A Comparison of Estimates of Michaelis-Menten Kinetic Constants from Various Linear Transformations

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(Received for publication, August 26, 1964)

If an enzymatic reaction follows Michaelis-Menten kinetics, a plot of the initial velocity of reaction, \( v \), against the concentration of substrate, \( C_S \), will give a rectangular hyperbola of the form

\[
v = \frac{V_{\max} C_S}{K_m + C_S}
\]

The parameters which characterize this equation, and which must ordinarily be estimated from the observed data, are \( V_{\max} \), the maximum initial velocity which is theoretically attained when the enzyme has been “saturated” by an infinite concentration of substrate, and \( K_m \), the Michaelis constant which is numerically equal to the concentration of substrate for half-maximal initial velocity. Since the relationship between the independent variable, \( C_S \), and the dependent variable, \( v \), is curvilinear, it has long been customary to facilitate estimation of the two parameters by plotting the experimental data according to one of the following three linear transformations of Equation 1.

\[
\frac{1}{v} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \frac{1}{C_S}
\] (la)

\[
\frac{C_S}{v} = \frac{K_m}{V_{\max}} + \left(\frac{1}{V_{\max}}\right) C_S
\] (lb)

\[
\left(\frac{1}{v}\right) = \left(\frac{1}{V_{\max}}\right) + \left(\frac{K_m}{V_{\max}}\right) \left(\frac{1}{C_S}\right)
\] (lc)

Of these transformations, by far the most popular has been 1c, which corresponds to the Lineweaver-Burk, or “double reciprocal” method of plotting kinetic data. In this method, the reciprocal of the initial velocity is plotted against the reciprocal of the substrate concentration; a straight line is fitted to the points, and \( V_{\max} \) is calculated as the reciprocal of the intercept of the line on the \( 1/v \) axis. The second parameter, \( K_m \), is obtained either by multiplying the slope of the line, \( K_m/V_{\max} \), by \( V_{\max} \) or by extrapolating the line to the \( 1/C_S \) axis where the intercept will be \(-1/K_m\).

Since Equations 1a, 1b, and 1c are all mere variants of Equation 1, it might at first seem that any one of them could be used to estimate \( K_m \) and \( V_{\max} \) with equal accuracy from a given set of experimental data. This would indeed be true if both \( v \) and \( C_S \) were errorless. In fact, however, \( C_S \) can ordinarily be controlled by the investigator quite precisely, \( v \) is subject to more or less experimental error. Under these circumstances, the three linear transformations no longer provide equally accurate estimates of the parameters, particularly if a straight line is fitted to the points by eye or by the method of least squares used without proper weighting. In Equations 1b and 1c, taking the reciprocal of \( v \) tends to give undue emphasis to the smallest values of \( v \) which are just the ones likely to have the greatest percentage error. Furthermore, in Equation 1a the dependent variable, \( v \), appears on both sides of the equation so that a plot of \( v \) against \( v/C_S \) will show some degree of inevitable correlation.\(^1\) The same is true of the independent variable, \( C_S \), in Equation 1b according to which \( C_S/v \) is to be plotted against \( C_S \). Finally, in plotting a graph according to Equation 1a, both plotted variables are subject to error, and the ordinary method of fitting a line to the points by the method of least squares is theoretically no longer applicable. The relative importance of these disadvantages has not yet been fully investigated mathematically. (However, see Wilkinson (1) for a comparison of Equations 1b and 1c.) As a result, arguments in favor of one or another of the linear transformations have hitherto been based largely on intuitive reasoning rather than on quantitative considerations (2-5).

In the study presented below, a digital computer has been used to generate random samples from populations of simulated data, and to estimate \( K_m \) and \( V_{\max} \) from each sample, by the method of least squares, without weighting, for each of the linear transformations. The distribution of the sample estimates of the parameters could then be compared with the “true” values, and the behavior of the various transformations could be assessed.

EXPERIMENTAL PROCEDURE

Survey of Current Practice—As a guide to choosing realistic values for the computer analysis, a survey was made of all papers presenting Lineweaver-Burk plots in six consecutive issues of The Journal of Biological Chemistry (October 1960 to March 1961, inclusive). From each of these 28 papers, the one graph lying closest to a 45° angle from the horizontal on the printed page was chosen because a 45° angle facilitated accurate measurements of both the ordinate and the abscissa for each point. These measurements were simply made in tenths of millimeters of distance from the origin of coordinates, the actual scales being of no importance for our purposes. Estimates were also made of the intercept of the fitted line and “\( V_{\max} \)” and “\( K_m \)” were calculated from these estimates.

\(^1\) Inevitable in the sense that even if \( v \) were completely unrelated to \( C_S \), the two variables being plotted (\( v \) and \( v/C_S \)) would be correlated with each other. In this instance the inevitable correlation, being positive, tends to weaken the observed correlation between the plotted variables because the theoretical relationship between \( v \) and \( v/C_S \) predicted by Equation 1a is negative. But in a plot of \( C_S/v \) as a function of \( C_S \), the inevitable correlation, being positive, tends to strengthen the observed correlation between the two variables because the theoretical relationship predicted by Equation 1b is also positive.

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The range of substrate concentrations used for 28 Lineweaver-Burk plots taken from 28 different papers published in six consecutive issues of the Journal of Biological Chemistry. Each horizontal line represents one of the plots, the short vertical bars showing the substrate concentrations, expressed as a proportion of the calculated value of $K_m$, and placed on a logarithmic scale. The next to the last line shows the mean length and mean midpoint of the 28 lines above. The last line shows the values chosen for the present analysis. Its midpoint is at the mean of the midpoints of the 28 lines. The mean midpoint corresponds to the ratio $C_d/K_m = 0.68$. The bottom line in Fig. 1 represents the values chosen for the present analysis. Its midpoint is very close to the mean midpoint of the 28 lines, and it contains five points, the mean number in the survey. These five points were chosen to be equidistant on a logarithmic scale, each substrate concentration being taken, for convenience, as twice the preceding one. The substrate concentrations thus cover a 16-fold range, instead of the mean 10-fold range calculated from the survey, but this discrepancy is certainly not large enough to make the chosen values unrealistic. The actual numerical values used in the computer analysis are given in Table I.

Assumptions Concerning Error—Throughout the present analysis, it is assumed that $C_d$ was controlled without error, but that the “observed” sample values of $v$ corresponding to any particular value of $C_d$ were normally distributed about the “true” or “population” value of $v$ calculated by means of Equation 1 from $C_d$ and from the true parameters, $K_m = 15$ and $V_{max} = 30$. From the 28 published graphs which were surveyed, it was impossible to make more than an educated guess about the magnitude of the error to which $v$ was subject, although it seemed fairly obvious that the range of magnitude was very large. Nor was it possible from the survey to tell whether the absolute magnitude of the error in a given experiment was reasonably constant for various values of $v$, or whether the error tended to vary with $v$ so that the percentage error remained constant.

Under these circumstances, it was arbitrarily decided to consider three situations: (a) small error of constant magnitude: $\sigma = \pm 0.2$ so that $\sigma/v$, the coefficient of variation, ranged from 0.047 for the lowest “true” value of $v$ to 0.0091 for the highest “true” value of $v$; (b) large error of constant magnitude: $\sigma = \pm 1.0$ so that $\sigma/v$ ranged from 0.23 to 0.046; and (c) large error, increasing with $v$. For convenience in programming, successive values of the variance, $\sigma^2$, differed by a factor of 2, starting with $\sigma^2 = 1.0$ for $v = 4.29$ and ending with $\sigma^2 = 16$ for $v = 21.82$. The resulting values of $\sigma/v$ (in sequence from lowest $v$ to highest $v$) were 0.23, 0.19, 0.17, 0.16, and 0.18. Although not identical, these values are sufficiently similar to illustrate the situation in which the percentage error is constant.

Computer Program—For each of the three types of error of $v$, an IBM 1620 computer was programmed to generate a normally distributed population of the values of $v$ with the desired standard deviation around each of the five “true” values of $v$ as means. An actual experiment was simulated by having the computer draw a single value of $v$ at random from each of the five populations. From each such set of five “experimental” values, the computer then calculated the parameters (slope and intercept) of the “best” straight line fitted to the unweighted points by the method of least squares, using in turn each of the linear transformations of Equation 1. Finally, from the parameters of the fitted line the “experimental” estimates of the Michaelis-Menten parameters, $K_m$ and $V_{max}$, were calculated. Five hundred replicate “experiments” were thus performed by the computer.
For comparison, parameters for least square lines were also calculated after the points had been appropriately weighted. The weight assigned to a given "experimental" value of v was inversely proportional to the square of the standard deviation to which the "observed" value after transformation would be subject.

RESULTS

1. Small Error of Constant Magnitude—The frequencies with which various values of the parameters Vmax and Km were obtained with each of the three methods of calculation is depicted in Fig. 2, and the means and standard deviations of the 500 "experimental" estimates for the three methods are given in Table II. When the parameters were estimated either by plotting v against v/C8 (top row, Fig. 2) or by plotting C8/v against C8 (middle row, Fig. 2), the "experimental" estimates were rather closely clustered about the true population parameters with only a slight tendency for the experimental values to be skewed toward high values. Estimates obtained by plotting C8/v against C8 were slightly better than estimates obtained by plotting v against v/C8. But the most remarkable result was the marked inferiority of the double reciprocal or Lineweaver-Burk method, in which 1/v is plotted against 1/C8. The Lineweaver-Burk method yielded estimates of Vmax and of Km, the variance of which was substantially greater than for the other two methods. Furthermore, the Lineweaver-Burk estimates tended to be considerably skewed toward falsely high values (Fig. 2).

2. Large Error of Constant Magnitude—With v subject to a constant standard deviation of ±1.0 instead of ±0.2, the inferiority of the Lineweaver-Burk method became even more striking (Fig. 3 and Table II). Indeed, a considerable proportion of the estimates of Vmax and of Km were abnormally large, or even negative, because the unweighted, least squares line now sometimes intersected the axis of ordinates (where 1/C8 = 0) close to, or even below, the origin. Intersection in the vicinity of the origin yields very small positive or negative values of 1/Vmax and, hence, very large positive or negative values of Vmax. Even a few such values so greatly influence the arithmetic mean (and the standard deviation) that these familiar indices of central tendency and dispersion become meaningless. For this reason, for the Lineweaver-Burk method, only the median has been entered in Table II, and no measure of dispersion is given. However, it is evident from Fig. 3 that with this method the scatter of the estimated values of Vmax and of Km was much greater than for the other two methods. Moreover, the skewness toward high estimates is impressive, even if we disregard

<table>
<thead>
<tr>
<th>Assumptions about the variance of v</th>
<th>Vmax and Km estimated by plotting</th>
<th>Statistics calculated from 500 &quot;experiments&quot;</th>
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<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
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<td>Small and constant variance (σ² = 0.04)</td>
<td>1/v vs. 1/C8</td>
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<td>C8/v vs. C8</td>
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<td>v vs. v/C8</td>
<td>30.0</td>
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<tr>
<td>Large and constant variance (σ² = 1.00)</td>
<td>1/v vs. 1/C8</td>
<td>*</td>
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<td>C8/v vs. C8</td>
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<td></td>
<td>v vs. v/C8</td>
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<tr>
<td>Large and increasing variance (σ² = 1.00 to σ² = 16.00)</td>
<td>1/v vs. 1/C8</td>
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<tr>
<td></td>
<td>C8/v vs. C8</td>
<td>29.3</td>
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<td></td>
<td>v vs. v/C8</td>
<td>26.9</td>
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<tr>
<td>Large and constant variance (weighted)</td>
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<td>29.7</td>
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<tr>
<td>Large and increasing variance (weighted)</td>
<td>1/v vs. 1/C8 or C8/v vs. C8</td>
<td>31.3</td>
</tr>
</tbody>
</table>

* Too large to be meaningful; see text.
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Fig. 3. The frequency distribution of \( V_{\text{max}} \) and of \( K_m \) estimated by three different linear transformations from 500 replicate “experiments” in which the error of \( v \) was assumed to be large and constant. Forty of the estimates of \( V_{\text{max}} \) and 35 of the estimates of \( K_m \) obtained by the Lineweaver-Burk method (bottom histograms) were larger than 100 or less than 0.

Fig. 4. The frequency distribution of \( V_{\text{max}} \) and of \( K_m \) estimated by three different linear transformations from 500 replicate “experiments” in which the error of \( v \) was assumed to be large, and increased roughly in proportion to \( v \). Forty-five of the estimates of \( V_{\text{max}} \) and 36 of the estimates of \( K_m \) by the Lineweaver-Burk method (bottom histograms) were larger than 100 or less than 0. Two of the estimates of \( V_{\text{max}} \) and four of the estimates of \( K_m \) obtained by plotting \( C_s/v \) against \( C_s \) (middle histograms) were larger than 100 or less than 0.

DISCUSSION

The most striking feature revealed by the present analysis is the great inferiority of the Lineweaver-Burk method for estimating the parameters \( V_{\text{max}} \) and \( K_m \) when applied to unweighted data. Even when the error in measuring \( v \) was assumed to be...
small, the Lineweaver-Burk method was decidedly the poorest of the three linear transformations. When the error of \( v \) was assumed to be large and constant, or large and increasing, a considerable fraction of the estimates of \( V_{\text{max}} \) and \( K_m \) were post- orerously large, or even negative, when the Lineweaver-Burk method was used. The main defect of the Lineweaver-Burk plot is that when the reciprocals of the variables are plotted, the smallest value of \( v \) plays an inordinately important part in determining the position of the fitted line. If this smallest value of \( v \) happens to be badly underestimated, the corresponding plotted value of \( 1/v \) will be far too large. As a result, the unweighted least squares line will be rotated counterclockwise about the point whose coordinates are the mean values of the plotted variables, and through which the least squares line must pass. If this rotation is great enough, the intercept of the line on the \( 1/v \) axis will be close to or below the origin of coordinates, thus yielding totally worthless estimates of \( V_{\text{max}} \) and \( K_m \).

It is not so easy to choose between the other two linear transformations. When the error of \( v \) was small, plotting \( C_s/v \) against \( C_s \) was somewhat better than plotting \( v \) against \( v/C_s \), although both methods yielded reasonably accurate estimates of the parameters. However, when the error of \( v \) was large and constant, or large and variable, the variances of the estimates obtained by plotting \( v \) against \( v/C_s \) were less than the corresponding variances obtained by plotting \( C_s/v \) against \( C_s \). If the principal intent of the investigator is to compare parameters estimated from two similar experiments (for example, one with, and one without an inhibitor), the method giving estimates with the smallest variances should presumably be chosen, even if it is somewhat biased. But if the principal object is to estimate the true values of \( K_m \) and of \( V_{\text{max}} \) as closely as possible, the investigator is not sure about the magnitude of the error to which \( v \) may be subject. For if the error of \( v \) is large, either method will provide good estimates of the parameters. But if the error of \( v \) is small, the better estimates are given by plotting \( v \) against \( v/C_s \).

**Effect of Proper Weighting**—The discussion thus far has dealt only with the application of the three linear transformations to unweighted observations. This disregard of proper weighting seems to be in accord with current practice, for in only one of the 28 papers surveyed was any attention given to the problem of weighting. Most investigators seem to content themselves with from the 500 replicate “experiments” how closely the true values were estimated by each of the three methods, without worrying about whether the observed deviations from the true values were due to symmetrical scatter, skewness, bias, or all three. Accordingly, for each type of error assumed for \( v \), and for each method of calculation, Table III gives the deviation from the true value (taken as equally large above and below the true value) which was actually observed to include various proportions of the 500 sample estimates. In reality, therefore, the entries in the table represent symmetrical confidence limits for the true value. The unsatisfactory performance of the Lineweaver-Burk method (whatever the error assumed for \( v \)) is once again evident. But of greater present interest is a comparison between the other two methods for various degrees of confidence. With the large error (constant or increasing), for 95% confidence or better, estimates derived by plotting \( v \) against \( v/C_s \) always gave smaller confidence limits than did plotting \( C_s/v \) against \( C_s \), although the reverse was often true for lower levels of confidence. In other words, although moderately poor estimates might be obtained somewhat more frequently by plotting \( v \) against \( v/C_s \), outstandingly poor estimates were obtained much more frequently with the \( C_s/v \) against \( C_s \) method.

In summary, if it be granted that large underestimates or overestimates are to be feared more than small, the \( v \) against \( v/C_s \) transformation should be preferred, at least when the experiment is not sure about the magnitude of the error to which \( v \) may be subject. For if the error of \( v \) is small, either method will provide good estimates of the parameters. But if the error of \( v \) is large, the better estimates are given by plotting \( v \) against \( v/C_s \).
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Fig. 5. What happens to the three linear transformations when a large error reduces a particular value of \( v \) by 2 units (i.e. by twice the "large and constant" standard deviation assumed in the present analysis). For the three graphs at the left this error affected only the smallest \( v \); for the three graphs vertically in the middle, only the middlemost \( v \); and for the three graphs at the right, only the largest \( v \). All other values of \( v \) were the theoretical true values listed in Table I. Note that in the top row of graphs, representing the Lineweaver-Burk transformation, the scale of ordinates for the graph at the left is half as large as for the other two. For a numerical analysis of the three graphs at the left, see Table IV.

<table>
<thead>
<tr>
<th>Method</th>
<th>Slope</th>
<th>Intercept</th>
<th>( \frac{1}{v} ) vs. ( \frac{1}{C})</th>
<th>( C\frac{v}{v} ) vs. ( C)</th>
<th>( v ) vs. ( v/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1/v ) vs. ( 1/C)</td>
<td>1.036</td>
<td>-0.0091</td>
<td>-114</td>
<td>860.0</td>
<td>6.88</td>
</tr>
<tr>
<td>( C\frac{v}{v} ) vs. ( C)</td>
<td>0.0262</td>
<td>0.712</td>
<td>27.2</td>
<td>81.3</td>
<td>3.45</td>
</tr>
<tr>
<td>( v ) vs. ( v/C)</td>
<td>-12.65</td>
<td>24.85</td>
<td>12.65</td>
<td>15.7</td>
<td>1.28</td>
</tr>
</tbody>
</table>

\( * t = (\text{slope})/(\text{standard error of slope}). \)

"fitting by eye," usually (so far as could be judged from the distribution of points about the fitted lines) giving approximately equal weight to each point. Such a line, fitted by a careful and experienced investigator, will usually be very close to the line calculated by the method of least squares from unweighted data.

In fact, however, the error to which the plotted points are subject is not, in general, constant, and far better estimates of the parameters will be obtained if each point is given a weight which is inversely proportional to the square of the error to which it is subject. For example, if \( v \) is subject to a constant error, and \( 1/v \) is being plotted against \( 1/C\), the correct weight will be approximately inversely proportional to the fourth power of the "true" value of \( v \). If the error of \( v \) is proportional to \( v \) (constant percentage error), and \( 1/v \) is being plotted against \( 1/C\), the correct weight will be approximately inversely proportional to the square of the "true" value of \( v \). In real experiments, however, the "true" values of \( v \) are not known and the weights are therefore calculated from the observed values. The beneficial effect of such weighting is illustrated in the lower part of Table II. With proper weighting, identical results are given by analyzing \( 1/v \) as a function of \( 1/C\) and by analyzing \( C\frac{v}{v} \) as a function of \( C\). Furthermore, the estimates of \( V_{\text{max}} \) and \( K_m \) have been substantially improved by the use of appropriate weighting. Yet in practice, the inconvenience of calculating and using proper weighting factors, often coupled with ignorance of just what errors influence \( v \), will probably continue to discourage the general use of weighting. It is all the more noteworthy that with a large and constant error affecting \( v \), plotting \( v \) against \( v/C\) (without any weighting) yielded estimates of the parameters which were not much inferior to those obtained by using the other two transformations with proper weighting. And when the variance of \( v \) was large and increasing (roughly a
constant percentage error of \( v \)), the variability of the estimates obtained by plotting \( v \) against \( v/C_S \) was actually less than when the other two methods were used even with proper weighting.

**Closeness of Fit versus Reliability for Estimating Parameters** — The closeness with which a straight line appears to fit a series of points when plotted on graph paper is influenced by many factors. Judging from some of the published graphs which were surveyed in this study, the use of big symbols for the points and the choice of scales which make the plotted line fall at an angle remote from 45° are still popular, although disingenuous, ways of making it appear that a series of unreliable points is well fitted by a straight line. In addition to these sleight-of-pencil tricks, it is worth comment that three or four unreliable points will seem to be “better” fitted by a line than will seven or eight equally unreliable points, merely because, with fewer points, there are fewer degrees of freedom available for variation about the fitted line. Finally, it should be obvious that the goodness of fit of a line does not depend merely upon the absolute magnitude of the deviations of the experimental points from the line in the vertical (dependent variable) direction, but also upon the range of the dependent variable.

Now let us consider how well the lines calculated (without weighting) from the three linear transformations fit the plotted points when one of the observed values of \( v \) is badly in error. In each graph of Fig. 5, four of the plotted points have precisely the true population values listed in Table I. The “true” relationship in any of the graphs may therefore be found by drawing a straight line through these points. But for purposes of illustration, the value of \( v \) for the remaining point is assumed to be less than its true value by 2.0, an error which is just twice the “large and constant” standard deviation assumed in the present analysis. For the three graphs at the left of Fig. 5, the smallest value of \( v \) is subject to this error; while for the three graphs vertically in the middle it is the midmost value, and for the three graphs at the right it is the largest value.

From Fig. 5, it is obvious that, as would be expected, the departure of the points from the fitted lines is greatest when the smallest value of \( v \) is subject to error, and least when the largest value of \( v \) is subject to the same error. But it is equally clear that the closeness with which the line fits the plotted points decreases from above downward, the closeness of fit being always best for the Lineweaver-Burk plot (top row) and worst for the plot of \( v \) against \( v/C_S \) (bottom row). To examine this matter more closely, consider specifically the three graphs at the left of Fig. 5. The Lineweaver-Burk plot seems to fit the points reasonably well. In contrast, the plot of \( C_S/v \) against \( C_S \) fits the points rather poorly, while the line for the \( v \) against \( v/C_S \) plot in the lowest graph is so very poor that its slope does not even differ significantly from zero as judged by the conventional \( t \) test (last two columns of Table IV). Yet the accuracy with which these three lines estimate \( V_{max} \) and \( K_m \) is in just the reverse order, being greatest for the \( v \) against \( v/C_S \) plot and least for the Lineweaver-Burk plot (Table IV). We are thus confronted with the paradox of obtaining the best estimates from the “worst fitting” line! The undeserved popularity of the Lineweaver-Burk method may well be based upon just this ability to provide what seems to be a good fit even when the experimental data are poor. The plot of \( v \) against \( v/C_S \), on the other hand, tends to exaggerate any departure of the points from the “true” line predicted by the Michaelis-Menten formulation because both plotted variables are influenced in the same direction by an error of \( v \). Thus, in addition to the merits previously discussed, this method of plotting often has the further important advantage of warning the investigator when his observations depart from the linear relationship which is to be expected on the basis of Michaelis-Menten kinetics.

**SUMMARY**

When an enzymatic reaction follows Michaelis-Menten kinetics, the equation for the initial velocity of reaction, \( v \), as a function of the substrate concentration, \( C_S \), is characterized by two parameters, the Michaelis constant, \( K_m \), and the maximum velocity of reaction, \( V_{max} \). These parameters are commonly estimated from one of three linear transformations of the original equation. The ability of each transformation to provide reasonable estimates of \( K_m \) and of \( V_{max} \) was investigated by programming a computer to calculate these parameters from each of 500 replicate “experiments” which differed from each other only because \( v \) (“measured” at each of five values of \( C_S \)) was subject to normally distributed error. Estimates of \( V_{max} \) and \( K_m \) obtained by the Lineweaver-Burk method of plotting \( 1/v \) against \( 1/C_S \) were by far the least reliable, whatever the error assumed for \( v \). Plotting \( C_S/v \) against \( C_S \) was slightly superior to plotting \( v \) against \( v/C_S \) when the error of \( v \) was small, but the reverse was true when the error of \( v \) was large and constant, or large and variable. Plotting \( v \) against \( v/C_S \) often has the further advantage of warning the investigator when his data deviate from the theoretical relationship, since it commonly tends to exaggerate such deviations. In contrast, the Lineweaver-Burk transformation tends to give a deceptively “good” fit, even with unreliable points. The marked inferiority of the Lineweaver-Burk plot strongly suggests that it should be abandoned as a method for estimating \( K_m \) and \( V_{max} \) from unweighted points, whether the points are fitted by eye or by the method of least squares.

**Acknowledgments** — We wish to thank Mrs. Anne Werkheiser, Department of Epidemiology, Roswell Park Memorial Institute, for making available her computer program which generates normally distributed random numbers, and Mr. Robert Moore, Department of Biostatistics, Roswell Park Memorial Institute, for programming all other computations for this study.

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