

Evaluation of Distribution-free Confidence Limits for Enzyme Kinetic Parameters

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Monte Carlo experiments have been used to test the robustness of distribution-free confidence limits for the parameters of the Michaelis–Menten equation (Porter & Trager, 1977). When used in conjunction with the modified form of the direct linear plot (Cornish-Bowden & Eisenthal, 1978), they prove to be more robust than least-squares confidence limits. In circumstances where the least-squares assumptions are correct, the distribution-free confidence limits define the parameters somewhat less precisely than the corresponding least-squares confidence limits, but this effect is negligible unless there are eight or fewer observations.

1. Introduction

The direct linear plot (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1974, 1978) provides a simple way of estimating the parameters K_m and V in the Michaelis–Menten equation, without the need for the rather sweeping statistical assumptions implicit in least-squares estimation. However, as originally described the method provides no simple information

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about the precision of the individual estimates of K_m and V , though it does provide a joint confidence region for K_m and V (Cornish-Bowden & Eisenthal, 1974), by a method similar to one proposed earlier by Daniels (1954) for use in straight-line regression. This omission can be remedied by means of rank correlation theory, as Sen (1968) has described, also in the context of straight-line regression. When applied to the Michaelis-Menten equation, a similar approach allows distribution-free confidence limits to be defined for K_m and V (Porter & Trager, 1977).

The method of Sen (1968) makes no assumption about the form of the distribution of the observations, but it does assume that each observation has the same distribution. This is unlikely to be exactly true for observations of initial velocities in enzyme kinetic experiments (Storer, Darlison & Cornish-Bowden, 1975; Siano, Zyskind & Fromm, 1975; Askelöf, Korsfeldt & Mannervik, 1976), and even if it were it would not remain so after transformation of the data into the two straight-line forms required for finding the confidence limits. So the extension of Sen's method to the Michaelis-Menten equation is not rigorous and the validity of the confidence limits cannot be regarded as proved. We have used Monte Carlo experiments to determine how seriously this lack of rigour affects the validity of the confidence limits, as compared with least-squares confidence limits obtained by assuming the error distribution. We have also compared the lengths of the confidence intervals given by the two methods with various types of error.

2. Theory

The method of Sen (1968) for estimating the slope of a straight line is related to the familiar method of least squares in the sense that both can be derived by assuming that there is no correlation between the independent variable and the errors in the dependent variable; they differ in the way they measure correlation. Consider a set of n observations (x_i, y_i) , for $i = 1-n$, that fit a straight line of true slope β and intercept α on the ordinate, with errors ε_i . Then, if a and b are estimates (not necessarily best-fit estimates) of α and β respectively, one can write

$$y_i = a + bx_i + e_i, \quad (1)$$

in which e_i , the difference between the observed and estimated values of y_i , is an estimate of the true error ε_i . In an experiment with random errors in y_i only one would expect there to be no correlation between the x_i and ε_i values. So one can define the best-fit estimate of the slope as the value \hat{b} that gives a value of zero for the correlation coefficient between the x_i values and the residuals e_i . If one uses the ordinary product-moment correlation

coefficient for this purpose one obtains an estimate of the slope that is numerically identical to the least-squares estimate. But one can minimize assumptions about the distribution of error by using the rank correlation coefficient τ proposed by Kendall (1938, 1970) instead. It is simple to calculate the distribution of τ in the case of zero correlation, because it is approximately normal even for rather small samples; it is also simple to find the value of b in equation (1) that makes τ zero, as it is the median of the set of b estimates obtained by putting all possible i, j pairs into the formula $b = (y_j - y_i)/(x_j - x_i)$. One can then assign confidence limits to β based on the theoretical distribution of τ in the case of zero correlation. The best-fit estimate \hat{b} and the confidence limits are independent of the intercept estimate a , because the value of τ is determined by differences $(e_j - e_i)$ that are all independent of a .

This analysis may be applied to the Michaelis-Menten equation by formulating it so that the desired parameter appears as the slope of a straight line. To obtain $1/\hat{V}$ the Michaelis-Menten equation must be written as follows:

$$s_i/v_i = (K_m/V) + (1/V)s_i + e_i \quad (2)$$

and to obtain \hat{K}_m/\hat{V} it must be written as follows:

$$1/v_i = (1/V) + (K_m/V)(1/s_i) + e'_i \quad (3)$$

In both of these equations v_i is the velocity observed at substrate concentration s_i , and K_m and V are the Michaelis constant and maximum velocity respectively. The parameters $1/V$ and K_m/V have several advantages over the more obvious choice of K_m and V , and indeed over other possibilities such as $1/K_m$ and V/K_m (Cornish-Bowden & Eisenthal, 1978), of which the most important is that estimates of them are median-unbiased, i.e. they are as likely to be too high as to be too low, provided that the observed velocities are median-unbiased with errors that do not exceed 100% in absolute magnitude. They also facilitate comparison with the weighted least-squares analysis of the Michaelis-Menten equation, for which it is also convenient to treat $1/V$ and K_m/V as primary parameters (Johansen & Lumry, 1961).

If the distribution of e_i in equation (2) is the same for each i the distribution of e'_i in equation (3) cannot be, and vice versa; so the method cannot give perfectly valid confidence intervals for both parameters, and may well not do so for either. (The reason why the method requires the errors to be drawn from the same distribution is that it depends on the supposition that if the true errors were known and were arranged in rank order than all possible orders would be equally likely. This is not strictly true if the errors are not all drawn from the same distribution, because in that case errors with variance higher than average will tend to have ranks near the beginning or end of the

ranking.) In principle this difficulty might be circumvented by weighting, but one might hope that the effect would be small enough for weighting to be unnecessary. As we shall show, this hope was fulfilled in our simulated experiments.

3. Implementation

The computer program described previously (Cornish-Bowden & Eisenthal, 1974) for finding median estimates of V and K_m has been modified in the light of the results of this paper and others (Porter & Trager, 1977; Cornish-Bowden & Eisenthal, 1978). The new version first estimates $1/V$ and K_m/V and their (approximately) 95% confidence limits and then calculates V , K_m and V/K_m from them. The confidence limits for V and V/K_m are obtained as the reciprocals of those for $1/V$ and K_m/V respectively. This procedure requires some comment because the distribution of V , for example, cannot be obtained by simply taking reciprocals of the distribution of $1/V$. However, if we are concerned simply to find an interval with a defined probability of containing the true value of V , we can find this by taking the reciprocals of the limits of the corresponding interval for $1/V$, provided that both limits have the same sign. This condition is satisfied if negative estimates of V are treated as if they were $+\infty$, as suggested previously (Cornish-Bowden & Eisenthal, 1978). Similar arguments apply to V/K_m , but the confidence limits for K_m cannot be obtained directly from those for $1/V$ and K_m/V ; instead they are found by calculating a series of estimates

$$K_{m(ij)} = (v_j - v_i) / [(v_i/s_i) - (v_j/s_j)]$$

from all possible non-replicate pairs of observations and taking the appropriate order statistics as confidence limits (Porter & Trager, 1977). Estimates corresponding to intersections in the third quadrant of the direct linear plot (both $K_{m(ij)}$ and $V_{(ij)}$ negative) are treated as $+\infty$, because such intersections are most likely to occur when both s_i and s_j are small compared with K_m (Cornish-Bowden & Eisenthal, 1978).

The program uses the normal approximation to the distribution of τ (Kendall, 1970), because it is easier to calculate than the exact distribution and, as we shall show in section 5, Results, it gives correct limits at the 95% level of confidence under virtually all circumstances. It has been necessary to generalize the routine used in the original program for finding a sample median: the new routine finds any defined set of order statistics from a sample of values.

The new program is available from A. C.-B. on request. It is written in FORTRAN. A slightly modified version is given in full by Henderson (1978).

4. Methods

Preliminary Monte Carlo experiments were done on an ICL (International Computers Ltd.) 1906A computer, but all of the results given in this paper were obtained with similar programs on an IBM (International Business Machines) 370 computer. In both cases the methods were essentially as described previously (Cornish-Bowden & Eisenthal, 1974); i.e. for each set of assumptions about experimental error 1000 experiments were simulated for each set of substrate concentrations considered, with random normal errors generated by the method of Box & Muller (1958) and introduced into the Michaelis-Menten equation in the various ways described previously (Cornish-Bowden & Eisenthal, 1974). For each simulated experiment the values of $1/V$ and K_m/V and their confidence limits were calculated by the distribution-free method (Sen, 1968; Porter & Trager, 1977) and by the method of least squares, and the numbers of experiments in which the true values were within the calculated confidence intervals were counted. There were no differences between the results from the two computers, apart from the expected slight sampling variations. In the absence of satisfactory documentation of the random number generator used with the IBM 370, uniformly distributed random numbers were generated in sets of 100 and permuted into an arbitrary sequence before use. This was done to eliminate correlation problems of the sort described by Neave (1973), and is similar to the remedy used by Andrews *et al.* (1972).

Least-squares estimates were defined differently from those used previously (Cornish-Bowden & Eisenthal, 1974), in the light of new information about the type of error that occurs in enzyme kinetic measurements (Storer *et al.*, 1975; Siano *et al.*, 1975; Askelöf *et al.*, 1976). Previously we followed Wilkinson (1961) and Cleland (1967) in assuming that the commonest error type would be "simple errors in v ", i.e. that all of the observed velocities would have the same standard deviation and that errors in the substrate concentrations could be neglected. All of the detailed studies (Storer *et al.*, 1975; Siano *et al.*, 1975; Askelöf *et al.*, 1976), however, as well as some others that have been described in outline (Weischet & Kirschner, 1976; Carper, Toews, Thompson & Buess, 1976) suggest that it is more realistic to take "relative errors in v " as the norm, i.e. to assume that all of the observed velocities have the same coefficient of variation. Accordingly, the least-squares results given in this paper were obtained with weights $1/v^2$ rather than 1 for the velocities (Johansen & Lumry, 1961; Wilkinson, 1961).

5. Results

(A) VALIDITY OF THE NORMAL APPROXIMATION TO THE DISTRIBUTION OF τ

The distribution of the observed rank correlation coefficient τ in the case where there is no underlying correlation is accurately approximated by a normal distribution, for non-replicate samples of 10 or more observations (Kendall, 1970). The exact distribution is tedious to calculate when there are replicates (Sillitto, 1947) and requires double precision arithmetic on 48-bit computers if there are 15 or more observations. It is therefore of some importance to know the accuracy of the normal approximation. Table 1 shows a comparison between the exact and approximate results for a 95% confidence interval, for all possible experimental designs with 5–20 observa-

TABLE 1

Validity of the normal approximation to the distribution of τ

For n observations ($5 \leq n \leq 20$), arranged either with no replicates, or with all duplicates, or with all triplicates, or with all quadruplicates, the Table compares the confidence for a $\geq 95\%$ confidence interval calculated from the exact distribution of τ (Sillitto, 1947) with the confidence for the same interval calculated from the normal approximation to the distribution of τ (Kendall, 1970). The interval specifies the first and last order statistics for confidence $\geq 95\%$. For example, if there are 8 non-replicate observations (line 6 of the Table), there are 28 intersections in the direct linear plot (Cornish-Bowden & Eisenthal, 1978) and the interval shown as 6–23 means that the 6th and 23rd members of the ranked set of 28 estimates of $1/V$ or K_m/V are $\geq 95\%$ confidence limits. In applying the approximate method, the 95% intervals were calculated as 94.82% intervals (± 1.945 standard deviations), to correct a slight tendency of the approximation to underestimate the probability. For large numbers of observations (more than 20) it would be slightly more accurate to use the usual value of ± 1.96 standard deviations. In all of the cases shown in this Table, and in the overwhelming majority of the other 1000 cases examined, the exact and approximate calculations specified the same intervals. The "design" specifies the arrangement of observations into replicate sets. For example, if 12 observations are grouped into 4 sets of triplicates the design is given as 4×3 (line 16 of the Table).

n	Design	Number of intersections	Interval	Confidence	
				exact	approximate
5	5×1	10	1–10	98.33	97.25
6	6×1	15	2–14	98.33	97.58
6	3×2	12	1–12	97.78	97.11
6	2×3	9	†	†	†
7	7×1	21	4–18	96.98	96.45
8	8×1	28	6–23	96.88	96.46
8	4×2	24	4–21	97.54	97.00
8	2×4	16	1–16	97.14	96.96
9	9×1	36	9–28	95.54	95.24
9	3×3	27	5–23	95.83	95.45
10	10×1	45	12–34	95.34	95.09
10	5×2	40	9–32	96.75	96.42

TABLE 1—*continued*

<i>n</i>	Design	Number of intersections	Interval	Confidence	
				exact	approximate
11	11 × 1	55	15-41	95.95	95.70
12	12 × 1	66	19-48	95.53	95.33
12	6 × 2	60	16-45	95.86	95.63
12	4 × 3	54	13-42	96.33	96.07
12	3 × 4	48	11-38	95.43	95.19
13	13 × 1	78	23-56	95.78	95.59
14	14 × 1	91	28-64	95.28	95.13
14	7 × 2	84	24-61	96.13	95.94
15	15 × 1	105	33-73	95.37	95.22
15	5 × 3	90	26-65	95.33	95.17
16	16 × 1	120	38-83	95.87	95.72
16	8 × 2	112	35-78	95.03	94.90
16	4 × 4	96	27-70	95.70	95.53
17	17 × 1	136	44-93	95.78	95.65
18	18 × 1	153	51-103	95.23	95.11
18	9 × 2	144	46-99	95.80	95.67
18	6 × 3	135	42-94	95.60	95.47
19	19 × 1	171	58-114	95.10	94.99
20	20 × 1	190	65-126	95.32	95.22
20	10 × 2	180	60-121	95.45	95.34
20	5 × 4	160	51-110	95.10	94.99

† For six observations arranged as two sets of triplicates there is no finite symmetrical interval with $\geq 95\%$ confidence.

tions that contain either no replicates, or all duplicates, or all triplicates, or all quadruplicates. It represents about 3% of a tabulation of all possible combinations of single observations, duplicates, triplicates, quadruplicates and quintuplicates for 5-20 observations. In the overwhelming majority of cases (including all of those in Table 1) the approximation provided the correct interval for $\geq 95\%$ confidence, and in all cases the calculated approximate confidence was very close to the correct value. So we see no reason why the approximate method should not be used routinely, and it is incorporated into the computer program described in section 3, Implementation.

(B) ROBUSTNESS OF LEAST-SQUARES AND DISTRIBUTION-FREE CONFIDENCE LIMITS

Table 2 shows the frequency with which the true values of K_m/V and $1/V$ fell within the calculated confidence limits in Monte Carlo experiments simulated under various conditions. The results shown form a small but typical fraction of the total number obtained. Although there are complexities in the details the general tenor of the results is clear. The least-squares

TABLE 2

Robustness of distribution-free and least-squares confidence limits

The table shows the percentage of Monte Carlo trials (out of 1000 in each case) in which the true values of K_m/V and $1/V$ fell within the putative distribution-free and least-squares $\geq 95\%$ confidence limits, for various ranges of substrate concentrations and various types of experimental error. In all cases the substrate concentrations were assumed to be known without error, and the error in v is shown as "simple" if each value was simulated with the same standard deviation and as "relative" if each v value was simulated with the same coefficient of variation. In all cases the least-squares results were calculated by assigning weight $1/v^2$ to each v value, i.e. assuming the errors to be "relative" whether they were in fact or not. The value of q defines the shape of the distribution curve, as described previously (Cornish-Bowden & Eisenthal, 1974): for $q = 1$ the errors were normally distributed; for $q = 4$ the distribution curve was mildly long-tailed. In all cases the coefficient of variation of the v value at half-saturation ($s = K_m$) was 5%.

Error type	q	n	range of s/K_m	Series†	Distribution-free			Least-squares	
					Ideal‡	K_m/V	$1/V$	K_m/V	$1/V$
Relative	1	5	0.3-1.5	arith.	98.33	97.3	98.2	94.0	94.9
Relative	4	5	0.3-1.5	arith.	98.33	98.4	98.9	95.4	95.9
Simple	1	5	0.3-1.5	arith.	98.33	97.3	97.9	87.7	93.3
Relative	1	10	0.2-2.0	arith.	95.34	91.3	94.6	93.9	94.2
Relative	4	10	0.2-2.0	arith.	95.34	93.0	95.7	94.4	95.3
Simple	1	10	0.2-2.0	arith.	95.34	88.4	93.4	78.1	89.3
Relative	1	10	0.1-1.0	arith.	95.34	92.6	96.3	95.8	95.8
Relative	1	10	0.5-5.0	arith.	95.34	93.4	95.2	95.3	95.6
Relative	1	10	0.2-2.0	geom.	95.34	92.5	94.6	94.9	95.0
Relative	1	10	0.2-2.0	harm.	95.34	95.8	92.8	96.3	95.6
Relative	1	10	0.2-2.0	dupl.	96.75	93.4	96.5	94.8	94.0
Relative	1	20	0.1-2.0	arith.	95.32	90.0	96.1	94.9	94.7
Relative	4	20	0.1-2.0	arith.	95.32	91.0	95.4	95.3	95.6
Simple	1	20	0.1-2.0	arith.	95.32	85.9	93.0	64.7	81.2
Relative	1	20	0.1-5.0	geom.	95.32	91.5	91.9	95.3	95.4
Relative	1	20	0.2-2.0	dupl.	95.45	92.5	95.7	95.9	95.9

† Arrangement of s values within the specified range: arith., arithmetic series; geom., geometric series; harm., harmonic series; dupl., duplicates in arithmetic series.

‡ It is impossible to define distribution-free confidence intervals with exactly 95% confidence, and the calculated confidence level of the shortest symmetrical interval with confidence $\geq 95\%$ is shown. The least-squares intervals were calculated to have exactly 95% confidence in all cases.

confidence limits are valid when the underlying assumptions are correct, but seriously invalid when they are not. The corresponding distribution-free confidence limits are much more robust: in the few cases where the results are poor the results with the least-squares limits are much worse. For example, in the worst case the distribution-free confidence intervals for K_m/V contained the true values in only 86% of experiments, but the least-squares intervals in the same case contained the true values in only 65% of experiments.

Although the least-squares confidence limits are not robust against departures from correct weighting, they seem to be robust against deviations from a normal distribution of errors, as also are the distribution-free confidence limits. For each set of conditions data were simulated both with a normal distribution of errors and with a mildly long-tailed distribution (with $q = 4$ in the terminology of Cornish-Bowden & Eisenthal, 1974, who also give details of how the distribution was obtained). In all cases the proportions of experiments in which the true values lay within the calculated limits were independent of the distribution, as may be seen from the three representative examples included in Table 2.

(C) LENGTHS OF LEAST-SQUARES AND DISTRIBUTION-FREE
CONFIDENCE INTERVALS

We have compared the lengths of least-squares and distribution-free confidence intervals for $1/V$ and K_m/V in a series of Monte Carlo experiments similar to those shown in Table 2. Obviously, both types of interval tend to decrease in length as n , the number of observations, increases. It also turns out that n is the most important determinant of the ratio of lengths, because the lengths of the distribution-free intervals increase very rapidly as n is made smaller. Figure 1 shows the ratio of lengths of the two types of interval for K_m/V as a function of n , for a series of trials in which the least-squares assumptions were correct. For small values of n , particularly 6 or less, the distribution-free intervals are far longer than the corresponding least-squares ones, to such an extent that it is unlikely that the distribution-free approach will become widely accepted for small n . But for moderate and large values of n , 10 and up, the ratio is not much greater than 1.0 and so the cost of using distribution-free intervals is slight. We should emphasize that the experiments shown in Fig. 1 were biased in favour of the least-squares approach, as the data were simulated with normally distributed relative errors in v . In other experiments with different error structures the results were similar to those in Fig. 1, i.e. the ratio decreased as n increased, but more favourable to the distribution-free approach: for example, for long-tailed data with $q = 4$ (Cornish-Bowden & Eisenthal, 1974), the distribution-free intervals for $n = 20$ were 25% shorter on average than the least-squares ones, instead of 7% longer as in Fig. 1.

When some observations are made in replicate there are fewer intersections in the direct linear plot than there would be for the same number of observations with no replication. However, there is no corresponding effect of replication on least-squares estimation. Consequently one might expect that there would be some cost of replication reflected in the relative lengths of the confidence intervals. In fact, although replication does increase the lengths

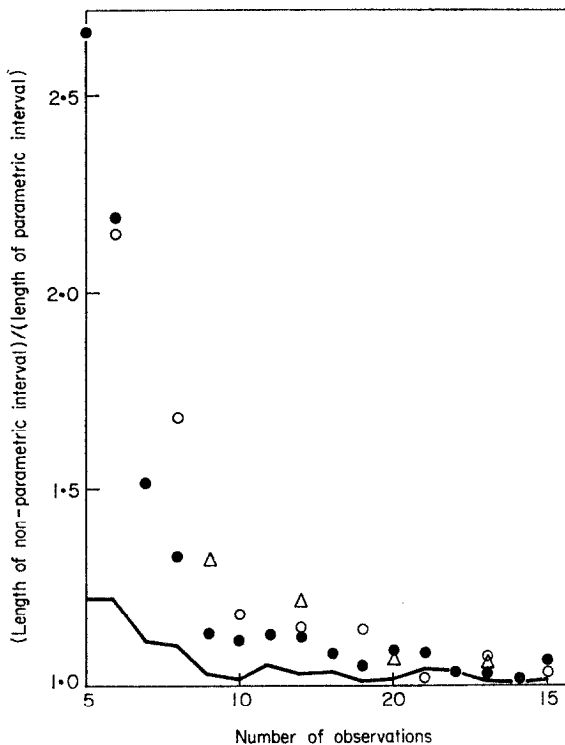


FIG. 1. Relative lengths of least-squares and distribution-free confidence intervals. The mean length of a $\geq 95\%$ distribution-free confidence interval for K_m/V in 100 Monte Carlo trials is divided by the mean length of the corresponding 95% least-squares confidence interval, and plotted against n , the number of observations. Substrate concentrations were arranged in arithmetic series in the range $0.2-2.0K_m$, either as singlets (●), duplicates (○) or triplicates (△). The irregular baseline shows the variation in the ratio of lengths (for singlet observations only) that would be expected if the extra length of the distribution-free confidence intervals were caused solely by the fact that in all cases these intervals were calculated with confidence greater than 95% by a small and irregular amount (see Table 1), whereas the least-squares intervals were calculated with confidence exactly 95% . The errors in v were normally distributed with coefficient of variation 5% .

of the distribution-free intervals, the effect is rather small (Fig. 1), small enough for sampling variations in the simulation to give points in unexpected relative positions for some values of n .

It is tempting to interpret the behaviour at small n as a reflection of the fact that the putative confidence of the distribution-free intervals is always somewhat greater than 95% , especially for small n , e.g. 98.33% for $n = 5$ or 6 . Although this effect is real, however, it is only a minor contributor to the long distribution-free intervals for small n , as can be seen from the baseline

in Fig. 1, which shows the ratio of lengths that would be expected for parametric confidence intervals calculated at slightly different levels of confidence. For example, one would expect a 98.33% confidence interval for a normally distributed variate to be about 22% longer than a 95% confidence interval.

(D) EFFECT OF MAGNITUDE OF EXPERIMENTAL ERROR

There is a tendency for the lengths of the distribution-free confidence intervals to increase more rapidly than the lengths of the corresponding least-squares intervals as the magnitude of the experimental error increases (Fig. 2). This effect is barely perceptible, however, unless the coefficient of variation exceeds about 15%. For errors smaller than this the results of the previous sections are essentially independent of the magnitude of the experimental error.

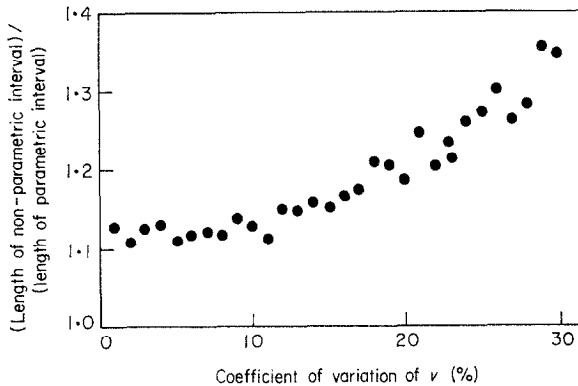


FIG. 2. Effect of magnitude of experimental error. The ratio of mean lengths of distribution-free and least-squares confidence intervals in 250 Monte Carlo trials is calculated as in Fig. 1 and plotted against the coefficient of variation of v . Each trial was carried out with ten data points at substrate concentrations in arithmetic series in the range $0.2-2.0 K_m$.

6. Discussion

The majority of published values of the parameters of the Michaelis-Menten equation have been obtained either from double-reciprocal plots with equal weight assigned to each $1/v$ value or from non-linear regression with equal weight assigned to each v value. If it is legitimate to generalize from the limited data available about the actual distribution of experimental error in enzyme kinetic experiments (Storer *et al.*, 1975; Siano *et al.*, 1975; Askelöf *et al.*, 1976), then these alternatives are about equally far from the correct weighting for least-squares analysis, and confidence limits or standard

errors calculated from them are invalid. One may hope to correct this difficulty by assigning each v value a weight $1/v^2$ (or each $1/v$ value a weight v^2), as in the least-squares calculations used in this paper. This approach has the advantage that the best-fit solution can be found in a single step without iteration (Johansen & Lumry, 1961; Cornish-Bowden, 1976), but it will work generally only if the experimental results cited above are generally applicable, and there is some evidence (Mannervik, 1975) that they are not. So it is inadvisable to use least-squares confidence limits unless there is some information about the actual error structure. In contrast, distribution-free confidence limits calculated as described in this paper behave satisfactorily with a wide variety of error structures and define the parameters almost as precisely as correctly weighted least-squares limits unless the number of observations is very small.

The great sensitivity of the lengths of the distribution-free confidence intervals to the number of observations has one important consequence for the treatment of replicate observations. One might be tempted to simplify the appearance of the direct linear plot by averaging replicate velocities before analysis. But it is clear from the results in Fig. 1 that this would be highly inadvisable unless there are measurements at at least ten substrate concentrations. The efficiency of the method depends on the existence of a substantial number of intersection points and it is definitely harmful to decrease this number by averaging replicates.

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