The Use of the Direct Linear Plot for Determining Initial Velocities

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A new method is described for estimating initial velocities of enzyme-catalysed reactions. It is simple to apply either graphically or numerically, and is particularly appropriate for experiments in which the initial straight part of the progress curve is very short or non-existent. It requires no more knowledge than is readily available about the details of the system, such as the extent of reaction at equilibrium, the rate of enzyme inactivation, the nature of product inhibition etc., unlike some other methods of analysing progress curves, which are often invalidated by small errors in the defining assumptions.

The direct linear plot (Eisenthal & Cornish-Bowden, 1974) provides a simple method of analysing initial velocities measured in enzyme kinetic experiments, and its statistical treatment (Cornish-Bowden & Eisenthal, 1974) is both easier to understand and less dependent on arbitrary assumptions than the widely used method of least squares. But effective analysis of initial velocities satisfies only one-half of the problem, because it presupposes a reliable method of estimating initial velocities from progress curves. This is difficult to do accurately unless there is a significant linear period at the beginning of each curve, and any systematic error that results from subjective bias is unlikely to be detected by subsequent statistical treatment.

There are three principal ways of estimating initial velocities, (i) estimation by eye with ruler and pencil, (ii) methods that use the integrated form of the rate equation (Jennings & Niemann, 1955) and (iii) methods involving best-fit polynomials (Booman & Niemann, 1956; Elmore *et al.*, 1963). Of these the first is by far the most widely used, but it is probably not the best, and evidence is given in the present paper that integrated rate equations provide the most reliable general approach. An extension of the direct linear plot to encompass integrated rate equations is described and discussed in relation to published methods.

It is not obvious that the information extracted from a progress curve should be confined to a measure of the initial velocity. Indeed, many of the first investigators of enzymes attempted to characterize the complete time-course of each reaction, but were frustrated by the complexities of enzymic catalysis. Walker & Schmidt (1944) showed how the constants of the Michaelis–Menten equation may be determined from a single progress curve by means of the integrated form of the equation. Unfortunately, their method and others of the same type depend crucially on the correctness of the

assumed rate equation, and give poor results in many common circumstances. Some of the reasons for these poor results are discussed in this paper.

Principle

The time-course of an enzyme-catalysed reaction in the steady state can often be represented by an equation of the following form:

$$V^{\text{app}} \cdot t = p + K_m^{\text{app}} \ln [p_\infty / (p_\infty - p)] \quad (1)$$

in which p is the product concentration at time t , p_∞ is the value of p at equilibrium, and V^{app} and K_m^{app} are constants with meanings that vary according to the mechanism of the reaction. The equation applies to several commonly encountered situations, and was quite widely known as an empirical equation before any clear information existed about the nature of enzymic catalysis (Henri, 1903; Barendrecht, 1904; Abderhalden & Michaelis, 1907; Philoche, 1908; Van Slyke & Cullen, 1914). Its simplest interpretation is that the decline in rate during an enzyme-catalysed reaction occurs solely because of depletion of one substrate, and that the rate is given at all times by the Michaelis–Menten equation

$$dp/dt = V(s_0 - p)/(K_m + s_0 - p)$$

where s_0 , the initial substrate concentration, is equal to p_∞ . In this case $V^{\text{app}} = V$, the maximum velocity, and $K_m^{\text{app}} = K_m$, the Michaelis constant, but no such correspondence applies in general, because eqn. (1) also describes more complex, and more common, situations, such as reactions subject to competitive product inhibition (Henri, 1903; Huang & Niemann, 1951; Schønheyder, 1952), reversible reactions (Alberty & Koerber, 1957), and reactions of more than a single substrate with one substrate at an appreciably lower concentration than any other (Laidler & Bunting, 1973).

Fig. 1. Direct linear plot of observations from a progress curve

Five observations in the range 10–50% of reaction are plotted as straight lines in parameter space. —, Intercepts of $-p/\ln[p_\infty/(p_\infty-p)]$ on the abscissa and of p/t on the ordinate. The five lines intersect at a common point with co-ordinates (K_m^{app}, V^{app}) . A straight line through the common intersection point with an intercept $-p_\infty$ on the abscissa makes an intercept v_0 on the ordinate (---).

Differentiation of eqn. (1) with respect to t shows that the initial velocity, v_0 , is given by $v_0 = V^{app} p_\infty / (K_m^{app} + p_\infty)$. In some simple cases this expression is equivalent to $v_0 = V_{s0} / (K_m + s_0)$, but it is more general and does not depend on the meanings of V^{app} and V_m^{app} or the value of p_∞ . Unlike most kinetic constants V^{app} and K_m^{app} are not restricted to finite positive values. For example, in a single-substrate reaction subject to competitive product inhibition with inhibition constant K_p , V^{app} is equal to $V/[1-(K_m/K_p)]$, which is infinite if K_p is equal to K_m and negative if K_p is less than K_m , a situation that is unusual, but not unknown (e.g., Philo & Selwyn, 1973).

Eqn. (1) defines a linear relationship between V^{app} and K_m^{app} (cf. Eisenthal & Cornish-Bowden, 1974) whereby any straight line in parameter space that passes through the point (K_m^{app}, V^{app}) makes intercepts $-p/\ln[p_\infty/(p_\infty-p)]$ on the K_m^{app} axis and p/t on the V^{app} axis. But it is known (Eisenthal & Cornish-Bowden, 1974; de Miguel Merino, 1974) that a straight line through the point (K_m^{app}, V^{app}) with intercept $-p_\infty$ on the K_m^{app} axis makes an intercept $V^{app} p_\infty / (K_m^{app} + p_\infty)$ on the V^{app} axis. Since this latter intercept is v_0 , the plot provides, in principle, a simple way of determining the initial velocity, as illustrated in Fig. 1.

In practice the method illustrated in Fig. 1 is open to the objection that the lines intersect at such a sharp angle that the point of intersection is ill-defined. This difficulty results from the use of perpendicular axes, which are necessary for estimating K_m^{app} and V^{app} but play no part in the estimation of v_0 . Since the values of K_m^{app} and V^{app}

are of no particular interest except as vehicles for calculating v_0 , for reasons that are discussed below, the difficulty can readily be removed by drawing the two axes at an acute angle to one another. This greatly improves the appearance of the plot, and dispenses with any need for graph paper, but has no effect on the distances of the intercepts from the origin, and consequently has no effect on the estimation of v_0 .

The intercepts $-p/\ln[p_\infty/(p_\infty-p)]$ are tedious to calculate without the aid of an electronic calculator, and so the method as described is unlikely to gain general acceptance for routine use in the laboratory. Fortunately there are several approximate forms of eqn. (1), which have been extensively discussed by Morgan (1972). The most suitable approximation in the present context is obtained by taking the first term of the infinite series

$$\ln(1+x) = 2 \left[\left(\frac{x}{2+x} \right) + \frac{1}{3} \left(\frac{x}{2+x} \right)^3 + \dots \right]$$

so that $-p/\ln[p_\infty/(p_\infty-p)]$ becomes $-p_\infty + \frac{1}{2}p$, which is not only an excellent approximation when p/p_∞ is small but is also very simple to evaluate.

Method

Approximate graphical method

For any progress curve of an enzyme-catalysed reaction, measure the product concentration p at several times t . About five values of p in the range $0.05p_\infty$ to $0.5p_\infty$ are appropriate, where p_∞ is the value of p at equilibrium, but the exact number and range are matters for convenience. Draw two axes inclined at an angle of about 20–30° to one another, labelled $-K_m^{app}$ (abscissa) and V^{app} (ordinate). For each measurement draw a straight line with intercepts $-p_\infty + \frac{1}{2}p$ on the abscissa and p/t on the ordinate. The lines should all intersect in the vicinity of a common point, which will usually be in the first quadrant, but may occasionally be in the third quadrant. Draw another straight line through the middle of the group of intersections, making an intercept $-p_\infty$ on the abscissa. (More precisely, this line should be drawn so that its intercept on the ordinate is the median of all the intercepts that would be obtained if straight lines were drawn in turn through every intersection point; but the gain in accuracy is not sufficient to justify the extra effort.) Read off the value of v_0 from the intercept on the ordinate. The plot is illustrated in Fig. 2.

This method ignores the fact that the systematic error inherent in the logarithmic approximation increases with the value of p , since this error is very small (see the Results section) and likely to be insignificant in comparison with random experimental error and draughting error.

'Exact' numerical method

For definitive processing of results by computer there is very little advantage in using an approximation for the logarithmic term, and the following method, which does not use one, is exact for any curve that obeys eqn. (1) exactly.

For each pair of points (t_i, p_i) and (t_j, p_j) on the progress curve, calculate the parameters $K_{ij}^{app.}$ and $V_{ij}^{app.}$ from the following equations:

$$K_{ij}^{app.} = (t_i p_j - t_j p_i) / \{t_j \ln [p_\infty / (p_\infty - p_i)] - t_i \ln [(p_\infty - p_j)]\} \quad (2)$$

$$V_{ij}^{app.} = \{p_i + K_{ij}^{app.} \ln [p_\infty / (p_\infty - p_i)]\} / t_i \quad (3)$$

and calculate an estimate v_{ij} of the initial velocity from

$$v_{ij} = V_{ij}^{app.} p_\infty / (K_{ij}^{app.} + p_\infty)$$

Determine the median of the v_{ij} values and use it as v_0 .

Results*Effect of inaccuracies in p_∞*

Various authors (Fleisher, 1953; Klyosov & Berezin, 1972; Newman *et al.*, 1974; cf. also Guggenheim, 1926) have pointed out that estimates of $K_m^{app.}$ and $V^{app.}$ in eqn. (1) are very sensitive to small errors in p_∞ , such as might arise from using an inaccurate value of the equilibrium constant. So it is important to determine whether the method given in the present paper is open to the same objection. Some results are given in Table 1 that show that it is not, and that instead estimates of v_0 are extraordinarily insensitive to errors in p_∞ , with percentage errors typically 0.005–0.001 times those in $K_m^{app.}$. Thus it is not necessary to have unrealistically precise knowledge of p_∞ for the method to be useful.

Inaccuracies inherent in the logarithmic approximation

Since the approximation $-p/\ln[p_\infty/(p_\infty - p)] \simeq -p_\infty + \frac{1}{2}p$ has an error of about 4% when $p/p_\infty = 0.5$, it might seem likely to generate significant errors in v_0 . This danger was investigated and the results, summarized in Table 2, show that even if the reaction is followed to 50% completion the errors in v_0 are very small and probably negligible in comparison with other sources of error.

Effect of progressive inactivation of the enzyme

Many enzymes become inactive at a significant rate when diluted to the very low concentrations commonly used in steady-state experiments. A

Fig. 2. Determination of the initial velocity from a direct linear plot

The two axes are drawn at an angle of 20–30° to one another. Observations (in the range 10–50% of reaction in this example) are plotted as straight lines (—) with intercepts $-p_\infty + \frac{1}{2}p$ on the abscissa and p/t on the ordinate. The initial velocity v_0 is found by drawing a straight line (---) through the middle of the family of intersection points with an intercept $-p_\infty$ on the abscissa; the intercept on the ordinate gives v_0 .

Table 1. Effect of errors in p_∞ on the estimation of $V^{app.}$, $K_m^{app.}$ and v_0

The Table shows the percentage errors in $V^{app.}$, $K_m^{app.}$ and v_0 caused by incorrectly assuming p_∞ to be equal to s_0 . Values of t corresponding to five values of p in the range $0.1s_0$ to $0.5s_0$ were calculated from eqn. (1) for various values of $s_0/K_m^{app.}$ and p_∞/s_0 . Although neglect of reversibility cannot cause underestimation of p_∞ , values of p_∞/s_0 greater than 1 were included because similar errors can occur for other reasons. For each combination of values, $V^{app.}$ and $K_m^{app.}$ were estimated as the medians of the sets of values of $V^{app.}$ and $K_m^{app.}$ respectively defined by eqns. (2)–(3), substituting s_0 for p_∞ throughout, and v_0 was estimated as the median value of $(V_{ij}^{app.} s_0) / (K_{ij}^{app.} + s_0)$.

p_∞/s_0	$s_0/K_m^{app.} = 0.1$			$s_0/K_m^{app.} = 0.5$			$s_0/K_m^{app.} = 2.0$		
	$V^{app.}$	$K_m^{app.}$	v_0	$V^{app.}$	$K_m^{app.}$	v_0	$V^{app.}$	$K_m^{app.}$	v_0
0.90	-182	-209	0.500	80	130	0.376	13	49	0.194
0.95	282	329	0.213	24	42	0.158	5	20	0.080
1.00	0	0	0.000	0	0	0.000	0	0	0.000
1.05	-42	-49	-0.162	-13	-23	-0.117	-4	-15	-0.058
1.10	-57	-66	-0.285	-21	-38	-0.205	-6	-27	-0.099

full treatment of this phenomenon is rather complex (see Laidler & Bunting, 1973) if one allows for different rates of inactivation of the free enzyme and its complexes with substrate. But insight can be gained from examination of the simplest assumption, whereby all forms of the enzyme inactivate according to first-order kinetics with the same rate constant, k_0 . The progress curve is then of the form:

$$V^{app} [1 - \exp(-k_0 t)] = k_0 p + \{k_0 K_m^{app} \ln[p_\infty/(p_\infty - p)]\} \quad (4)$$

Table 3 shows some representative results from an investigation of the effects of such inactivation on the estimation of K_m^{app} and V^{app} . As little as 2% inactivation during 50% of reaction can produce significant errors in the estimates of K_m^{app} and V^{app} ; but, in contrast, as much as 25% inactivation during 10% of reaction is possible without generating intolerable errors in v_0 .

Selwyn (1965) has described a beautifully simple method for detecting progressive inactivation of an enzyme. If several progress curves are obtained under identical conditions, apart from the initial enzyme concentration e_0 , then plots of p against $e_0 t$ at various values of e_0 should be superimposable, regardless of the form of the rate equation, unless there is significant inactivation of the enzyme during the reaction. This method is certainly sensitive enough to detect the large amount of inactivation necessary to prevent accurate estimation of v_0 by the method described.

Effect of deviations from eqn. (1)

Although eqn. (1) applies quite widely, there are a few important situations that it does not describe exactly. For example, product inhibition may well

be mixed rather than competitive (Walter & Frieden, 1963) and in this case the rate equation is of the form:

$$\frac{dp}{dt} = \frac{V(s_0 - p)}{K_m(1 + p/K_p) + (s_0 - p)(1 + p/K_p')} \quad (5)$$

where K_p and K_p' are the competitive and uncompetitive inhibition constants respectively, and the other symbols are as defined above. Integration gives:

$$Vt = (1 - K_m/K_p)p + p^2/2K_p' + K_m(1 + s_0/K_p) \ln[p_\infty/(p_\infty - p)] \quad (6)$$

which differs from the form of eqn. (1) by virtue of the term in p^2 . But the deviations from eqn. (1) in practice are generally rather slight, and similar in character to those that result from the use of an inaccurate value of p_∞ or neglecting inactivation. Thus essentially the same results apply as those that we have considered already: estimates of K_m^{app} and V^{app} are grossly distorted, but there is no corresponding

Table 2. *Validity of the logarithmic approximation*

The Table shows the percentage errors in v_0 caused by using the approximation $-p/\ln[p_\infty/(p_\infty - p)] \approx -p_\infty + \frac{1}{2}p$, for various values of p_∞/K_m^{app} and various extents of reaction. In each case estimates of v_0 were calculated from five equally spaced values of p in the range given.

Range of p/p_∞ (%)	Percentage error in v_0		
	$p_\infty/K_m^{app} = 0.1$	$p_\infty/K_m^{app} = 0.5$	$p_\infty/K_m^{app} = 2.0$
1-5	0.006	0.004	0.002
2-10	0.024	0.017	0.009
5-25	0.179	0.131	0.065
10-50	1.017	0.744	0.370

Table 3. *Effect of enzyme inactivation on estimation of V^{app} , K_m^{app} and v_0*

The Table shows the percentage errors in estimates of V^{app} , K_m^{app} and v_0 caused by neglecting enzyme inactivation, for various values of p_∞/K_m^{app} , the extent of reaction, and the amount of inactivation. In each case values of t corresponding to five values of p in the stated range were calculated from eqn. (4) with a value of k_0 selected to give the desired amount of inactivation at the end of the run. V^{app} and K_m^{app} were estimated as the medians of the sets of values defined by eqns. (2)–(3) and v_0 was estimated by the 'exact' numerical method described in the Method section.

Range of p/p_∞ (%)	Inactivation at end of run (%)	$p_\infty/K_m^{app} = 0.1$			$p_\infty/K_m^{app} = 2.0$		
		V^{app}	K_m^{app}	v_0	V^{app}	K_m^{app}	v_0
2-10	2	-184	-202	0.011	40	121	-0.006
2-10	5	-122	-134	0.041	280	839	-0.000
2-10	10	-109	-120	0.135	-286	-858	0.050
2-10	25	-103	-113	0.809	-129	-388	0.580
10-50	2	67	73	0.041	5	14	-0.041
10-50	5	-814	-892	0.115	12	37	-0.091
10-50	10	-189	-208	0.276	30	90	-0.144
10-50	25	-120	-132	1.095	193	579	-0.009

distortion with v_0 . Similar conclusions apply to at least two other types of reaction, namely those in which two or more substrate concentrations vary appreciably (Schwert, 1969), and those accompanied by significant volume changes, as in pH-stat experiments (Gingold, 1974); but there is no need to discuss these further, since no new principles emerge.

It would be misleading to imply that there are no reactions at all that cannot be adequately described by eqn. (1). The most common of these is a reaction assayed by a coupled-enzyme technique in which, for one reason or another, the coupling enzyme is not present in large excess. In this case the kinetics are rather complex (see Storer & Cornish-Bowden, 1974) and are characterized by a period of acceleration before the 'initial' rate is reached. Even if the lines of the plot intersect at a common point (which will be in the second quadrant in this instance) they can only provide an estimate of the rate at zero time, which is unlikely to be of interest. Initial periods of acceleration can also result from special properties of the enzyme, such as activation by a product (Massey & Veeger, 1961) or 'hysteresis' (Frieden, 1970). In any such case the simple approach described in the present paper is unlikely to be appropriate.

Discussion

Comparison with the method of Walker & Schmidt (1944)

Walker & Schmidt (1944) estimated the kinetic parameters of histidine ammonia-lyase by means of eqn. (1), by assuming that the reaction was irreversible and that product inhibition was insignificant, so that $V = V^{app}$ and $K_m = K_m^{app}$. This approach is superficially very attractive, since it promises values of V and K_m from far fewer measurements than are commonly thought necessary: in principle only a single curve need be studied, with a single value of s_0 , and a plot of p/t against $\ln[p_\infty/(p_\infty - p)]/t$ should be a straight line with slope $-K_m$ and intercept V on the ordinate. But product inhibition is far too common for this method to have any wide validity. Even if the product inhibition is slight, K_m^{app} may differ greatly from K_m , and the results for V are little better. If the inhibition is competitive with inhibition constant K_p , K_m^{app} is equal to $K_m(1 + p_\infty/K_p)/(1 - K_m/K_p)$, which exceeds K_m by 75% if $K_p = 5K_m$ and $p_\infty = 2K_m$. For the discrepancy to be acceptably small, say less than 5%, K_p must exceed $61K_m$ if $p_\infty = 2K_m$. So significant errors are possible even if product inhibition is too weak to be easily measured. Similar objections apply to other methods of analysing progress curves that underestimate the importance of product inhibition, such as those of Lee & Wilson (1971) and Atkins & Nimmo (1973).

Not only are values of K_m^{app} and V^{app} difficult to interpret with any confidence, but even their estimation is fraught with serious difficulties, being frustrated by imprecise knowledge of p_∞ , any uncompetitive component in the product inhibition, variation of more than one substrate concentration, progressive inactivation of the enzyme, or changes in volume. Klyosov & Berezin (1972) have described an adaptation of the method of Guggenheim (1926) to overcome the need for an accurate value of p_∞ . But, although their method does largely achieve that aim, it can only yield satisfactory results if all of the other sources of difficulty can be eliminated as well. By contrast, estimation of v_0 , either by the direct linear plot or by the related methods of Foster & Niemann (1953) and Jennings & Niemann (1955), requires no more accuracy in p_∞ and knowledge of the mechanism than are readily obtainable. The price that must be paid for the increased accuracy is that much less information is obtained from each curve, but a precise and reliable value of v_0 is surely preferable to highly dubious estimates of V^{app} and K_m^{app} (or even more dubious estimates of V and K_m). This conclusion is not new: it was reached 20 years ago by Niemann and his associates (Foster & Niemann, 1953; Applewhite & Niemann, 1955; Jennings & Niemann, 1955), but it has not been generally recognized. It may well be that the low repute of methods based on integrated rate equations (see, e.g., Gutfreund, 1965) is a consequence of the many published methods that give very poor results outside the limited context in which they have been tested.

Comparison with the methods of Jennings & Niemann (1955)

The results discussed so far argue strongly for methods that attach no significance to the values of V^{app} and K_m^{app} other than as vehicles for calculating v_0 accurately. But they do not provide any reason for preferring the direct linear plot to the three methods discussed by Jennings & Niemann (1955). The advantages of the new method are statistical in character and will be discussed in relation to the first of the earlier methods (Foster & Niemann, 1953; Applewhite & Niemann, 1955), which is typical of the three. In this method p/t is plotted against $\ln[p_\infty/(p_\infty - p)]/t$, and v_0 is found by extrapolating the line back until it intersects a straight line through the origin of slope p_∞ , at a point with co-ordinates $(v_0/p_\infty, v_0)$. One might suppose that the extrapolation would be most accurate if it were made as short as possible by using observations near the beginning of the curve. But both p/t and $\ln[p_\infty/(p_\infty - p)]/t$ are indefinite when $t = 0$ and are subject to large errors in the early part of the reaction. This means that some

extrapolation is inevitable even when it is experimentally possible to observe the beginning of the reaction.

One way out of this difficulty might be to fit the untransformed progress curve to the appropriate equation by the method of least squares, as suggested by Fernley (1974). But this is rather difficult and is of no help in providing a simple graphical method, since it requires computation. In any case, the error in p itself is likely to vary in a complex way: at the beginning of a reaction there are effects of incomplete mixing, small temperature inaccuracies, instrumental fluctuations etc., which become negligible as the system settles down; but other errors in p increase systematically during the reaction, as inaccuracies in the defining assumptions, such as neglect of inactivation of the enzyme and changes in the ionic strength or volume, accumulate. It may well be difficult or impossible to devise a weighting scheme that adequately reflects these variations. For a population of excellent observations mixed with a few very poor ones a non-parametric method of analysis, such as the one suggested in this paper, may be expected to give more satisfactory results than a parametric method that depends on arbitrary assumptions about the distribution of error.

Comparison with simple methods

One of the main advantages of the direct linear plot in obtaining kinetic parameters from initial-velocity measurements is that it is simpler than any of the more widely used methods. No such advantage can be claimed for its use in estimating the initial velocity from a progress curve, which is certainly more laborious than estimating the initial slope of the curve with a ruler and pencil. It is therefore necessary to consider when simple methods are likely to be satisfactory.

A steady-state experiment is ideally carried out in such a way that the initial conditions apply throughout the period of measurement, without perceptible depletion of substrate, inactivation of the enzyme, or accumulation of product. When this ideal is achieved, the progress 'curve' is straight, apart from high-frequency noise, throughout the period of measurement, and estimation of v_0 from the constant slope is at least as accurate as any more elaborate method. But it is much more usual for the progress curve to be perceptibly curved, and in this case estimation of the initial tangent is a subjective operation and generally leads to underestimation of the true initial velocity (Walter & Barrett, 1970).

Lee & Wilson (1971) have proposed a simple way of avoiding the need to estimate the initial tangent, whereby the mean velocity over an extended period is measured and assumed to be the initial

velocity corresponding to the mean substrate concentration in the same period. Although this method is remarkably accurate under some special circumstances, it ignores both product inhibition and enzyme inactivation, and neither can be allowed for without losing the essential simplicity of the method.

Comparison with polynomial curve-fitting

Booman & Niemann (1956) considered that, in cases where the proper integrated rate equation was unknown or too cumbersome for convenient application, the use of a simplified equation would lead to bias in the estimate of the initial velocity. This is a reasonable supposition *a priori*, but it does not seem to be borne out by the results described in the present paper, which suggest that the estimation of v_0 from an integrated rate equation can tolerate severe errors in the parameters and form of the equation. Nonetheless it is appropriate to discuss the alternative proposed by Booman & Niemann (1956), in which a progress curve is fitted to a polynomial equation, which may be differentiated with respect to time to give the velocity at any instant.

Booman & Niemann (1956) and Elmore *et al.* (1963) have described methods for fitting a series of polynomials of increasing order to a set of points from a progress curve, stopping the process when there ceases to be any significant improvement in fit on increasing the order. But Knowles (1965) has argued that this unrestricted approach permits very poor estimates of the initial velocity, because there is no reliable way of recognizing and ignoring insignificant fluctuations of the curve. He has therefore restricted the fitting process to a quadratic equation, and Lowe & Williams (1966) have similarly restricted it to a cubic equation. Experience in this laboratory (J. A. Cole, B. Compton & A. Cornish-Bowden, unpublished work) indicates that such restrictions are especially desirable when analysing curves with substantial instrumental noise.

There are several reasons for problems with polynomials. First, differentiation of a function increases the proportion of any noise that may be present (Lanczos, 1957). Secondly, estimation of an initial velocity requires extrapolation outside the range of observation, a process that is particularly hazardous with high-order polynomials, which characteristically optimize the fit at the sampling points at the expense of uncontrolled deviations elsewhere, especially at points out of range. Thirdly, all practical polynomial-fitting procedures involve assumptions about the weights of the observations, and these are difficult to define so that they accurately reflect the actual variation of variance. A single poor

observation at the beginning of the period of measurement may be very damaging, but can only be omitted at the expense of increased extrapolation. Finally, an integrated rate equation is at best an exact expression of reality and is usually a good approximation, whereas a polynomial, especially one of low order, cannot express a progress curve exactly. Philo & Selwyn (1973) observed an extreme example of this when they found that even an eleventh-order best-fit polynomial (expressing p in terms of t rather than the more usual converse) differed appreciably from a progress curve calculated from eqn. (1) with no experimental error. In general, whatever restrictions one places on polynomial-fitting one cannot avoid the dilemma of a choice between a low-order polynomial that is too inflexible to fit exactly even in the absence of noise and a high-order polynomial that is so flexible that it cannot reject ridiculous solutions.

Conclusions

Most of the situations common in steady-state kinetics yield integrated rate equations of the same form as eqn. (1) or very similar to it. Moreover, small inaccuracies in the defining assumptions generate large errors in the coefficients of eqn. (1) but small or negligible changes in the curve that it defines. One of the main problems is provided by uncertainty in the value of p_{∞} . This can sometimes be overcome by treating it as an unknown (Newman *et al.*, 1974), but this results in a very ill-conditioned system, i.e. one in which very many combinations of parameter values can account adequately for the same data: this explains why Newman *et al.* (1974) observed large increases in the standard errors of the kinetic parameters when they treated p_{∞} as an unknown. So it is difficult to avoid the conclusion of Foster & Niemann (1953) that integrated rate equations should not be used for direct evaluation of enzyme kinetic parameters. Instead they are better used for obtaining more accurate values of the initial velocity than are available from simple methods. The method described in this paper provides an alternative to those discussed by Jennings & Niemann (1955) and avoids the need to make crucial decisions about weighting on the basis of inadequate knowledge. It is somewhat simpler than the earlier methods to apply numerically with the aid of a calculator or computer, and is also very convenient to use as a graphical method.

In situations where the simplest integrated rate equation, eqn. (1), does not apply exactly, it seems nonetheless to be more reliable than a best-fit polynomial as a predictive equation, because it is capable of fitting more closely than a low-order polynomial and lacks the capacity of a high-order polynomial for accommodating insignificant fluctuations.

The method described in this paper seems particularly appropriate for use in very difficult experimental situations when the reaction under investigation cannot be followed continuously but must instead be assayed by analysing samples at intervals. If only a small number of points, say two or three, can be sampled in each reaction, and yet the rate decreases significantly during the experiment, eqn. (1) is likely to provide the only possible basis for making a realistic assessment of the initial velocity; certainly there would be no justification for making any simpler assumption, and insufficient information for fitting a polynomial.

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