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The Direct Linear Plot

A NEW GRAPHICAL PROCEDURE FOR ESTIMATING ENZYME KINETIC PARAMETERS

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A new plot is described for analysing the results of kinetic experiments in which the Michaelis–Menten equation is obeyed. Observations are plotted as lines in parameter space, instead of points in observation space. With appropriate modifications the plot is applicable to most problems of interest to the enzyme kineticist. It has the following advantages over traditional methods of plotting kinetic results: it is very simple to construct, because it is composed entirely of straight lines and requires no calculation or mathematical tables; the kinetic constants are read off the plot directly, again without calculation; it may be used during the course of an experiment to judge the success of the experiment, and to modify the experimental design; it provides clear and accurate information about the quality of the observations, and identifies aberrant observations; it provides a clear indication of the precision of the kinetic constants; constructed with care, it provides unbiased estimates of the kinetic constants, the same as those provided by a computer program; it may be used to simulate results for illustrative purposes very rapidly and simply.

The behaviour of many physical and biological systems can be described in terms of a hyperbolic relationship between a measured response and a controlled variable. In biochemistry this is most often encountered in the field of enzyme kinetics. The steady-state kinetics of the great majority of the enzyme-catalysed reactions that have been studied are adequately described in terms of mechanisms that predict a hyperbolic relationship between the steady-state velocity, v, and the concentration, s, of a substrate, cofactor or reversible modifier. The most familiar expression of this relationship is the Michaelis–Menten equation:

$$v = \frac{Vs}{K_m + s} \qquad (1)$$

where V and K_m are constants, known as the maximum velocity and the Michaelis constant respectively. A plot of v against s is a rectangular hyperbola through the origin, with asymptotes $v = V$ and $s = -K_m$. Physical necessity restricts measurement of v to finite positive values of s. Consequently it is not possible to measure V and K_m accurately from such a plot, because the asymptotes cannot be approached closely enough.

Several ways of obviating this difficulty have been proposed over a period of many years. Michaelis & Menten (1913) made use of the fact that a plot of v/V against $\log s$ has a point of inflexion where $s = K_m$ and that the maximum slope at this point is 0.576. This method is statistically unobjectionable, but it has not found favour with later workers, possibly because the plot is curved. However, a logarithmic scale for concentration does have important advantages, and is particularly useful if measurements are made over a very wide range of concentrations, and if departures from hyperbolic behaviour are exhibited (see Weber & Anderson, 1965).

Most biochemists have preferred to use one of the three linear plots introduced by Woolf (cited in Haldane & Stern, 1932), which are derived from the three linear transformations of eqn. (1) shown in eqns. (2–4):

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V} \cdot \frac{1}{s} \qquad (2)$$

$$\frac{s}{v} = \frac{K_m}{V} + \frac{1}{V} \cdot s \qquad (3)$$

$$v = V - K_m \cdot \frac{v}{s} \qquad (4)$$

Of these, the double-reciprocal plot, where $1/v$ is

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plotted against $1/s$, has been by far the most widely used, although the other two, particularly the plot of v against v/s, have attracted enthusiastic proponents (e.g. Hofstee, 1959). Hanes (1932) drew attention to the statistical dangers that attend any transformation of eqn. (1), but his warning appears to have been totally ignored, and it was not until the work of Johansen & Lumry (1961) and Wilkinson (1961) that the statistical properties of the linear plots were seriously examined. These workers, and also Dowd & Riggs (1965), showed that all three linear plots were statistically objectionable, and that the double-reciprocal plot was by far the worst.

In an attempt to avoid these difficulties, Wilkinson (1961) proposed a computational procedure for fitting experimental data to eqn. (1) directly. It is by no means clear that this approach is entirely satisfactory in practice, because it requires several unsupported assumptions to be made about the distribution of experimental error, as we discuss in the following paper (Cornish-Bowden & Eisenthal, 1974). Moreover, it is quintessentially a computational method, with no usable graphical equivalent, and is thus unsuited to routine laboratory use.

All of the methods currently in use for estimating kinetic parameters require some calculation to be made. In this paper we describe a simple graphical procedure for estimating V and K_m which is not only statistically sound, but which also requires no calculation of any kind. It is also applicable to many experiments more complex than those described simply by eqn. (1), including experiments involving multiple substrates, and the common types of inhibition. We shall refer to it as the direct linear plot, because it represents each observation by a straight line, and it yields the values of V and K_m directly.

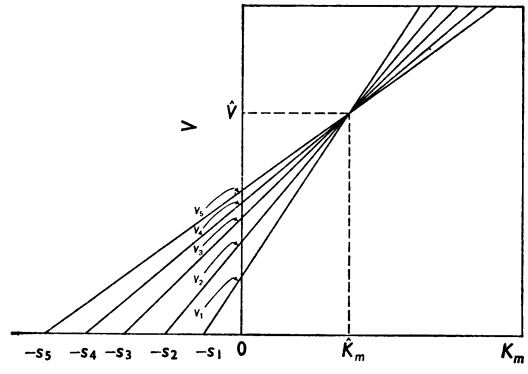

Fig. 1. *Determination of $\hat{V}$ and $\hat{K}_m$ by the direct linear plot*

Each line represents one observation of s and v, and has intercepts $-s$ and v on the K_m and V axes respectively. The point of intersection of the lines gives the co-ordinates of the best-fit values, $\hat{K}_m$ and $\hat{V}$.

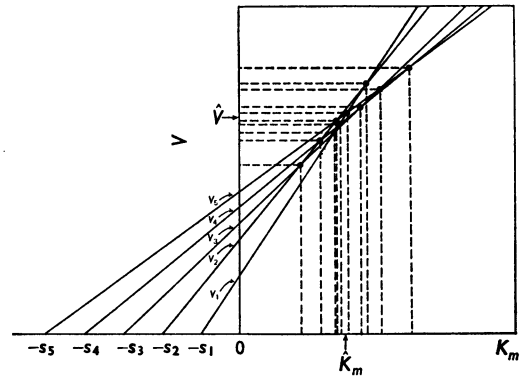

Fig. 2. *Effect of experimental error*

The unique intersection point of Fig. 1 degenerates into ten points [i.e. $\frac{1}{2}n(n-1)$, with $n=5$] when the lines are subject to error. Each intersection provides an estimate of K_m and an estimate of V. The best estimates, $\hat{K}_m$ and $\hat{V}$, are taken as the medians of the two sets of estimates.

Method

Set up axes, K_m and V, corresponding to the familiar x and y axes, respectively. For each observation (s, v), mark off the points $K_m = -s$ on the K_m axis, and $V = v$ on the V axis, and draw a line through the two points, extending it into the first quadrant. When this is done for all observations, the lines intersect at a common point, whose co-ordinates (K_m, V) provide the values of K_m and V that satisfy the Michaelis–Menten equation exactly for every observation. This is illustrated in Fig. 1 for an experiment of five observations.

When the observations are subject to error (i.e. always), there is no unique intersection point for all of the lines; but it is easy to locate $\hat{V}$ and $\hat{K}_m$, the best values of V and K_m, by a method illustrated in Fig. 2. Each intersection is considered to provide an estimate of V and an estimate of K_m. These estimates can be marked off on the axes if desired, as shown. Then the median (i.e. the middle) value from each series is taken to be the best estimate $\hat{V}$ or $\hat{K}_m$. If there are an even number of values the median is taken as the mean of the middle two estimates. If there should exist any three lines that appear to intersect at a common point this point is treated as three points rather than one in finding the median, because there would be three intersection points if the resolution were sufficient to reveal them. Similarly, the common intersection of four lines is treated as six intersections, etc. The total number of intersections is always $\frac{1}{2}n(n-1)$, except in the rare case where some lines are exactly parallel. But if the experimental precision is poor, there will certainly be some intersections off-scale,

usually at large positive values of K_m and V. The number of such intersections can always be deduced from the slopes of the lines, but it may often be easier to count intersections from the low end of the scale, and to stop when half of the total have been counted. The reasons for choosing the median as the best estimate are elaborated in the following paper (Cornish-Bowden & Eisenthal, 1974). Here it will suffice to emphasize that the choice of the median is of paramount importance, and that the mean is entirely unsuitable as an alternative.

The point of intersection of two lines is most precisely defined if they intersect at right angles, and least precisely if they intersect at a very acute angle. It follows therefore that the most precise estimates of V and K_m are obtained if the range of s and v is as great as possible [provided that eqn. (1) is obeyed over the whole range]. Similarly, to ensure the maximum precision of each intersection, it is desirable for the values of v to be evenly spaced within the range. It is much easier to space s evenly, however, since s is directly chosen by the experimenter, and this is likely to be adequate in practice.

Theory

Eqn. (1) may be rearranged into the form:

$$\frac{V}{v}-\frac{K_m}{s}=1 \qquad (5)$$

which is of the general form:

$$\frac{x}{a}+\frac{y}{b}=1 \qquad (6)$$

i.e. the equation of a straight line in xy space with intercepts a on the x axis and b on the y axis. Thus V and K_m are linearly related for given values of v and s, even though v and s themselves are not. Eqn. (5) defines a straight line plotted in VK_m space. In VK_m space the axes V and K_m replace the more familiar v and s. In general, any point in vs space can be represented as a line in VK_m space (cf. Dolby, 1960).

For each observation (s,v) there exists a straight line in VK_m space with intercepts $-s$ on the K_m axis and v on the V axis. This line relates all values of V and K_m that satisfy eqn. (1) exactly for the particular values of s and v. It follows that the co-ordinates of the point where the lines intersect provide the only values of V and K_m that satisfy eqn. (1) for every observation.

In the case where the observations are subject to error, it may be questioned whether it is right to give equal weight to all intersections in determining the best-fit values, $\hat{V}$ and $\hat{K}_m$, since they are plainly not all equally precise. This is an important consideration, but in practice excellent results can be obtained without weighting, because the median of a sample, unlike the mean, is not greatly affected by weighting (Bowley, 1928).

Applications

The most obvious use of the direct linear plot is in the estimation of V and K_m in cases where eqn. (1) is obeyed. But its application is by no means limited to this, because many situations in enzyme kinetics can be expressed by equations of the same form. For example, most of the common equations for inhibition, activation, pH-dependence, multiple-substrate reactions etc. can be written in the form of eqn. (7):

$$v=\frac{V^{\text{app.}}s}{K_m^{\text{app.}}+s} \qquad (7)$$

where $V^{\text{app.}}$ and $K_m^{\text{app.}}$, the 'apparent' values of V and K_m, are not true constants, but depend on certain variables (inhibitor concentration, pH etc.) which are held constant while s is varied. Since eqn. (7) is of exactly the same form as eqn. (1) the direct linear plot may be used to determine $\hat{V}^{\text{app.}}$ and $\hat{K}_m^{\text{app.}}$ in exactly the way that we have described.

Inhibition

The way in which $V^{\text{app.}}$ and $K_m^{\text{app.}}$ vary is a function of the type of mechanism obeyed. For example, in simple competitive inhibition, $V^{\text{app.}}$ is constant and equal to V, but $K_m^{\text{app.}}$ varies according to eqn. (8):

$$K_m^{\text{app.}}=K_m\left(1+\frac{i}{K_i}\right) \qquad (8)$$

where i is the inhibitor concentration, and K_i is the inhibition constant. In uncompetitive inhibition both $V^{\text{app.}}$ and $K_m^{\text{app.}}$ vary with i, but in constant proportion, so that $V^{\text{app.}}/K_m^{\text{app.}}$ is constant, and equal to V/K_m. The direct linear plot distinguishes between these two cases in a very straightforward manner: if plots are made at various values of i, the common intersection point shifts parallel with the K_m axis and away from the V axis as i is increased, if the inhibition is competitive, but directly towards the origin if the inhibition is uncompetitive. These two asymptotic cases are illustrated in Fig. 3, together with the intermediate directions of shift characteristic of mixed inhibition. In a real experiment the common intersection point is not precisely defined, on account of experimental error, and so the exact direction in which it is shifted cannot be precisely determined. Thus it may be impossible to discriminate conclusively between mixed inhibition and any one of the pure inhibition types, i.e. competitive, pure non-competitive and uncompetitive inhibition. This is also of course true of all other plots, but it is seen particularly clearly in the direct linear plot, with a clear indication of the amount of uncertainty about the type of inhibition. An example is shown in Fig. 4, for the results of an experiment in which v was measured in the presence and absence of inhibitor at five different substrate concentrations: although a slight degree of

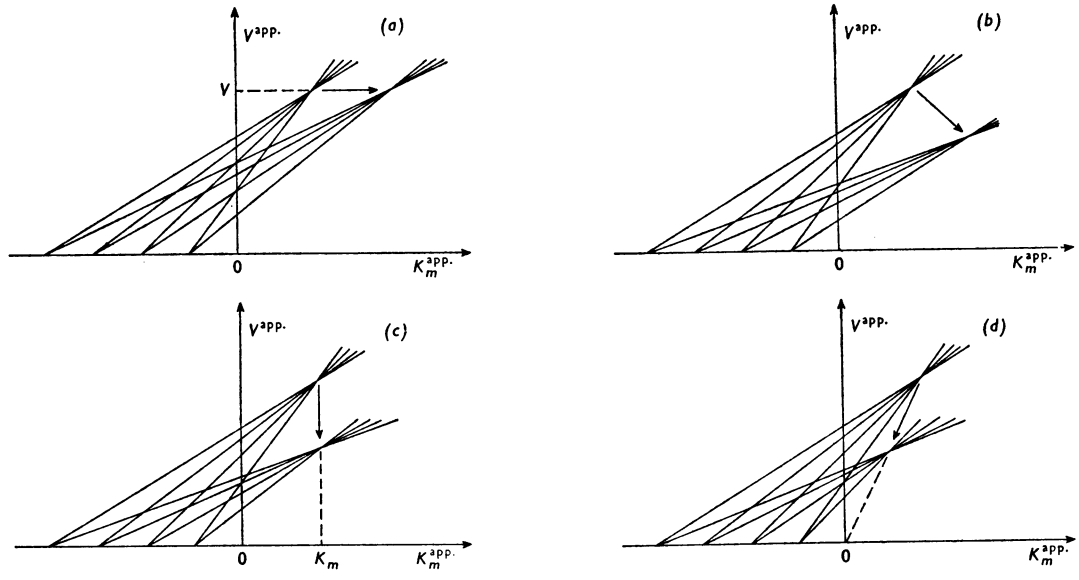

Fig. 3. *Diagnosis of inhibition types*

For each of the simple inhibition types, the arrow indicates the direction in which the common intersection point ($\hat{K}_m^{app.}$, $\hat{V}^{app.}$), of the direct linear plot shifts as the inhibitor concentration is increased. (*a*) Competitive; (*b*) mixed; (*c*) pure non-competitive; (*d*) uncompetitive.

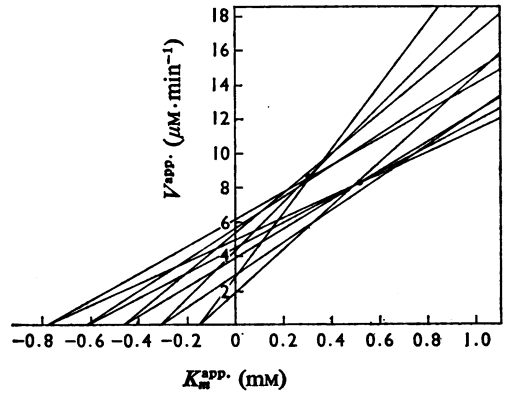

Fig. 4. *Diagnosis of competitive inhibition in a real experiment*

Data of Cornish-Bowden (1967) for the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine by acetyl-L-phenylalanyl-D-phenylalanine at 37°C, pH2.1, are plotted. Full experimental details have been given by Knowles *et al.* (1969). For each value of s, the higher value of v was observed in the absence of inhibitor, the lower value in the presence of 0.525mM-inhibitor. For each set of lines the point having the co-ordinates of the median estimates $\hat{K}_m^{app.}$ and $\hat{V}^{app.}$ is shown as a filled circle.

mixed inhibition is consistent with the data, there can be little doubt that the inhibition is almost wholly competitive, and a much more precise and extensive experiment would be required to justify any alternative claim. Schlamowitz *et al.* (1969) have discussed the problem of discriminating between competitive inhibition and the limiting case of mixed inhibition, and have argued against the use of certain plots for diagnostic purposes. However, the difficulty is inherent in the definition of competitive inhibition, and is not an artifact of the method of analysis. Consequently the direct linear plot is no more able to resolve this problem than any other plot, though it does give a clear indication of the amount of uncertainty.

Two-substrate reactions

The general equation for the steady-state velocity of an enzyme-catalysed reaction involving two substrates is shown in eqn. (9):

$$v = \frac{Vab}{K_i^A K_m^B + K_m^B a + K_m^A b + ab} \qquad (9)$$

where V, K_i^A, K_m^A and K_m^B are the kinetic constants and a and b are the concentrations of the two substrates.

Although this is strictly the equation for reactions which proceed through a ternary complex, the equation for the substituted-enzyme (or Ping Pong) mechanism may be obtained from eqn. (9) by putting $K_i^A = 0$. In its general form, eqn. (9) is not suitable for plotting in two dimensions, but if one substrate concentration is varied at a constant value of the other, it becomes of the form of eqn. (1). For example, if b is held constant and a is varied, eqn. (9) becomes

$$v = \frac{V^{app.}a}{K_m^{app.}+a} \quad (10)$$

where

$$V^{app.} = \frac{Vb}{K_m^B+b} \quad (11)$$

$$K_m^{app.} = \frac{K_i^A K_m^B + K_m^A b}{K_m^B+b} \quad (12)$$

$$V^{app.}/K_m^{app.} = \frac{(V/K_m^A)b}{K_i^A K_m^B/K_m^A+b} \quad (13)$$

Notice that, although eqn. (12) defines a three-parameter hyperbola (i.e. one that does not pass through the origin), eqns. (10), (11) and (13) are all of the form of eqn. (1). So $\hat{V}^{app.}$ and $\hat{K}_m^{app.}$ can be found from a direct linear plot when v is measured at various values of a, and two secondary plots are then available to give the values of the kinetic parameters: a plot of V against K_m^B, with intercepts $V^{app.}$ and $-b$; and a plot of V/K_m^A against $K_i^A K_m^B/K_m^A$, with intercepts $V^{app.}/K_m^{app.}$ and $-b$. An analogous series of plots is possible if b is treated as the variable substrate instead of a.

The substituted-enzyme mechanism

The appropriate equations for the substituted-enzyme mechanism may be obtained very simply by setting K_i^A to zero in eqns. (9)–(13). Then it may be seen that a primary plot of $V_m^{app.}$ against $K_m^{app.}$, and one secondary plot, of V against K_m^B, are possible when a is the variable substrate, similar to those for ternary-complex mechanisms. A second secondary plot is not required, because $V^{app.}/K_m^{app.}$ is independent of b (and equal to V/K_m^A) for this mechanism.

Diagnostic applications in the two-substrate case

The primary plot of $V^{app.}$ against $K_m^{app.}$ can be used diagnostically to discriminate between the substituted-enzyme and ternary-complex mechanisms. Elimination of b between eqns. (11) and (12) yields:

$$K_m^{app.} = K_i^A + \frac{(K_m^A - K_i^A)}{V} \cdot V^{app.} \quad (14)$$

This equation shows that the locus of the common intersection point at various values of b is a straight line. It is a finite line, extending from the point $(K_i^A, 0)$ for $b \to 0$ to (K_m^A, V) for $b \to \infty$. For the substituted-enzyme mechanism the terminus for $b \to 0$ is the origin, and so the locus of the common intersection point is a straight line from the origin. These conclusions are illustrated in Fig. 5.

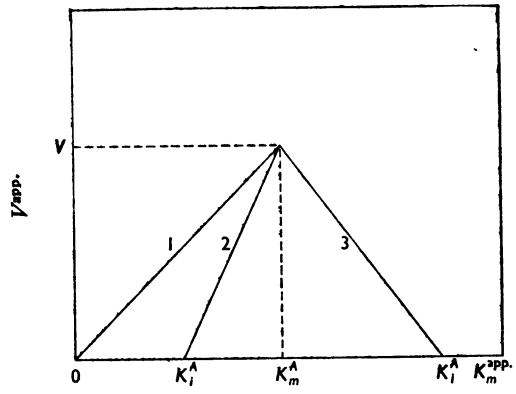

Fig. 5. *Diagnosis of two-substrate mechanisms*

The solid lines represent the loci of the common intersection points, $(\hat{K}_m^{app.}, \hat{V}^{app.})$ at different values of the constant substrate concentration, b, for three cases: 1, substituted-enzyme mechanism; 2, ternary-complex mechanism, with $K_i^A < K_m^A$; 3, ternary-complex mechanism, with $K_i^A > K_m^A$. In each case the terminus for $b \to \infty$ is (K_m^A, V); for the substituted-enzyme mechanism, the terminus for $b \to 0$ is the origin; for the ternary-complex mechanism it is $(K_i^A, 0)$. All of the lines are finite, i.e. they do not extend beyond the termini.

Discussion

Cleland (1963) has advised that enzyme kinetic constants be evaluated by means of computer programs, but that the double-reciprocal plot of $1/v$ against $1/s$ be used to display results, to identify poor observations, and to detect lack of fit. This advice has been widely followed, but it leads to the very unsatisfactory consequence that, unless the results are unusually precise, there is very little relation between the method of estimation and the method of display: kinetic constants determined by the method of Wilkinson (1961) do not in general appear to be correct when presented in the form of a double-reciprocal plot. Moreover, as we demonstrate in the following paper (Cornish-Bowden & Eisenthal, 1974), the information provided by the double-reciprocal plot about the presence of poor observations can be so misleading as to be worthless. We can see no justification for the continued use of the double-reciprocal plot for any purpose.

The direct linear plot has several advantages, not only over the double-reciprocal plot, but over the other well-known plots as well. Since it requires no calculation or mathematical tables, it is particularly

convenient for routine use in the laboratory. In preliminary studies to determine the appropriate range of s values, the observations can be recorded on the plot as quickly as they are measured, revealing the approximate values of the kinetic constants almost instantaneously. Further, the location of the median involves counting rather than calculating, and so the graphical procedure can, with care, be an exact replica of the computational procedure.

The direct linear plot focuses attention directly on the values of V and K_m (or other kinetic parameters), and provides more information about the uncertainty in these values than other plots. This has the consequence that it is difficult to avoid crowding when displaying the results of several experiments, where, for example, two concentrations have both been varied over several values. Although Fig. 4 demonstrates that two series of observations can be clearly displayed in one plot, that is likely to be the limit. So, for presenting results for publication, it is likely that one of the traditional plots will continue to be useful. The plot of s/v against s, with its analogues in multiple-substrate studies, is probably the best for this purpose.

We have discussed the direct linear plot hitherto as a means of evaluating enzyme kinetic constants. But it can also be used as an aid in the planning of experiments: if the approximate values of the kinetic constants are known, one can draw a series of lines through the expected common intersection point, to assess the values of s needed to give selected values of v, or vice versa. The method is simple enough for this to be done as an experiment proceeds, so that one can readily use the first results to plan the remainder of the experiment. When this method is used it is advisable to begin with s values at the upper and lower ends of the intended range, to obtain a well-defined intersection point. The plot also provides a simple and quick method of simulating data for illustrative purposes.

The account of the direct linear plot in this paper has been confined to applications that are likely to be of general usefulness to enzymologists. There remain some possible applications that have not been discussed, e.g. in relation to integrated rate equations. In other areas of biochemistry, the direct linear plot can be applied to binding experiments, where it has advantages over conventional graphical methods (Scatchard, 1949) for estimation of binding parameters. These do not by any means exhaust the possibilities for a plot of this type: almost any equation of two parameters can be plotted in parameter space rather than observation space.

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