen (1980) to the effect that the fumarase-catalysed interconversion of L-malate and fumarate obeys hyperbolic kinetics provided the substrate solutions are correctly prepared.

Effect of a significant aqueous rate component at the start of an enzyme-catalysed reaction

Newman *et al.* (1974) have presented the integrated form of the rate equation which describes this situation. The aqueous component is represented by a first-order term added to the simple Michaelis equation. Those authors examined some consequences of the presence of a first-order term in the rate equation, albeit by computer-simulation analysis. The half-time equation for such an enzyme-catalysed reaction is given by eqn. (6):

$$t_{\frac{1}{2}} = \frac{1}{V_{app.} + kK}\left[\frac{V_{app.}}{k}\ln\left(\frac{k[S_0] + kK_m(app.) + V_{app.}}{k[S_0]/2 + kK_m(app.) + V_{app.}}\right) + K_m(app.)\ln 2\right] \quad (6)$$

where k is the apparent first-order rate constant for the aqueous contribution in the particular experimental conditions. Eqn. (6) predicts a non-linear half-time plot, since the local substrate concentration is in the log term. At low substrate

Table 4. *Kinetic parameters of the fumarase-catalysed interconversion of fumarate and* L-*malate determined by half-time analysis of progress-curve data as compared with literature values determined by using the initial-velocity method*

The kinetic parameters were determined from secondary plots essentially as described by Yun & Suelter (1977).

	Half-time plot method*		Initial-velocity† method
	L-Malate→fumarate	Fumarate→L-malate	
K_{Malate}	4.4 mM	3.6 mM	4.1 mM
$K_{Fumarate}$	0.19 mM	0.18 mM	0.21 mM
$\frac{V_{Fumarate}}{V_{Malate}}$	0.20	0.23	0.27

* 15 mM-Phosphate, pH 8.0, 25°C.
† 15 mM-Phosphate, pH 8.0, 25°C [Alberty *et al.* (1954)].

concentration, when $k[S_0] \ll V + kK_m(\text{app.})$, eqn. (6) can be simplified to yield eqn. (7):

$$t_{\frac{1}{2}} = \frac{1}{(V_{app.} + kK_m(\text{app.}))}\left[\frac{V_{app.}}{2}\frac{[S_0]}{(V_{app.} + kK_m(\text{app.})}\right] \quad (7)$$

Eqn. (7) predicts a linear region in a half-time plot at low local $[S_0]$, even in the presence of gross concave-down curvature at high $[S_0]$. This has been found to be so in all cases we have studied. The linear region has, in our experience, invariably proved sufficient for the determination of 'correct' values of $V_{app.}$ and K_m(app.) from the slope and intercept of the tangent to this linear region. An example of this is shown in Fig. 2 for the α-chymotrypsin-catalysed hydrolysis of Moc-Gly-ONp. The tangent values of the parameters have been used, together with eqn. (5), to calculate the line through the experimental points.

In order to demonstrate this effect it was necessary to use a low enough enzyme concentration such that the aqueous rate could compete, cf. Fig. 1(*a*). Thus the usual method of eliminating curvature due to this source is to increase the enzyme concentration until such curvature becomes insignificant or to lower the initial substrate concentration. In some circumstances the pH value of the assay may be altered such that the aqueous rate component becomes less significant.

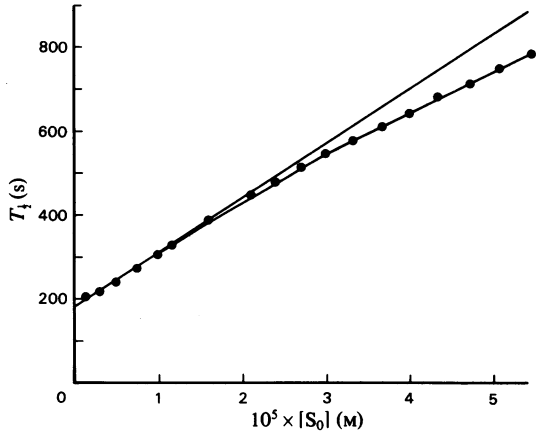

Fig. 2. *Non-linearity in a half-time plot due to a significant aqueous contribution to the total rate at the start of the reaction between α-chymotrypsin and Moc-Gly-ONp*

The slope and intercept of the tangent to the points at low local $[S_0]$ shown in the Figure were used in conjunction with $k_{obs.} = 1.68 \times 10^{-4}\,s^{-1}$, the observed aqueous hydrolysis rate constant under these conditions (see below) to estimate the unperturbed values of the Michaelis parameters (see the text); $V_{app.} = 3.46 \times 10^{-8}\,M \cdot s^{-1}$, $k_{cat.,app.} = 0.056\,s^{-1}$; K_m(app.) $= 9.4 \times 10^{-6}$ M. These parameters were used to calculate the theoretical curve which fits the experimental points as shown using eqn. (5). The reaction was monitored at 420nm with an initial $[S_0] = 57\,\mu M$ in 0.1M-phosphate buffer, pH6.98, containing 10% acetonitrile (25°C).

Comparison of the half-time method with other methods of analysing progress-curve data

Cornish-Bowden (1975, 1977) has provided useful discussions of the relative merits of the various methods that have been used for the analysis of progress curves in terms of integrated rate equations. We shall here concern ourselves with the comparison of some of these methods, and some more recently published, with the half-time method. The material contained in the Theory section and in the present section makes plain the essential simplicity of the half-time method.

The half-time method is related to that proposed by Yun & Suelter (1977), although the relationship is not readily apparent at a first reading. Those authors showed that double-reciprocal plots constructed by using chord-slope estimates of the initial velocities essentially by the method of Lee & Wilson (1971) provide good estimates of the Michaelis parameters provided certain rules are followed. The substrate concentration relevant to a particular chord-slope estimate of an initial velocity is taken as the mean of the substrate concentrations used to estimate the chord slope. Provided $\Delta[S]/[S_0] < 0.5$, i.e. that the difference between the concentrations used to estimate the chord slope is not greater than half the mean local substrate concentration, then the approximation is good. Further, those authors have shown that if $\Delta[S]/[S_0]$ is maintained constant, as indeed it is in the half-time method, then their method becomes exact. The method of Yun & Suelter (1977) is described in terms of the measurement of chord slopes as in the Guggenheim (1926) treatment of first-order reactions. If this method is followed, $\Delta[S]/[S_0]$ does not remain constant, and it is necessary to apply a correction factor to the substrate concentration values for exactness to be preserved. Alternatively the measurements may be devised such that $\Delta[S]/[S_0]$ remains constant, but this is a cumbersome procedure using chord-slope estimates. As mentioned above, the half-time method inherently implies use of constant $\Delta[S]/[S_0]$ and is plotted in a half-reciprocal form that results in minimal statistical bias in graphical plots (Dowd & Riggs, 1965).

Effect of errors in $[P_\infty]$ values

As shown in Table 2 for half-time analysis, the effect of errors in $[P_\infty]$ is serious, the degree being

quantitatively similar to that found by Newman *et al.* (1974) for linearizing transformations of eqn. 1. Non-linear regression analysis of half-time plots in which $[P_\infty]$ was included as a variable led to an improved fit in terms of the sum of squares, but resulted in best-fit $[P_\infty]$ values that were ill-defined. We conclude, as did Cornish-Bowden (1975), that in most circumstances non-linear regression is not an appropriate form of analysis for this type of problem, owing to extreme ill-conditioning of the system, quite apart from the computational complexity involved.

Direct linear form of the half-time plot

The advantages of the use of non-parametric statistical methods have been discussed at length by Cornish-Bowden & Eisenthal (1974). Table 3 reveals that good agreement is seen between linear regression of half-time plots and non-parametric statistical analysis of the direct linear form.

Computational methods

The present and forthcoming availability of relatively inexpensive microprocessor systems which may be interfaced with spectrophotometers (see e.g. Davies & Devia, 1981) requires that suitably simple computational methods should be available for analysis of progress-curve data. The half-time method is simple to compute, but suffers from the disadvantage, as do all other methods so far proposed, that a reliable value of $[P_\infty]$ must be obtained before computation commences. In practice this has proved to be only a minor limitation in view of the rapidity of the half-time method as compared with initial-velocity studies.

Some simple rules that must be followed for the reliable analysis of enzyme kinetics using progress-curve techniques

On the basis of our experiences in the analysis of progress-curve data we are able to propose a set of simple rules. The relatively simple nature of these rules should serve to encourage more widespread use of this time- and material-saving technique.

1. The initial $[S_0]$ should be $1–3 \times K_m$ ($0.5–5\,K_m$ is usable) and $>100 \times [E_0]$.

2. The pH value of the assay must be checked at the beginning and end of each reaction; these values should ideally be identical, but must anyway be very close.

3. The reaction must be run at several (three to five) values of the initial $[S_0]$ consistent with rule 1 above in order to assess whether the reaction is subject to product inhibition.

4. The reaction must be allowed to proceed until a reliable estimate of $[P_\infty]$ is obtained.

5. A wavelength is chosen such that the recorder pen traverses about 90% of the available chart width during the reaction. If possible use the 0–1.0 absorbance scale and select a pair of chart speeds such that the reaction is about 90% complete in 30cm and a stable $[P_\infty]$ reached in about 60cm. This will optimize the signal-to-noise ratio and readability of the chart display.

6. The reaction must be carried out at two or more enzyme concentrations with appropriately adjusted chart speeds such that the traces will be exactly superimposable if the enzyme retains full activity during the run (half-time plots are non-linear if first-order enzyme activity loss occurs).

7. The enzyme concentration should be chosen such that any spontaneous (non-enzymic) component of the total rate is minimized in a manner that is consistent with satisfactory display of the complete reaction on the chart recorder.

Some comments upon the rules

Ideally rules 1–7 should be applied rigorously in all circumstances, but usable results are obtainable in some cases under less-than-optimal conditions. For example, we have obtained half-time plots in which the initial $[S_0] = 0.2K_m$. These plots are subject to considerable scatter, but the Michaelis parameters may nonetheless be estimated with reasonable confidence. Also, although we have been unable to completely eliminate pH change during those papain-catalysed reactions which were started at high initial $[S_0]$, the resulting half-time plots allow reasonable estimates of the Michaelis parameters to be made.

Rule 4 regarding $[P_\infty]$ estimation cannot, however, be relaxed at all, since the effect of error in this parameter is magnified by approximately an order of magnitude in K_m. Modern spectrophotometers, in particular those that employ holographic gratings, have a very low noise performance and are essentially drift-free. This allows $[P_\infty]$ values to be estimated very accurately *provided* the reaction is allowed to each completion. The low noise level of these instruments allows some relaxation of rule 5, since satisfactory performance is available at larger scale expansions.

As a result of this and other (e.g. Yun & Suelter, 1977) investigations of progress-curve analysis, it is apparent that there is little to be feared provided one is familiar with the pitfalls. The essential simplicity and exactness of the half-time method commends its use in all situations in which progress-curve analysis is appropriate.

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