OBSERVATIONS ON PROGRAMS TO ESTIMATE THE PARAMETERS OF ENZYME KINETICS

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This paper gives a critical account of the two major programs so far published specifically to estimate the parameters of enzyme kinetics. The dangers of submitting data to the programs without proper checks are discussed, and a screening test is described to identifty sets of data which may not be best fitted by a rectangular hyperbola.

The problem of obtaining the best estimates of the constants A and B of eq. (1)

$$v = \frac{A[S]}{B + [S]}, \quad (1)$$

preferably with statistical confidence limits, from a set of experimental data, is one of permanent interest to enzymologists. Many people have pointed out that the inversion procedure of Lineweaver and Burk [1] is often unsatisfactory, because most relative error attaches to the observations of v corresponding to small [S], and it is these which have most weight in fitting the regression coefficients to the inverted data. In 1961 Wilkinson [2] showed that if eq. (1) was transformed into

$$[S]/v = B/A + [S]/A, \quad (2)$$

the values of $1/v$ were effectively weighted by being multiplied by the corresponding values of [S], and a much more reliable fit to the data could be obtained.

More recently Cleland [3] and Hanson, Ling and Havir [4] have published computer programs which obtain estimates of A and B by direct iterative fitting to eq. (1), using initial estimates of the parameters from other sources. Both programs require linearization of eq. (1). Cleland's program uses least-squares fitting of the parameters, while that of Hanson et al., which is based on a comprehensive paper on the fitting of data to hyperbolic equations by Bliss and James [5], uses a maximum-likelihood method. Both methods assume that the experimental error is associated exclusively with the measurement of v, i.e., that [S] and the other experimental variables are always controlled with complete accuracy, although Bliss and James state that even if this assumption is not completely true it is not likely to affect the operation of the procedure very much. Finally, Cleland's program includes a rather sophisticated subroutine for obtaining initial estimates of A and B, based on a straightforward inversion of eq. (1), while in the program of Hanson et al., the preliminary estimates are normally obtained from elsewhere, presumably graphically, and entered with the experimental data.

We have had some experience with both these programs, and would like to offer some observations on their relative advantages and disadvantages, together with an attempt to overcome a more insidious difficulty.

With reasonably accurate data, there is probably little to choose between either of these programs and Wilkinson's method. Bliss and James in fact took the latter's data (7 points) and showed that their estimate of K_m was 0.5966 ± 0.0683 and of V_{max} 0.6904 ± 0.0368, compared with the estimates of 0.595 ± 0.064 and 0.690 ± 0.036 given by Wilkinson. Both these sets of estimates are more accurate than would normally be required by many people. The disadvantage of Hanson et al.'s program that initial estimates of A and B must be obtained previously is more apparent than real. The convergence of the method is so good that a very rough estimate of B

alone will suffice, and indeed, its value may initially be set at 0. By comparison, the initial estimates of B in Cleland's program must lie between 0.1 and 1.5 times the true value. One great advantage of the least-squares method is that it can be extended to more complex equations, such as

$$V = \frac{A[S_1][S_2]}{B + C[S_1] + D[S_2] + [S_1][S_2]} . \quad (3)$$

It is not difficult to write subroutines to produce estimates of the 4 parameters A, B, C and D from a set of experimental results obtained by using a mesh of concentrations of the 2 substrates, although it must be said that no-one has yet investigated the extent to which such multiple linearization is justifiable, in any real situation involving enzyme data. The Bliss and James procedure appears to be limited to the simple hyperbolic case. For this reason we have tended to use more extensively the Cleland program.

Before going on to discuss a danger which we see as inherent in the use of programs of this type, it is perhaps useful to point out that it is not essential to use initial steady-state conditions for the derivation of kinetic parameters, and Woolf [6] has written a program which fits a progress curve of the reaction to the integrated form of the rate equation. There seems one practical objection to the use of this method; in our experience the enzyme always progressively loses activity, to a greater or lesser extent, in the measuring vessel. The rate of this loss is always difficult to estimate accurately, and in effect one is distorting one's estimate of the parameters by using a rate equation which does not take account of it. The inaccuracy is only insignificant in experiments of short duration.

The danger which we see in the use of Cleland's program, in particular, is that it may be used in a mechanical fashion without ensuring that the basic postulates are being obeyed. Cleland [3] apparently envisages that his program will mainly be used for obtaining very accurate estimates of parameters in extended studies of a single enzyme, using large numbers of experimental points. In such studies, the investigator will certainly have made a preliminary analysis of his results by graphical means, or have other supporting evidence, to show that the results really fit a curve corresponding to eqs. (1) or (3) or another. However, all investigations which make use of enzyme kinetics are not like this. For example, a worker in this laboratory, studying the distribution of glutamine synthetase in various tissues of the rat, produced no less than 54 7-point sets of data, including runs in the presence and absence of an inhibitor to test whether the enzyme was identical in all tissues studied. The runs were made in replicate, but could not be lumped together because several different animals were used as sources of the enzyme. The results were analysed by a Cleland program, and it would have been unreasonable to expect that each set of data should have been inspected graphically before entering it. There must be many investigations of this kind, in which a computer program is invaluable for working up the experimental results. Yet, apart from the possibility of a sigmoid velocity-substrate curve arising from transformations between different forms of an enzyme [7], a number of workers have pointed out that in certain circumstances the velocity-substrate curve need not be hyperbolic, even when multiple forms of an enzyme are not involved [8–10]. It seems to us that it is unwise to assist in the wide distribution of programs which analyse data on a fixed basis, without including a warning device to indicate that a particular set of data may not be obeying the postulate. In the remainder of this paper we describe a modification of the Cleland program to include such a warning device. For the problem of estimating the parameters of enzymes known not to give rectangular hyperbolae see [12].

The two most likely deviations from a hyperbolic curve are a sigmoid shape at low substrate concentrations, and falling off of the curve at high [S] because of substrate inhibition. No simple screening device is likely to work equally well in both situations, but deviations due to the latter cause are almost certain to be observed while the data are being obtained, while sigmoid behaviour at low [S] cannot be detected by simple inspection of the data. The program as it stands will, of course, fit a hyperbola to a set of data which lie on an S-shaped curve, with a corresponding increase in the residual variance, but the significance of this as it appears in the output is unlikely to be appreciated by the user, particularly if the data are rather rough (see fig. 1).

If the coordinates of a mildly sigmoid curve (e.g., fig. 1) are inverted, the new curve of $1/y$ against $1/x$

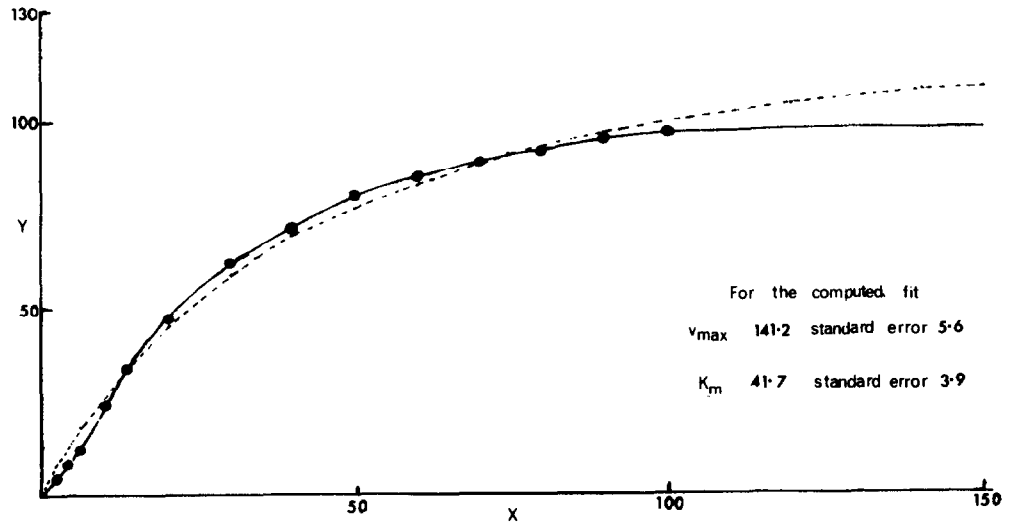

Fig. 1. Sigmoid curve with "experimental" points (see text). This is curve I of fig. 32 of Barcroft [11]. The original coordinates were: abscissa, oxygen tension; ordinate, percentage saturation. The dashed line shows the hyperbola fitted to the points by the program of Cleland [3].

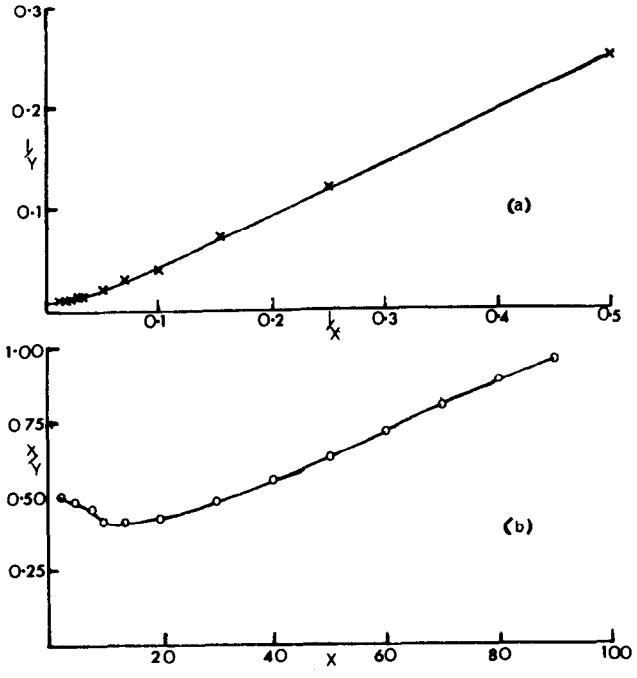

Fig. 2. (a) "Lineweaver-Burk" plot of the points shown in fig. 1. (b) "Wilkinson" plot of the points shown in fig. 1.

has a slight but definite inflection (fig. 2a). The curve of x/y against x, on the other hand, is parabolic, with a clearly defined minimum (fig. 2b). This may be rationalized by saying that if fig. 2a may be represented, over a limited range, by a quadratic equation, e.g.,

$$(1/y) = P + Q(1/x) + R(1/x)^2 , \qquad (4)$$

the corresponding transformed equation is that of a parabola:

$$(x/y) = Px + Q + R(1/x) . \qquad (5)$$

Given the shape of the original curve, the minimum of this function is almost certain to lie in the first quadrant.

Thus a good criterion for departure from a hyperbolic curve in the original data might be to test the plot of $[S]/v$ against $[S]$ for departure from linearity. For reasons discussed below, this might best be done by fitting a quadratic, rather than specifically an equation of the type of (5). If the coefficient of the squared term were found to be not statistically significant, a new estimation of the two linear coefficients would quickly provide initial values for the iteration procedure.

The procedure for obtaining initial estimates of A and B in the Cleland program is based on the regression of $1/v$ against $1/[S]$, but the values of $1/v$ are weighted by the appropriate value of v^4. This is reasonable if it can be assumed that the variance of the experimental observations is constant throughout the range of [S] used.

It has recently been shown that if the variance of v is not constant, but randomly distributed (which probably accords best with the intuitive thinking of most experimenters) then, if there are replicate (particularly duplicate) observations of v for all or most of [S] values, simple linear regression provides better estimates of $1/A$ and B/A then either weighted regression or maximum likelihood [13]. In the present instance, however, we contented ourselves with ascertaining whether estimates of A and B provided by the Wilkinson method would be satisfactory substitutes for those provided by the procedure just described. This was done by taking 9 Lineweaver-Burk plots at random from the laboratory records, marking on them from 6 to 15 points at intervals suggested by the experimental protocols, and inverting the coordinates of these points to provide ideal values of "v" and "[S]" To the "v" values were then added deviations from a table of random normal deviates. For each set of points the deviates were multiplied by a different factor to simulate "rough" or "smooth" sets of experimental data. Finally, these simulated data points were converted into 9 sets each of "$1/v$, $1/[S]$" and "$[S]/v$, [S]" values. The regression coefficients were then estimated for each of the two sets of values, and compared with the slopes and intercepts of the original lines from which the data had been constructed.

In general, the v^4 weighting procedure provided better estimates than the Wilkinson method, although in one instance a simple unweighted regression gave better estimates than either. The Wilkinson estimates were, however, still good enough to be used as initial values in the iteration procedure.

The next step was to test the sensitivity of the method in detecting a non-hyperbolic curve. A haemoglobin saturation curve was used, since it will be generally accepted to be sigmoid in shape, but the actual curve chosen had a rather shallow inflection (fig. 1). The appearance of a significant quadratic term was investigated in both the "$1/v$ versus $1/[S]$"

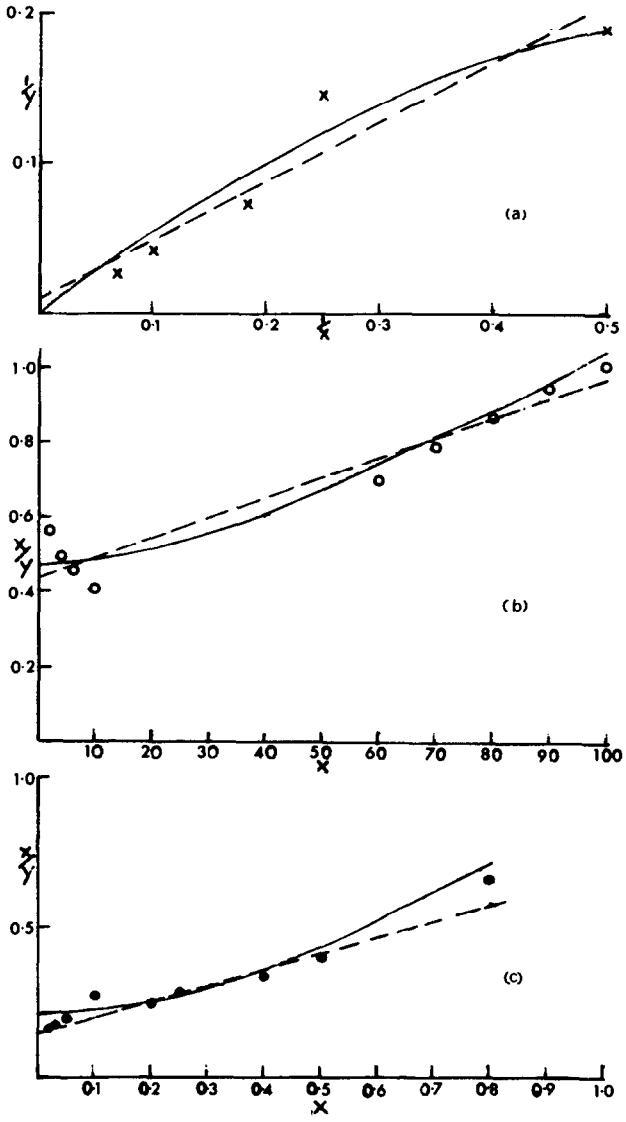

Fig. 3. (a) A false negative test on a Lineweaver-Burk inversion. The quadratic curve is convex upwards (cf. fig. 2a), and the points would be equally well fitted by a linear equation (dashed line). (b) A false negative test on a Wilkinson inversion. A quadratic curve has been drawn through the points, but the regression analysis states that they would be equally well fitted by a linear equation (dashed line). (c) A false positive test (Wilkinson inversion). The points were constructed by adding random deviates to values of y obtained from the straight line (dashed), but they are significantly better fitted by the quadratic curve.

and "[S]/v versus [S]" forms of the inverted data.

Fourteen "experimental" points were selected along the curve, as shown in fig. 1, and a set of 9 from these 14 points was taken to constitute one set of experimental observations. No random deviates were added. There are 2002 possible sets of 9 points, and of these 45 sets were put through the testing procedure. They were chosen by means of a table of random numbers, but the 14 points were first grouped in such a way that each occurred with roughly equal frequency in the overall selection. The significance of the quadratic term was established by the usual F test ($F > 5.95$ for significance at the 5% level, with 6:1 degrees of freedom).

The "[S]/v versus [S]" data showed significant departure from linearity at at least the 5% level in all 45 instances. The "$1/v$ versus $1/[S]$" data failed to show a significant quadratic term in 4 of the 45 sets, although in other cases the F-ratio was a shigh as 800. When random deviates were added to the "v" values as described earlier, the straightforward inversion test failed on 40% of the sets of data tried, even with very small variance around the theoretical curve (fig. 3a). The "[S]/v versus [S]" test failed once in all tests; this was largely because a quadratic equation does not fit this particular curve very well (fig. 3b). A more reliable test is actually the ratio of the coefficients of the [S] and $[S]^2$ terms, which was found to be < 0.5 in every test (including that shown in fig. 3b).

Finally, the occurrence of false positive tests was investigated on 18 sets of simulated linear data, constructed as described earlier. One significant quadratic term appeared (fig. 3c): differentiation between the two situations is very easy on inspecting the graphs.

This screening test is obviously rather crude. It could be improved, for the particular deviation from the Michaelis-Menten curve which was investigated, by fitting an equation such as (5), but the quadratic was retained because it can also give some indication of departure from expected behaviour in the region of large [S]. The test is also only valid for the simplest version of the program, in which only 2 parameters are to be estimated. Nevertheless, it seems to us to be useful if the program is to have its widest distribution, to every biochemist whose labours would be shortened by it. We are preparing a modification of the original program, to print a diagnostic comment and stop the computation if the test is positive, for distribution to interested users.

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