Robust regression of enzyme kinetic data

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A method described previously [Cornish-Bowden & Endrenyi (1981) *Biochem. J.* **193**, 1005–1008] for fitting theoretical equations to enzyme kinetic data without prior knowledge of weights or error distribution has been tested by computer simulation. With the equations for various kinds of linear inhibition as an example, the method performed well under all of the conditions examined, giving results that were often much better than those given by widely used least-squares alternatives, and were never appreciably worse. Although equations for two-substrate kinetics were not explicitly tested, the results for inhibition equations can be generalized to include two-substrate equations because the two are formally equivalent for simulation purposes. As a check on the results with inhibition equations the method was also tested for fitting bell-shaped pH-activity profiles and gave correspondingly good results.

INTRODUCTION

In a previous paper (Cornish-Bowden & Endrenyi, 1981) we described a new method for fitting equations to enzyme kinetic data. This was designed not only to be robust against outliers, utilising the 'biweight' method of Mosteller & Tukey (1977) for this purpose, but also to avoid the need for prior knowledge of the proper weights, a normal requirement in least-squares calculations. In studies with the Michaelis–Menten equation, it performed very effectively in a wide range of error conditions, and proved to be both robust against outliers and capable of providing a useful approximation to the correct weighting function. Insensitivity to outliers and independence of weighting information are also properties of the computationally far simpler median estimates (Cornish-Bowden *et al.*, 1978) derived from the direct linear plot (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1974, 1978), and indeed the new method and the median method performed about equally well in the Michaelis–Menten case. However, the median method cannot readily be generalized to equations of more than two parameters, whereas the new method can in principle be applied to any equation normally analysed by linear or non-linear regression. The corresponding computer program, which is now published in full (Cornish-Bowden, 1985), is not fully general, being restricted to equations of not more than four parameters and three variables that are linear when written in reciprocal form, but it is nonetheless applicable to nearly all of the equations commonly used in steady-state enzyme kinetics, including those for the common types of inhibition, two-substrate kinetics, bell-shaped pH-activity profiles etc.

We did not previously examine how well the new method performed in practice when applied to equations more complex than the Michaelis–Menten equation. We have now done so, and describe in this paper the results of simulation studies with equations for inhibition and pH-dependence, showing that the robustness against outliers and independence from prior weighting information observed with the Michaelis–Menten equation (Cornish-Bowden & Endrenyi, 1981) also apply to these more complex cases.

METHODS

Robust regression

As the method of robust regression tested in the present paper has been described previously (Cornish-Bowden & Endrenyi, 1981), and the computer program based on it has been published in full (Cornish-Bowden, 1985), the description here is limited to what is necessary for defining terms and symbols.

The function used for assigning a weight to an observed rate v_i is derived from the assumption that the variance $\sigma^2(v_i)$ of v_i is given by the following equation:

$$\sigma^2(v_i) = \sigma_0^2 + \sigma_2^2 v_{i, \text{true}}^2 \quad (1)$$

in which $v_{i, \text{true}}$ is the true rate, and σ_0 and σ_2 are constants. Although all three of the quantities on the right-hand side of eqn. (1) are unknown in a real experiment, the true rate can be replaced by the best-fit calculated rate $\hat{v}_i$, and the 'sigma ratio', σ_0/σ_2 , can be estimated as described previously (Cornish-Bowden & Endrenyi, 1981) by considering the observations in pairs. Then a weight w_i for use in the regression can be calculated from the following equation:

$$w_i = \frac{(\sigma_0/\sigma_2)^2 + \bar{v}^2}{(\sigma_0/\sigma_2)^2 + \hat{v}_i^2} \quad (2)$$

The numerator of this expression can have any constant value without affecting the final estimates, but it is convenient to give it the value shown, with $\bar{v}$ defined as the mean of all the observed v_i values, to generate a numerically convenient range of w_i values.

The actual weight given to a rate v_i in the final regression is not simply w_i , because to introduce robustness against outliers it is necessary to modify it according to the biweight method of Mosteller & Tukey (1977), and the final weight W_i is defined as follows:

$$W_i = \begin{cases} w_i(1 - u_i^2)^3 & \text{if } |u_i| < 1 \\ 0 & \text{if } |u_i| > 1 \end{cases} \quad (3)$$

where u_i is a measure of the deviation of the i th observation from the best-fit model, defined as follows:

$$u_i = w_i^{1/2}(v_i - \hat{v}_i)/6S \quad (4)$$

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where S is the median absolute value of $w_i^{\frac{1}{2}}(v_i - \hat{v}_i)$, and the constant 6 is chosen so that values of $|u_i|$ greater than 1 will be rare.

Simulation studies

The robust regression method has been compared with least-squares analysis by means of Monte Carlo trials with computer-simulated data. Various computers have been used for this purpose, initially an IBM 360/70 at the University of Toronto and an ICL 1906A at the University of Birmingham, but most recently a Honeywell Multics at the University of Birmingham. Although

shown, but were similar.) Although the results shown in Fig. 1 refer only to the estimation of K_m , the other parameters V , K_{ic} and K_{iu} were also examined and gave results in agreement with those for K_m .

Robustness against outliers

To test for robustness against outliers, simulations were carried out in which the population of errors was a mixture between two normal populations, so that each observation had a probability p of being drawn from a population with a q -fold higher standard deviation than that of the majority. The variance of a rate v with true value v_{true} could be expressed as follows:

$$\sigma^2(v) = \begin{cases} (\sigma_0^2 + \sigma_2^2 v_{\text{true}}^2) / [(p(q^2 - 1) + 1)] & \text{with probability } (1 - p) \\ q^2(\sigma_0^2 + \sigma_2^2 v_{\text{true}}^2) / [(p(q^2 - 1) + 1)] & \text{with probability } p \end{cases} \quad (6)$$

various different methods of random-number generation were used there were no significant differences between the results obtained with different computers. All of the results reported were obtained with the Honeywell Multics, using routines from the NAG Library for generating random numbers from specified distributions.

The denominator in these expressions has the effect of maintaining the variance of the combined population at a constant value when p and q are varied. The value of p was varied from 0 to 0.2 at constant $q = 5$, and in a second series q was varied from 1 to 5 at constant $p = 0.1$. (Normally distributed errors are given by either $p = 0$ or $q = 1$.) The values of σ_0 and σ_2 were 0.5 and 0.05

RESULTS

Precision and accuracy of the parameters estimated by the robust method when the correct weighting function is unknown

Fig. 1 summarizes the results of fitting sets of data by three methods when the true sigma ratio varied from zero ('relative errors') to infinity ('simple errors'). Every set of data was generated from the following equation for mixed inhibition:

$$v = Vs / [K_m(1 + i/K_{ic}) + s(1 + i/K_{iu})] \quad (5)$$

where v is the rate at substrate concentration s , inhibitor concentration i , and true parameter values as follows: limiting rate $V = 100$, Michaelis constant $K_m = 1$, competitive inhibition constant $K_{ic} = 1$ and uncompetitive inhibition constant $K_{iu} = 5$. All values are expressed in arbitrary (but consistent) units. Each experiment was simulated with all possible combinations of five s values (0.2, 0.5, 1.0, 2.0, 5.0) and five i values (0.0, 1.0, 2.0, 5.0, 8.0), making 25 observations per experiment. Errors were introduced with σ_0 varying in ten stages from 0.0 to 1.0 and σ_2 varying simultaneously from 0.05 to 0.00 in such a way that the standard deviation of a rate with true value 20 remained constant at 1.0 (or 5%). Each experiment was simulated 250 times, and the results shown in Fig. 1 thus represent a summary of 2500 trials.

By all criteria the robust method performs very well in the simulation shown in Fig. 1. At the extremes, where one or other of the least-squares methods is ideal, the robust method shows only slightly more scatter of results than the ideal method, and over almost the whole range of intermediate cases it is clearly superior to both least-squares methods. Not only does it show a narrower distribution in the central region, as represented in Fig. 1 by the gaps between the quartiles, but it also shows a lower incidence of highly erratic values. Moreover, although median bias is small compared with the semi-interquartile range for all methods in all of the cases shown, the largest values for median bias do not occur with the robust method. (Results for mean bias are not

Fig. 1. Performance of fitting methods with different weighting functions

Experiments were simulated according to eqn. (5) with true parameter values $V = 100$, $K_m = 1$, $K_{ic} = 1$ and $K_{iu} = 5$, for all 25 combinations of five s values (0.2, 0.5, 1.0, 2.0 and 5.0) and five i values (0.0, 1.0, 2.0, 5.0 and 8.0). Normally distributed errors were introduced with variances given by eqn. (1) with $\sigma_0 = \sin \theta$ and $\sigma_2 = 0.05 \cos \theta$, for θ varying from 0° ('relative errors') to 90° ('simple errors') as indicated. Each experiment was analysed by the robust method (labelled 'Robust'), by least squares assuming relative errors ('Relative LS') and by least squares assuming simple errors ('Simple LS'). Each experiment was repeated 250 times and the distributions of values of K_m are shown as quartile plots (Tufté, 1983): for each distribution the central point defines the median and the flanking lines extend from the minimum to the lower quartile and from the upper quartile to the maximum; thus 50% of values fell within the gap between the flanking lines, and the narrower the gap the more precise the method.

respectively, and the experimental design was the same as in Fig. 1, i.e. the same 25 combinations of substrate and inhibitor concentrations were used.

The results of this simulation (Fig. 2) were essentially as one would predict: the robust method gave excellent protection against outliers, but the least-squares method, regardless of how it was weighted, did not. As with the previous study, the other three parameters were also examined and their behaviour was similar to that of K_m .

Standard deviations

As an alternative to the graphical type of comparison between methods of estimation used in Figs. 1 and 2, one can also compare them in a more numerical way by considering the standard deviations of the estimated parameters. Fig. 3 shows the results of the simulations of Figs. 1–2 considered in this way. It may be seen that this approach leads to the same conclusion as before: that although circumstances exist in which one of the least-squares calculations provides a slightly more precise result than the robust method, there are no circumstances where the robust method is markedly inferior and many where it is very much better than either or both of the others.

It is striking, and at first sight surprising, that none of the methods gets worse as p or q increases (i.e. as the proportion or standard deviation of outliers increases), and indeed the robust method becomes steadily better. The reason for this is that eqn. (6) is written in such a way that the variance of the combined populations of outliers and non-outliers remains constant when p or q is changed. Thus the quality of the non-outliers must improve as the effect of outliers increases. The robust method proves able to capitalize on this improvement, by taking more account of the 'good' observations than of the 'bad', whereas a least-squares calculation cannot do so.

If the values plotted in Fig. 3 are squared they provide an indication of the number of observations needed to achieve a given precision for the different methods. In the worst case for the robust method (for $\theta = 10^\circ$ in Fig. 3a), it has a standard deviation 1.19-fold greater than that for the least-squares calculation, assuming relative errors. If this value were exact it would indicate that one would need to make about 42% ($1.19^2 = 1.416$) more observations to achieve the same precision with the robust method as one could obtain with the better least-squares calculation, or, expressing the same result in a different way, the robust method has about $100/1.416 = 71\%$ of the efficiency of the better least-squares method. This is, however, the worst case; in no other case is the efficiency of the robust method less than 76% of that of the better of the least-squares methods. On the other hand, the efficiency of the least-squares methods can be as low as 18% of that of the robust method, even in the absence of outliers. To put this into a practical perspective, an efficiency of 18% means that one would need to make about 140 observations in order to achieve the precision with the poor method of analysis that one would have with 25 observations with the better method.

Correlation between parameter estimates

It is well known that in fitting experimental data to the Michaelis–Menten equation the estimates of the parameters V and K_m are normally very highly correlated. This is a consequence of the fact that one is restricted by

Fig. 2. Effect of outliers

Experiments were simulated as in Fig. 1, except that the values of $\sigma_0 = 0.5$ and $\sigma_2 = 0.05$ were held constant, and outliers were introduced according to eqn. (6) with probability p and relative magnitude q , for p varying from 0 to 0.2 at constant $q = 5$, and also for q varying from 1 to 5 at constant $p = 0.1$. In each case the distribution of estimates of K_m in 250 experiments is shown as a quartile plot, as in Fig. 1, with maxima off-scale (greater than 1.7) represented by arrowheads; a number 2 above the arrowhead indicates a value between 2 and 3, a number 3 indicates a value between 3 and 4, whereas no number indicates a value between 1.7 and 2. The combinations $p = 0$ at $q = 5$ and $p = 0.1$ at $q = 1$ both represent the same case, but both are shown as an indication of reproducibility of the simulation. Note that the medians and quartiles for these two combinations agree very well but the extreme values do not: this is to be expected and emphasizes that the extreme values should be interpreted with caution. The combination $p = 0.1$ at $q = 5$ appears in both series but was simulated once only, i.e. the same data are plotted twice in this case so that both series should be complete.

physical constraints to a very small part of the total curve defined by the equation (one cannot use negative substrate concentrations, and even in the positive region one is in practice confined to a small range), and cannot be overcome. The usual definition of K_m as the substrate concentration at which $v = V/2$ illustrates how the estimated value of K_m depends on the estimate of V . Similar considerations apply to equations of more than two parameters, and in general in enzyme kinetics one can expect to find non-trivial correlation between every pair of parameter estimates. This is illustrated by the 'rugplot'

Fig. 3. Standard deviations

The standard deviations of estimates of K_m are plotted for the three simulations considered in Figs. 1 and 2. The three methods are indicated by the following symbols: ●, robust method; ○, relative least squares; □, simple least squares.

Fig. 4. Correlation between parameter estimates

The parameter values obtained in 25 experiments simulated as in Fig. 2, with $p = 0.1$, $q = 3$ are shown as a 'rugplot' (Tuft, 1983), in which each panel shows a pair of parameters plotted against one another. Each value is

(Tuft, 1983) in Fig. 4, in which the four parameter values obtained in 25 simulated experiments are plotted against one another in each of the six possible combinations.

In examining Fig. 4, one must distinguish between aspects that are special for the particular experimental design used and equation fitted, and those that are general. The details of the plot are all to some degree special to the particular simulation, and the fact that K_{ic} is more precisely evaluated than K_{iu} is a consequence of the experimental design, which was deliberately chosen so that one inhibition constant would be more precise than the other. The general features, which would have been observed regardless of which method of fitting was used, are that V is more precise than the other parameters, and that strong correlation is evident throughout the plot. The skewness of each distribution (for each parameter the median is nearer to the minimum than to the maximum, and nearer to the lower quartile than to the upper) is also a general feature, and has the important practical consequence that gross errors in parameter values are much more likely to be positive than to be negative.

Model discrimination with the robust method

In multiple linear regression the problem of choosing between alternative models can be regarded as solved, as the theory of F tests has been extensively developed (see Draper & Smith, 1981). Similar use of F tests in comparing non-linear models is commonplace and, although the theoretical foundation of such tests is

plotted as a percentage of its true value, i.e. in each panel the true values are at co-ordinates (100, 100), and the same scale is used throughout. The minimum, maximum, median and quartiles of the individual parameters are shown as fringes between the panels. [Tuft (1983) suggests using the fringes to plot the whole set of values of each variable, but this produces excessive crowding when there are as many as 25 values to be plotted.]

Table 1. Effect of fitting data to the wrong model

The Table shows the parameter values $\pm$ standard errors, and the effective sum of squares ($SS_{\text{eff.}}$), number of degrees of freedom (d.f.) and mean square (M.S.) obtained when the following sets of data were fitted by the robust method to the equations for competitive inhibition, uncompetitive inhibition, and mixed inhibition. For each of five s values, the values of v at the five i values 0.0, 1.0, 2.0, 5.0 and 8.0 were as follows (each set being in order of increasing i): $s = 0.2$, $v = 17.377, 9.251, 5.857, 3.325, 1.859$; $s = 0.5$, $v = 36.103^*, 20.660, 14.337, 7.511, 5.593$; $s = 1.0$, $v = 52.734, 32.371, 21.808, 12.865, 8.228$; $s = 2.0$, $v = 65.511, 45.645, 35.328, 18.451, 13.019^*$; $s = 5.0$, $v = 87.594, 64.363, 48.740, 32.602, 22.936$. The points were generated from eqn. (5) with true parameter values $V = 100$, $K_m = 1$, $K_{ic} = 1$, $K_{iu} = 5$, $\sigma_0 = 0.5$, $\sigma_2 = 0.05$ and each observation had probability 0.1 of having a 3-fold higher standard deviation ($p = 0.1$, $q = 3$). The asterisks (*) above identify the points that were generated as outliers, but this information was not used in fitting the data. The high precision to which the v values are expressed is not intended to imply that such precision could be approached in a real experiment; rather it is to permit the numerical details of the calculation to be checked.

	V	K_m	K_{ic}	K_{iu}	$SS_{\text{eff.}}$	d.f.	M.S.
Competitive	88.6 ± 5.0	0.710 ± 0.097	0.612 ± 0.070	—	88.104	18.7	4.718
Uncompetitive	126.9 ± 15.8	1.909 ± 0.493	—	1.293 ± 0.266	902.91	20.7	43.68
Mixed	101.8 ± 3.0	0.940 ± 0.057	0.919 ± 0.058	4.75 ± 0.51	16.265	18.3	0.890

insecure, they provide a useful guide in practice. Studies by Burguillo *et al.* (1983) have indicated that enzyme kineticists can in practice place some trust in F tests regardless of the theoretical objections to them.

There are no established methods corresponding to the F test for deciding which equation fits a set of data best if the equations are obtained by a robust procedure. Nonetheless, the robust method discussed in this paper does generate a sum of squares defined as follows:

$$SS = \sum W_i(v_i - \hat{v}_i)^2 \quad (7)$$

We shall refer to this as the 'effective sum of squares'. One can also define an 'effective number of observations' $n_{\text{eff.}}$, defined as follows:

$$n_{\text{eff.}} = \sum W_i / w_i \quad (8)$$

for use in calculating an 'effective number of degrees of freedom' as the effective number of observations less the number of parameters. In classical regression analysis one calculates the number of degrees of freedom simply as the difference between the numbers of observations and parameters. However, the biweight method assigns zero weight to some observations and very small weight to others, and it seems likely therefore that a classical calculation applied to the results of a robust regression would exaggerate the amount of information in the data. If eqn. (8) is used to define the effective number of observations, those observations that have zero weight are not counted at all, whereas those that have decreased weight are counted as less than one observation each.

Using the definitions suggested, one can easily carry out the same sort of tests with the results of a robust regression as one would do in classical regression. It is, however, one thing to specify a procedure, but quite another to have any confidence in the results. Confidence in a procedure that does not rest on a secure theoretical foundation can only come from long experience and thorough testing, and consequently the approach discussed in this section is offered only as a suggestion for future study. Nonetheless, the sort of results that can be expected are illustrated in Table 1, which compares the results of fitting data generated from eqn. (5), the equation for mixed inhibition, to the correct equation and to the equations for competitive inhibition and uncompetitive inhibition.

At least two generalizations can be made about the

results of fitting data to various models. First, parameter values estimated for models that are special cases of the correct model tend to be both biased and imprecise, and the degree of bias may be much larger than the standard error estimated from the data (e.g. in Table 1 the true value of K_{ic} exceeds the value estimated assuming competitive inhibition by more than 5 times the estimated standard error). Secondly, the effective sum of squares and mean square do provide a realistic indication of the quality of fit. However, the effective number of degrees of freedom may decrease by much more than 1.0 when an important missing parameter is introduced: for example, going from uncompetitive inhibition to mixed inhibition introduces a single extra parameter, K_{ic} , but the effective number of degrees of freedom decreases by 2.4 for this case in Table 1. This type of behaviour may sometimes be a useful indicator in itself of an improvement in fit, but it makes it very difficult to suggest what degrees of freedom should be used in checking the significance of the decrease in the effective sum of squares by an F test. For the present, therefore, we believe that it is safest not to conclude that an improvement is significant unless it is so overwhelmingly clear that an F test could only confirm what is obvious from inspection.

As an alternative to the variance behaviour defined by eqn. (1), a power relationship between the error variance and the true response (the true rate in the case of enzyme kinetics) has often been suggested (Tukey, 1957; Box & Cox, 1964; Box & Hill, 1974). If evaluations of two models yielded different powers in this relationship the resulting weights would have different physical dimensions and the weighted sums of squares obtained in the two calculations could not be directly compared. In contrast, the weighting scheme that we have proposed remains dimensionally consistent even when the estimated sigma ratios are different for the two models. This permits comparison of two models by the procedure described above. Nonetheless, the consequences of quantitative comparisons between effective sums of squares defined by eqn. (7) but based on different sets of weights, W_i , remain to be explored.

Residual plots

Residual plots are always a valuable adjunct to any fitting exercise, as they can often reveal features that are

Fig. 5. Residual plots as a method of model discrimination

The residual plots shown were obtained by fitting the data set defined in the caption to Table 1 by the robust method to the equations for (a) mixed inhibition (correct model), (b) competitive inhibition and (c) uncompetitive inhibition. The originals from which the Figures were drawn were rapidly traced from computer print-outs with no attempt at high accuracy or a tidy appearance, such features being of no importance in residual plots.

difficult to perceive in columns of numbers, and they offer a useful alternative to the type of comparison between models shown in Table 1. There are various choices for both the ordinate and the abscissa, but we find that it is informative to plot $\text{arctan}(3u_i)$ against the calculated rate, where u_i is the residual defined by eqn. (4). As this is not an immediately obvious choice, it perhaps requires some comment. Even with normally distributed data, the

largest residual is often much larger than all the others, and consequently if the simple residual u_i is plotted one must choose a scale such that not all of the points are plotted or all are plotted but most are compressed against the axis. Neither of these results is satisfactory, but the problem can be overcome by using the arctangent transformation, which has very little distorting effect on small residuals but allows very large, or even infinite, values to be plotted. The factor 3 has the effect of scaling the residuals in such a way that more than half of them are plotted with less than 10% distortion from strict proportionality.

When the simulations considered in Table 1 are plotted in this way (Fig. 5), one may see that there is a pronounced systematic character to the scatter of points fitted to the least satisfactory model (uncompetitive, Fig. 5c), an opposite tendency in the intermediate case (competitive, Fig. 5b), and a random scatter with the correct model (mixed, Fig. 5a). Although one might not be certain that the scatter in Fig. 5(b) is partly systematic if one considered it in isolation, comparison with Fig. 5(a) leaves no doubt.

The suggested arctangent transformation of residuals is primarily useful for graphically exploring the validity of various models, as illustrated in Fig. 5, but it is less suitable for detection of unusual, outlying, observations. For this purpose plotting the weighted residual (without the arctangent transformation) is likely to be better (e.g. Box & Hill, 1974; Hawkins, 1980; Belsley *et al.*, 1980; Beckman & Cook, 1983; Endrenyi, 1984). In general, one should not expect one method of plotting to yield more than one kind of information effectively, and it is nearly always helpful to make several different residual plots of the same data.

The labelling and choice of scales in Fig. 5 also perhaps require comment, as they may appear to contravene normal good practice for preparing graphs. However, it is important to realize that the value of a residual plot lies in the immediate visual impression that it gives, not as a source of exact numerical information. Time spent on careful drawing of points and labelling and choice of axes is, in general, time wasted, time that could be much more profitably used in making additional plots, for example plots of the same residuals against a different abscissa variable. Thus, although the plots shown in Fig. 5 have been prepared by an artist for publication, they were prepared from originals traced in less than 2 min each from computer print-outs. For non-publication purposes, line-printer representations of residual plots are all that are required, although as many line printers print rather faintly it is useful to emphasize the plotted points by blackening them by hand: this may avoid overlooking points that are not in prominent positions, for example those close to the axes.

Effect of fitting the wrong equation

We have considered (Table 1 and Fig. 5) the effect of fitting data to equations that are special cases of the correct equation. There are, however, two other ways in which one can choose the wrong model: it may be more general than is needed, so that the correct model is a special case of the model fitted; or the fitted model may simply be wrong, so that neither it nor the correct model is a special case of the other. We do not need to consider the latter case in an extreme form, because it would be unlikely to yield any useful information, and anyway one

Table 2. Fitting competitive data to the uncompetitive equation

The Table shows the parameter values estimated when a series of experiments simulated according to the equation for competitive inhibition were fitted to the equation for uncompetitive inhibition, which not only includes a redundant parameter K_{iu} but also lacks a necessary parameter K_{ic} . The true parameter values were $V = 100$, $K_m = 1$, $K_{ic} = 1$, and the error characteristics were as in Fig. 4.

	V	K_m	K_{iu}
Minimum	127	2.26	1.64
Lower quartile	134	2.55	1.95
Median	141	2.71	2.16
Upper quartile	146	2.97	2.28
Maximum	171	3.56	2.59

does not often encounter attempts to fit, for example, Beer's law to Michaelis-Menten data, even in student examinations. The more realistic case is where the two equations are 'overlapping', as for example the equations for competitive and uncompetitive inhibition have two parameters in common and the two that they do not share have meanings that are similar to one another. Thus we can study both of these remaining cases by examining the effect of fitting data generated from the equation for competitive inhibition to the equations for mixed inhibition and uncompetitive inhibition.

The effect of fitting an equation that is too general was studied by fitting competitive-inhibition data both to the equation for mixed inhibition and to the correct equation. The three parameters common to both equations, V , K_m and K_{ic} , were all estimated quite well when the more general equation was fitted, with only slightly more scatter. The estimates of the redundant parameter, K_{iu} , were grossly scattered, and were all numerically large enough compared with the highest i value to have only a trivial effect on the calculated rates. The values ranged from -1503 to $+748$, the numerically smallest value being 23.0 . Although this range may seem an exception to the general observation that parameter estimates are positively skewed, it is not an exception if one considers the negative values as 'beyond infinity' rather than as 'below zero' (cf. Cornish-Bowden & Eisenthal, 1978), an interpretation supported by the fact that there are no values between -25 and $+23$, although there are five (out of 25) in the range $+23$ to $+50$.

In summary, fitting a model that is too general introduces no perceptible bias into the parameters estimated, but it decreases the precision with which they are estimated and it increases (slightly) the correlation between the estimates. The cause of the increased correlation is not obvious, but the decreased precision is easily rationalized, as it is clear that introducing an extra parameter into a model will allow the same fit to the data to be achieved with less precise values of the genuine parameters.

The effect of fitting a model that is simply wrong is illustrated in Table 2: the estimates of both V and K_m are severely biased, as are also those of K_{iu} if one regards them as attempts to estimate K_{ic} . Although these characteristics would introduce serious inaccuracies and misinterpretations if unnoticed, it ought to be relatively

easy to detect that an incorrect model has been fitted by means of the sort of methods illustrated in Table 1 and Fig. 5. Thus the problems should be easily avoidable in practice if care is taken with model discrimination.

Two-substrate kinetics

The most important alternative to the inhibition equations as a basis for investigating the behaviour of the robust method with equations of more than two parameters would be the commonly encountered equations for two-substrate reactions, such as the following equation:

$$v = Vab/(K_{iA}K_{mB} + K_{mB}a + K_{mA}b + ab) \quad (9)$$

which defines the initial rate of an enzyme obeying a compulsory-order ternary-complex mechanism or a random-order ternary-complex mechanism with substrates binding at equilibrium (see, e.g., Cornish-Bowden, 1979), or the corresponding equation for a substituted-enzyme (ping pong) mechanism, which is the same as eqn. (9) except that the term $K_{iA}K_{mB}$ is missing. In either case a and b are the concentration of two substrates A and B and the other symbols represent parameters to be estimated. However, although eqn. (9) is superficially different from eqn. (5), they are in reality interchangeable by the transformations $s \rightarrow a$, $i \rightarrow 1/b$, $K_m \rightarrow K_{mA}$, $K_{ic} \rightarrow K_{mA}/K_{iA}K_{mB}$ and $K_{iu} \rightarrow 1/K_{mB}$, and a simulation of one equation is exactly equivalent to a simulation of the other. For example, the simulation shown in Fig. 1 is exactly equivalent to a simulation of eqn. (9) with all 25 combinations of five a values (0.2, 0.5, 1.0, 2.0 and 5.0) and five b values (saturating, 1.0, 0.5, 0.2 and 0.125), for true parameter values $V = 100$, $K_{mA} = 1$, $K_{mB} = 0.2$, $K_{iA} = 5$.

Bell-shaped pH-activity profiles

For the reasons set out in the previous section, studies of the common two-substrate equations do not provide a satisfactory basis for extending investigation of the properties of the robust method. It is more informative to consider the equation for the Michaelis function (Michaelis, 1922):

$$k = \bar{k}/(1 + h/K_1 + K_2/h) \quad (10)$$

in which k is a measured kinetic constant, such as the catalytic constant or specificity constant of an enzyme-catalysed reaction obeying Michaelis-Menten kinetics, $h = 10^{-\text{pH}}$ is the hydrogen-ion activity and $\bar{k}$, K_1 and K_2 are parameters to be estimated. This equation describes a bell-shaped dependence of k on pH, and has several useful features for illustrative purposes: it is an example of an equation with three parameters but only one independent variable, which facilitates systematic study of the effects of varying the experimental design or the number of observations; its dependent variable is itself estimated from previous analysis rather than directly measured, and is consequently liable to be subject to larger and more unpredictable errors than a measured rate; it is written in terms of concentrations and dissociation constants but experimentally and descriptively most workers prefer pH and pK values. The last of these characteristics presents no problem for the computer program (Cornish-Bowden, 1985) implementing the robust method, which allows concentrations to be input as their logarithms or negative logarithms, and allows some or all of the parameters to be converted into their

Fig. 6. Effect of deteriorating design

The inter-quartile range of estimates of pK_1 (the least well-defined parameter) is plotted against the number of observations (bottom axis) and the smallest experimental pH (top axis), for simulated experiments generated from eqn. (10), the equation for a bell-shaped curve, for true parameter values, experimental designs and error behaviour as given in the text. Each design was simulated 100 times, and each set of data was fitted by the robust method (●), relative least squares (○) and simple least squares (□).

logarithms or negative logarithms. We shall therefore discuss the results in terms of pH and pK values.

For simulating eqn. (10) we have considered a case where the true parameter values are $k = 10$, $pK_1 = 5$ and $pK_2 = 7$, and for the full design there are 15 observations with pH values in arithmetic progression from 3.9 to 8.1. As this design extends more than 1 pH unit beyond the pK values on both sides, one would expect it to be near-optimal. The errors were set at appreciably larger values than those used for the inhibition studies, with $\sigma_0 = 0.050$, $\sigma_2 = 0.1$, $p = 0.2$ and $q = 3$ (20% outliers with 3-fold higher standard deviation). For studying the degeneration of the estimation methods as the number of observations n decreases and the design deteriorates, n was decreased from 15 to 6, with omission of the lowest pH value each time; thus in the final case $n = 6$ and pH ranged from 6.6 to 8.1.

The results of this study are shown in Fig. 6 for n from 9 to 15. For n less than 12 all methods gave occasional negative values of $1/kK_1$ and (much less often, and only for n less than 8) $1/k$, which were converted into $+10^{-10}$ before conversion into values of k and pK_1 . They were frequent enough to make the results for n less than 9 meaningless, even in the form of inter-quartile ranges, and these are therefore omitted from Fig. 6. For n from 9 to 11 negative values were rare enough for the inter-quartile ranges to be meaningful, though they should still be treated with caution because the quartiles depend to some degree on how the negative values are treated.

As values of n less than 12 correspond to experiments where none of the pH values was less than pK_1 , one might expect that all methods of estimating pK_1 would degenerate as n is decreased below 12, and it is clear from Fig. 6 that this is exactly what happens: there is a clear boundary at around $n = 11$ or 12, so that there is a clear

advantage in having a design that extends beyond the pK values, but little advantage in extending it far beyond.

What is perhaps surprising is that the robust method does not degenerate any faster than the least-squares methods as n decreases: over the whole range considered it provides either the best results or results that are almost as good as the best. This is somewhat surprising because one would expect that both the biweight method and the method for estimating the sigma ratio would depend to some degree on the presence of an adequate number of 'good' observations to provide the necessary information.

DISCUSSION

It is clearly impossible to test the behaviour of the robust method of fitting data in all of the situations that could possibly arise. Even for the Michaelis-Menten equation, with only two parameters to be estimated and only one independent variable, there are many more kinds of error behaviour, experimental designs, different numbers of observations etc. than one could hope to investigate in a reasonable amount of time. With four parameters to estimate and two independent variables to vary, and the need to discriminate between different equations that might plausibly fit the data, the problem is at least an order of magnitude more complex, and the simulations we have described cannot be considered a definitive test of the robust method. Perhaps future theoretical analysis of the properties of the robust estimators may make simulation unnecessary, and it is encouraging that the results of such theoretical analysis of the median estimators of the Michaelis-Menten parameters (Cornish-Bowden & Eisenthal, 1974, 1978) are beginning to be available (Dalgaard & Johansen, 1985), but for the present we must rely on simulation for testing the new robust method. Fortunately, the consistently good behaviour of the robust method in all of the cases that we have examined provides a basis for believing that it should be the method of choice in all cases where there are an adequate number of observations, unless independent information exists about the appropriate weights to use. When such information does exist, or when there is good reason to believe that outliers are not present in the data, the same computer program (Cornish-Bowden, 1985) can still be used, because it allows the weighting function to be specified in the data rather than estimated during execution, and it allows least-squares estimation to be used in place of the biweight method when required. From the user's point of view, therefore, the robust method is no more difficult or time-consuming to use than any other computer-based method. Indeed, as all of the commonly encountered equations used in steady-state kinetics (inhibition, activation, two-substrate reactions, pH-activity profiles etc.) are defined within the program, it may well be less so.

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